TARGETED THERAPIES FOR GLIOBLASTOMA: A CRITICAL APPRAISAL

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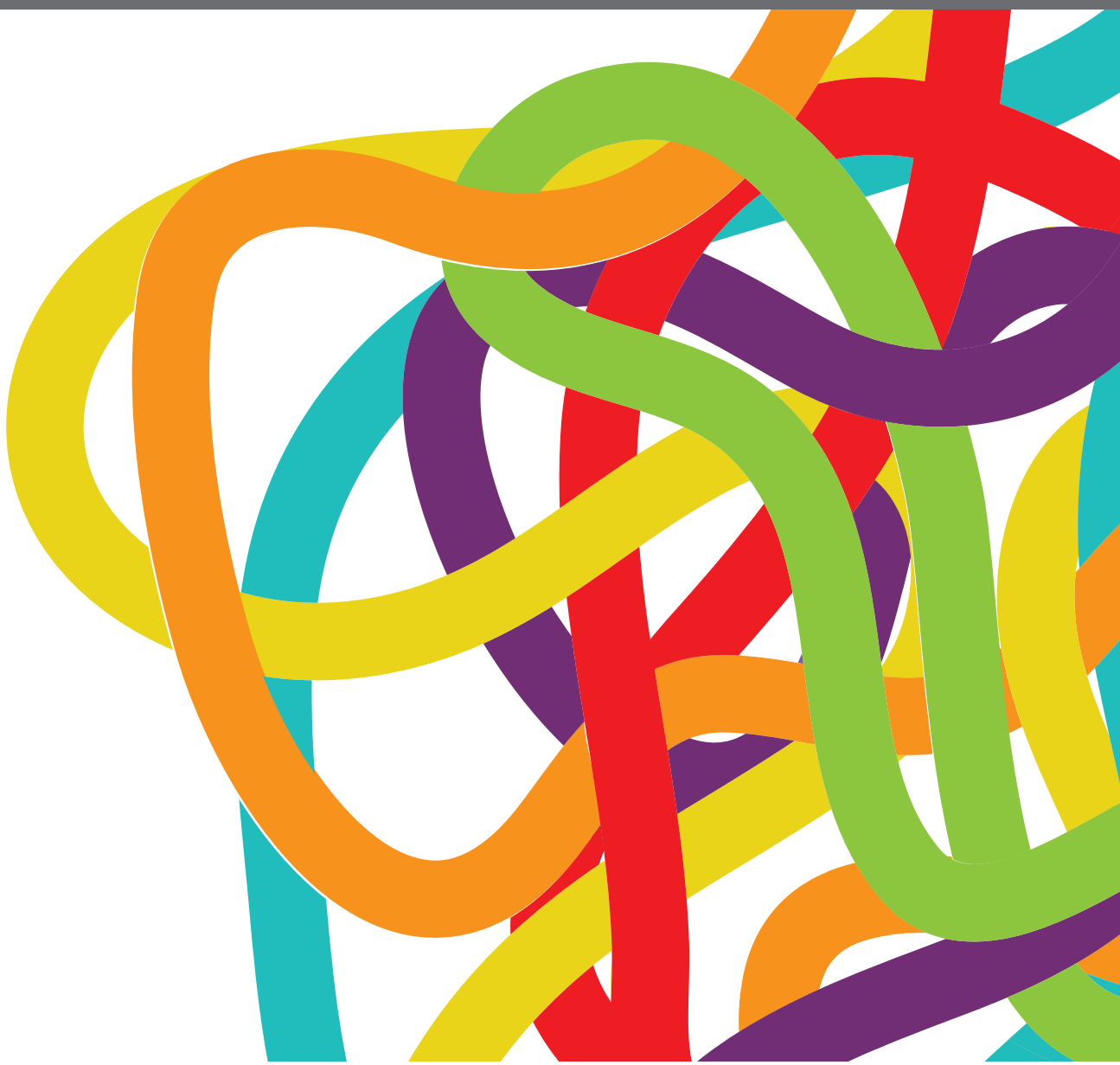

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TARGETED THERAPIES FOR GLIOBLASTOMA: A CRITICAL APPRAISAL

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EDITORIAL
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Editorial: Targeted Therapies for Glioblastoma: A Critical Appraisal

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Editorial on the Research Topic

Targeted Therapies for Glioblastoma: A Critical Appraisal

High grade gliomas including glioblastoma (GBM) in adults and diffuse intrinsic pontine glioma (DIPG) in children are fatal brain tumors with <5% of patients surviving 5 years after initial diagnosis and treatment. Targeted agents hold promise as monotherapy or as sensitizing strategies to improve response to traditional chemo-radiation. However, despite intense research endeavors and numerous clinical trials, no targeted agents have been FDA approved in the past decade. Clonal heterogeneity, acquired or inherent resistance to available therapies, restricted drug delivery, resistant stem-like cells, and immune-evasive properties in these tumors have been subject to intense study. Advances in technology including next-generation sequencing- has allowed comprehensive mapping of genetic alterations such as single nucleotide polymorphism, fusions, and copy number variations, and alterations to the epigenetic landscape including DNA methylation and histone post-translational modifications (PTMs). This approach has led to identification of several therapeutic targets and potential biomarkers, resulting in a number of new investigational treatment modalities. These include inhibitors of signaling, cell cycle and DNA damage repair pathways, and angiogenesis, in addition to ongoing evaluations of novel gene-, viro-, and immuno-therapies. However, as highlighted in the series of articles (referenced) and compiled in this eBook, despite significant progress in understanding pathology and molecular underpinnings, clinical development of novel therapeutics has faced challenges that hinder overall progress.

INHIBITORS OF SIGNALING AND ANGIOGENESIS

A number of therapeutic agents targeting growth factor receptors and downstream pathways, cell cycle, epigenetic modulators, angiogenesis, and antitumor immune responses have been tested. Targeting of receptor tyrosine kinases (EGFR, FGFR, PDGFR, and cMET) or their downstream signaling pathways (PI3K/AKT/mTOR and MAPK) using small molecule kinase inhibitors, antibodies, or antibody drug conjugates (ADCs) have been extensively studied (Jain). However, except a few documented cases of response, both kinase inhibitors and monoclonal antibodies or antibody-drug conjugates (ADCs) targeting receptor tyrosine kinases have failed to prolong overall or progression-free survival (PFS) of patients with GBM or DIPG. Adding to the pipeline of signaling inhibitors, Sheng et al., show that the drug importazole, by disruption of

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interaction between RAN-GTPase and KPNB1, inhibits growth of GBM cells. Therefore, targeting of RAN-GTPase with importazole appears to be a promising strategy for GBM (Sheng et al.). However, analysis of brain pharmacokinetics and *in vivo* efficacy of importazole should be carefully determined to allow further development of importazole as targeted therapy for GBM. Similarly, angiogenesis inhibitors evaluated as monotherapy or in combination therapies in clinical trials of GBM also had no significant benefit in overall survival, although combining bevacizumab with the standard of care led to an increased PFS in subset of patients enrolled in recent clinical trials involving patients with newly diagnosed GBM. Determining predictive biomarkers may help fully harness the benefits of anti-angiogenic agents. Malo et al. report that the antiangiogenic agents potentiate immune responses, which probably leads to the improved PFS in subset of patients on clinical trials of antiangiogenic therapy. Dissecting the molecular basis of immune modulation by anti-angiogenic therapy is therefore relevant to delineate biomarker(s) of response.

TARGETING EPIGENETIC MODIFIERS

Epigenetic modifications including DNA methylation and histone PTMS influence nearly all aspects of gliomagenesis, progression, and recurrence. Epigenetic modifications in solid tumors are gaining relevance as biomarkers and drug targets (Romani et al.). Although therapies targeting epigenetic regulators or chromatin remodeling complexes remain at early stages of development, the DNA methylation studies have helped delineate MGMT promoter hypermethylation as a robust biomarker for TMZ-based chemotherapy. Encouraged with the success of HDAC inhibitors (HDACi) in hematologic malignancies, HDACi therapies have been explored for GBM and DIPG. However, despite promising results in preclinical models, success of HDACi in clinical trials of GBM and DIPG has been modest. Radio-sensitizing effects of HDACi Panobinostat and valporic acid in phase-I clinical trials appear to be promising, but more studies are needed to support further development. BET inhibitors and EZH2 inhibitors are other epigenetic modifiers recently entered in clinical trials in GBM. While many more small molecules targeted at epigenetic pathways are on the horizon, recent discovery that majority of DIPG tumors harbor H3K27M histone protein mutation that causes global loss of H3K27me3, was of particular interest because pharmacologic restoration of H3K27me3 levels by GSK-J4, a prototype inhibitor against the H3K27me3 demethylase JMJD3, has shown excellent anti-tumor activity. However, clinical trials employing GSK-J4 have yet to be launched. Besides traditional epigenetic machinery, neomorphic IDH1 mutations result in production of 2-hydroxy glutarate, which is a strong epigenetic modulator. Targeting mutant IDH1 with IDH1-inhibitors has shown promising results in hematological malignancies and opens the way for clinical testing in GBM and low-grade gliomas harboring IDH1 mutations.

INHIBITORS OF DNA REPAIR

Dysregulation of DNA repair pathways in tumor cells undermines the benefit of genotoxic therapies. Therefore, targeting DNA repair pathways is a rational strategy to improve the response to standard chemo-radiation therapy in GBM and DIPG. Progress has been made in understanding pathways of the DNA repair involved in resistance to chemo-radiation, leading to the discovery of range of druggable targets including MGMT and PARP. Therapeutic strategies aiming to improve response to TMZ using inhibitors of MGMT was discontinued due to severe myelosuppression in patients (Romani et al.). Since PARP plays pleiotropic role in DNA damage repair mechanisms, PARP-inhibitors (PARPi) have emerged as promising sensitizing strategy. After disappointing results from early clinical trials in recurrent GBM, and reports of limited *in vivo* sensitizing effects of PARP inhibition in TMZ-resistant GBM, several new clinical trials have been launched to evaluate PARP inhibitors in newly diagnosed GBM (Gupta et al.). Some of these trials have integrated MGMT promoter methylation as biomarker to distinguish TMZ-sensitive population. While outcome from ongoing clinical trials will determine the future of PARPi in GBM, Gupta et al. have described variables that may influence the success of PARPi in GBM.

RE-PURPOSING DRUGS KNOWN TO CROSS BLOOD BRAIN-BARRIER (BBB)

FDA-approved drugs with evidence of penetration into the central nervous system (CNS) have potential as chemo-sensitizing strategy. Harder et al. report that propentofylline, previously tested in patients with vascular dementia and Alzheimer's disease suppresses pro-tumorigenic functions of microglia by targeting TROY, an orphan receptor in the Tumor Necrosis Factor Receptor (TNFR) signaling. Similarly, pimozide, an antidepressant and antipsychotic drug, and the chlorpromazine, an antipsychotic drug, inhibit multiple pro-tumorigenic activities in GBM cells (Harder et al.). Interestingly, clinical data supports chloroquine as sensitizer of standard chemo-radiation in GBM. However, in light of reports suggesting that autophagy-inhibiting effect of chloroquine is largely dispensable for tumor suppression, understanding autophagy-independent activities of chloroquine may help define potential biomarkers (Weyerhäuser et al.). The anti-diabetic biguanide class of drugs (including metformin) is interesting because biguanides selectively inhibit chloride intracellular channel1 (CLIC1), which is an emerging prognostic and predictive biomarker, as well as a promising therapeutic target in GBM (Barbieri et al.). However, repurposing of these drugs for the treatment of GBM will require optimization of cancer-relevant regimen and better mechanistic understanding.

TARGETED IMMUNOTHERAPY

Immunotherapy is one of the most promising new cancer treatment approaches, and the recent reports challenging the

long held opinion that CNS is an "immune privileged site" led to investigations aimed at boosting host immunity. While the immunosuppressive tumor microenvironment prevents immune response in GBM, manipulating the host immune system using immune check point blockade (ICBs) is considered a reasoned strategy. As summarized by Romani et al., clinical trials evaluating ICBs as single agent or in various combinations with standard cytotoxic, targeted or other immunological therapies are ongoing. Although results of a large phase III trial are disappointing, but not surprising given the fact that gliomas carry a substantially low tumor mutational burden, an important feature associated with anti-tumor immunogenicity. Results of some phase I/II trials of ICBs combined with Bevacizumab and radiotherapy (RT) appear encouraging, which is likely due to enhanced immune response with RT and/or bevacizumab (Malo et al.). However, further studies may be required to analyze effects of RT, which can be an independent synergistic facilitator of response to immunotherapy Rajani et al., especially in genetically unstable tumors, where enhanced TMB with RT is possible. In context of recurrent tumors, where RT is precluded, using oncolytic agents in combination with ICBs may facilitate antitumor response. The impacts of prior brain RT in recurrent tumors is poorly understood, though increasing evidence suggest that RT-induced changes in brain may contribute to recurrence and aggressiveness of GBM (Gupta and Burns). Whether containment of CNS injury responses in brain after RT improves response to ICB therapy has to be carefully assessed. Epigenetic mechanisms by regulating expression of PD-1 and PD-L1, can modulate response to ICBs (Chin et al.). Therefore, targeting epigenetic pathways involved in PD-1 and PD-L1 upregulation can promote anti-tumor immunity and may synergize immunotherapy drugs (Chin et al.).

ADOPTIVE IMMUNOTHERAPY

Defective antigen processing, T-cell receptor signaling, co-stimulatory signaling or immune-surveillance capacity of natural killer (NK) cells may disrupt immune response even in presence of adequate TMB. Adoptive transfer of immune cells, trained or modified to attack cancer cells, has emerged as an attractive immunotherapy strategy. In this line of therapeutics, dendritic cell (DC) vaccines, activated NK-cells and chimeric antigen receptor (CAR) expressing T cells (CAR-T) or CAR expressing NK cells (CAR-NK) are under intense investigation.

DC Vaccines

DCs being the most prominent antigen presenting cells (APCs) are essential for sustained T cell and NK cell response. DC vaccines involve autologous transfer of DCs incubated with glioma stem cells or mixture of GBM associated peptides or tumor-specific peptide such as EGFRvIII extracellular domain. Early stage clinical trials of DC vaccines have yielded promising results in select groups of patients with GBM but have not met primary endpoint to extend overall survival time. Whether combining DC vaccines with the ICBs, improves overall response remains to be tested (Jain; Romani et al.; Rajani et al.).

CAR-T Cells

Since the use of genetically engineered T-cells expressing CARs (fusing extracellular antigen recognition domain directed against tumor specific antigens with transmembrane and intracellular domain of T-cell receptor), has been FDA approved for hematologic malignancies. A number of preclinical and clinical studies have been evaluating this strategy in solid tumors. At least 3 independent phase-I trials have demonstrated feasibility, safety, and encouraging signs of efficacy of CART cells directed against EGFRVIII, HER2, or IL13Ra2, well-known surface antigens in subgroups of GBM. While promising results have generated enthusiasm for CAR-T cell therapy of brain tumors, expanded search for CAR targets, improved trafficking and optimization of dose, frequency and schedule of administration, will be key to advancement of CAR-T cell therapy. Considering low engraftment, lack of proliferation or effector function of T-cells in brain tumor microenvironment, CAR-T cell therapy alone may not be sufficient. Combining CAR-T cell therapy with ICBs, oncolytic agents and/or lymphodepleting chemotherapy should be a more comprehensive and efficacious approach.

NK and CAR-NK Cells

NK cells in immunosuppressive environment of brain tumors lack immune-surveillance capacity. Therefore, transferring *ex-vivo* activated NK cells appears to be a promising approach to brain tumors. In a phase I clinical trials, autologous transplantation of *ex-vivo* activated NK cells (with IL-2 or IL-15) into the resection cavity of GBM patients, has shown anti-tumor activity. Similarly, allogeneic transplantation with continuously expanding NK-92, a constitutively active human NK cell line, has been safely applied that showed clinical response in a subset of patients. Similar to CAR-T cells, NK cells engineered to express CARs have been developed for targeted lysis of cancer cells. As a proof of principle study robust antitumor efficacy of NK-92 cells expressing an ErbB2-specific CAR have previously been demonstrated in syngeneic mouse models. While activated NK or CAR-NK cells appear to obviate several challenges of DC vaccine and/or CART cell therapy, the ongoing clinical trials will ultimately determine the fate of NK or CAR-NK cell-based treatments for human gliomas.

IMPROVEMENTS IN DRUG DELIVERY

Exclusion of toxins from entering the brain is one unique tissue BBB (Harder et al.; Himes et al.). Despite tumor vasculature being underdeveloped and leaky, throughout history, one of the leading challenges in treating brain tumors has been delivery of drugs past the BBB. For DIPG tumors, this might be harder, as there is evidence indicating that the BBB is even more privileged. Finding BBB-penetrating drugs, which can maintain effective steady state concentrations without causing toxicity to normal tissue, is vital but serious limitation to the development of targeted therapies. Developing new and safer methods of drug delivery to disrupt or bypass the BBB is an area of intensive research and multiple methods including convection enhanced delivery (CED), focused ultrasound (FUS), vasoactive peptides,

osmotic agents, and polymeric nanoparticles encapsulation are being developed (Harder et al.).

Himes et al. demonstrate that despite technical challenges, placement of CED catheters into the brainstem of small animals is safe. This is in line with the phase I safety trial in patients with DIPG tumors, where CED of the radionuclide [^{124}I]-8H9 was well-tolerated. Several ongoing clinical trials continue to investigate CED of various promising drug formulations for DIPG and GBM treatment that brings hope to patients. However, developing CED as a routine procedure is an ongoing challenge that requires further refinements in hardware technology and the understanding of CED pharmacology. Although preclinical and clinical studies of CED continue to enhance the pipeline of targeted agents for both DIPG and GBM, the invasive and highly technical nature of the procedure remains an obstacle.

Macromolecular drug delivery systems, such as liposomes and polymers, increase efficacy, stability, and plasma half-life of anticancer drugs while reducing toxicity to healthy tissues. Drug delivery through macromolecular carriers mostly relies on the passive targeting via the enhanced permeability and retention effect. Raucher et al. describe the use of macromolecular carriers that deliver and/or release drugs in response to internal or external stimuli. Additional studies are required to understand the pharmacology of macromolecular carriers, and refine assays to precisely measure toxicity of these promising macromolecular carriers.

Tumor-tropic properties of neural stem cells (NSCs) permit their use as delivery vehicles to selectively target therapeutic gene products to brain tumor cells (Gutova et al.). The clinical trials to date with the allogeneic, clonal HB1.F3.CD21 NSC line have demonstrated safety, injections through intracranial tracts (ICT) are technically challenging. Gutova et al. have developed intracerebral/ventricular (IVEN) method of delivery to overcome the challenges in ICT route of delivery. NSCs delivered by IVEN route in mice with intracranial GBM xenografts, migrated to contralateral brain and localized within tumors. Robust migration of clinically relevant HB1.F3.CD21 NSCs toward invasive tumors shows the feasibility of IVEN to deliver NSCs in to brain tumors and is likely to have impact on gene therapy based treatments of brain tumors.

CONCLUSIONS AND FUTURE PERSPECTIVES

The lack of bioactive brain penetrant-targeted molecules and inadequate considerations to genomic/molecular features of tumors may be partly responsible to systemic failure of targeted therapies in clinical trials. Although all targeted agents may have gone through preclinical testing to justify evaluation in clinical trials, repeated clinical failures of novel investigational drugs highlight the importance of comprehensive preclinical assessment of brain pharmacokinetics and efficacy evaluation involving genetically engineered animal models or larger panels of orthotopically implanted PDXs rather than justifying clinical trials based on *in vitro* cytotoxicity data or *in vivo* efficacy evaluation in limited number of xenografts established from cell lines. Integration of technological advances in drug delivery, patient stratification based on matching molecular characteristics and robust prognostic and predictive biomarkers in modern clinical trial designs will be crucial to successful translation of promising targeted therapies.

AUTHOR CONTRIBUTIONS

SG: conception, design, and writing. SK, DD, and JS: reviewed and helped to revise the manuscript.

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ORIGINAL RESEARCH
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Immunomodulation Mediated by Anti-angiogenic Therapy Improves CD8 T Cell Immunity Against Experimental Glioma

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Glioblastoma (GBM) is a lethal cancer of the central nervous system with a median survival rate of 15 months with treatment. Thus, there is a critical need to develop novel therapies for GBM. Immunotherapy is emerging as a promising therapeutic strategy. However, current therapies for GBM, in particular anti-angiogenic therapies that block vascular endothelial growth factor (VEGF), may have undefined consequences on the efficacy of immunotherapy. While this treatment is primarily prescribed to reduce tumor vascularization, multiple immune cell types also express VEGF receptors, including the most potent antigen-presenting cell, the dendritic cell (DC). Therefore, we assessed the role of anti-VEGF therapy in modifying DC function. We found that VEGF blockade results in a more mature DC phenotype in the brain, as demonstrated by an increase in the expression of the co-stimulatory molecules B7-1, B7-2, and MHC II. Furthermore, we observed reduced levels of the exhaustion markers PD-1 and Tim-3 on brain-infiltrating CD8 T cells, indicating improved functionality. Thus, anti-angiogenic therapy has the potential to be used in conjunction with and enhance immunotherapy for GBM.

Keywords: glioblastoma, anti-angiogenic therapy, immunotherapy, vaccine, combination therapy

INTRODUCTION

Glioblastoma (GBM) is a lethal cancer of the central nervous system (CNS). Patients diagnosed with GBM have a median expected survival of about 15 months following diagnosis with treatment (1, 2). As it currently stands, there is no cure for GBM, and even with surgical resection of the tumor, a patient will universally recur and succumb to disease. Therefore, there is a clear need for the development of new therapies for GBM treatment.

One such therapeutic strategy that has been rising in popularity are immunotherapies, which aim to target the immune system to respond to the tumor. Immunotherapies provide a facet of precision not possible with surgical techniques, which are unable to target the invasive edges of the tumor, or chemotherapies, which nonspecifically target all dividing cells (3). As a result, numerous research groups are testing a variety of immunotherapy strategies against GBM tumors, both in pre-clinical models and in clinical trials (2). In particular, strategies to activate tumor

antigen-specific CD8 T cells, which will then kill tumor cells using cytotoxic granules, have been promising (4). While these therapies have demonstrated some success, there are still no curative strategies for GBM. This is primarily due to the immune suppressive nature of the tumor microenvironment, and the global immune dysregulation patients present with despite immunotherapy treatments.

To simultaneously bypass the immune suppressive tumor environment and stimulate anti-tumor immune responses, concomitant therapies have become highly prevalent. These treatment regimens often combine a therapy that is currently in use with a novel immunotherapy, including vaccination (2). Importantly, the synergy between many of these combination treatments has not been defined. For example, combining anti-angiogenic therapies, often used in patients with recurrent GBM following surgical resection, with immunotherapies, improves survival in pre-clinical models (5). However, the extent to which anti-angiogenic therapy blocking vascular endothelial growth factor (VEGF) impacts the immune response to GBM directly, is unclear. Studies in other tumor models and in *in vitro* assays have suggested a regulatory role of VEGF on the immune system (6, 7). These studies in particular demonstrate a role for VEGF on retention of dendritic cells, a potent antigen presenting cell (APC), in state of reduced activation. This would in turn reduce T cell activation and subsequently negate the impact T cell-based immunotherapy strategies, including tumor antigen-specific vaccination.

We hypothesized that blockade of VEGF using the clinically available anti-angiogenic therapy, VEGF-Trap (Eylea/Aflibercept), we would improve dendritic cell maturation and in turn improve antitumor T cell responses in a murine model of GBM, the GL261-quad cassette syngeneic glioma. Our group has previously demonstrated that treatment with VEGF-Trap, which is a VEGF receptor (VEGFR) fusion protein conjugated to a human IgG Fc region, results in similar outcomes as GBM patients treated with bevacizumab anti-angiogenic therapies as measured by T1- and T2-weighted magnetic resonance imaging (MRI) and histology (5). Likewise, VEGF-trap treatment improves survival in GL261-quad cassette bearing animals (5). Importantly, VEGF-Trap is used in place of bevacizumab due to improved cross-reactivity with murine VEGF (8).

To address this hypothesis, we first assessed the expression of VEGFRs on the surface of dendritic cells, which we contend are the most potent APC to generate CD8 T cell responses in the CNS (9). We also treated GL261-quad cassette bearing animals with VEGF-Trap weekly and assessed the quality of dendritic cell activation in the tumor draining lymph nodes (TLDNs) 14 days post treatment. We also evaluated the proportion of tumor antigen-specific CD8 T cells in the CNS of these animals.

MATERIALS AND METHODS

Acute Viral Infection and Vaccination

Six- to eight-week-old C57BL/6 mice were infected intracranially (i.c.) with Theiler's murine encephalomyelitis virus (TMEV) as previously described (9–11). Animals were anesthetized with 1–2% isoflurane, then received a single dose of 2×10^5 plaque forming units (PFU) of TMEV in the right hemisphere of the brain. VEGFR expression was measured in the draining lymph nodes and brain 5 and 7 days following infection.

GL261 Cell Culture and Implantation

The GL261-quad cassette cell line has been transgenically modified to express four model antigens: $OVA_{257-264}$, $OVA_{323-339}$, human $GP100_{25-33}$, and I-E^a_{52-68}, in addition to a luciferase transgene to assess tumor burden. 6×10^4 GL261-quad cassette cells were implanted by stereotactic injection as previously described (5, 10). Six- to eight-week-old female C57BL/6 animals were anesthetized with 20 mg/kg ketamine and 5 mg/kg xylazine to minimize discomfort during the procedure. Cells were injected at a concentration of 6×10^4 GL261 cells per 1 μL phosphate buffered saline (PBS). Injection rate was 0.2 μL per minute. The site of injection was 1 mm lateral, 2 mm anterior of the bregma with a depth of 3 mm from the surface. All animal experiments were approved by and performed in accordance with the Mayo Clinic Institutional Animal Care and Use Committee.

Bioluminescence Imaging

GL261-quad cassette-bearing animals were assessed for tumor burden using bioluminescence imaging as previously described (5, 10). Animals were intraperitoneally injected with 150 mg/kg D-luciferin sodium salt in PBS (Gold Biotechnology, Olivette, MO). Animals were anesthetized with 1–2% isoflurane before and throughout imaging. Animals were scanned using an IVIS Spectrum system (Xenogen Corp., Amameda, CA, USA) running Living Image software. Bioluminescence intensity (photons/s) was recorded in a circular region of interest surrounding the head. Animals with average bioluminescence intensity above 10^5 photons/s were considered tumor bearing and treated with VEGF-Trap or PBS. All animal work was completed in accordance to the Mayo Clinic Institutional Animal Care and Use Committee guidelines.

Anti-angiogenic Therapy Treatment

VEGF-Trap/Aflibercept (Regeneron Pharmaceuticals, Rensselaer, NY, USA) was administered at a dose of 12.5 mg/kg in PBS in a total volume of 100 μL intravenously (i.v.) by injection into the tail vein 2 weeks post-tumor injection. Treatment was continued weekly until animals were euthanized for flow cytometry analysis. Control mice received 100 μL PBS i.v., at the same time points.

Flow Cytometry

Lymph nodes and spleens were harvested in RPMI and pressed through a 70 μm filter to achieve a single cell suspension for compensation control samples. Brains were harvested and manually homogenized using a dounce homogenizer as previously described (12). Brain samples were filtered through a 70 μm filter to achieve a single cell suspension into a 50% percoll solution. Samples were centrifuged at 7,840 g. The myelin

debris layer formed at the top of the gradient was aspirated. All samples were washed twice and plated in a 96-well v bottom plate. Peptide:MHC tetramers were constructed by our lab and samples were stained at a 1:100 dilution of tetramer for 30 min at room temperature in the dark. Following a wash, antibodies against CD45, CD11c, CD11b, I-A^b, CD80, CD86, VEGFR2, Nrp-1, CD4, CD8α, PD-1, and Tim-3 were used for staining at a 1:100 dilution for 30 min on ice in the dark (BD Biosciences, San Jose, CA; Tonbo Biosciences, San Diego, CA) in addition to Ghost Red 780 Viability Dye used at a 1:1000 dilution (Tonbo Biosciences, San Diego, CA). Samples were fixed with 2% paraformaldehyde. Samples were subsequently run on a BD LSRII flow cytometer equipped with FACSDiva software (BD Biosciences, San Jose, CA). Samples were digitally compensated using single-stained controls and analyzed by FlowJo v10 software (FlowJo LLC, Ashland, OR).

Statistical Analysis

All data are presented as mean ± standard error of the mean (SEM). Significance was determined using a Mann-Whitney Rank Sum Test. GraphPad Prism 7.0 (La Jolla, CA) were used for all statistical analysis.

Data Availability

All data generated during this study are available from the corresponding author on reasonable request.

RESULTS

Dendritic Cells Express VEGFRs in the Inflamed CNS

To address the impact of VEGF signaling on the immune system, we first sought to identify the cells through which VEGF would signal. To address this question, we used infection with Theiler's Murine Encephalomyelitis Virus (TMEV) as a model of CNS inflammation. Intracranial infection with TMEV results in extensive immune cell expansion in the deep cervical lymph nodes and subsequent immune cell infiltration into the CNS (13). We therefore assessed VEGFR expression on CD11c$^+$ dendritic cells, compared to CD11c$^-$ immune cells, 5 and 7 days post infection. Expression of VEGFR2, considered the primary signaling receptor for VEGF, and Neuropilin-1, known as a co-receptor for VEGF signaling. We found that CD11c$^+$ dendritic cells express low but detectable levels of VEGFR2 in the deep cervical lymph nodes 5 days post infection, and express higher levels of VEGFR2 in the brain 5 days post infection (**Figures 1A,B**). By 7 days post infection, CD11c$^+$ cells in the brain express high levels of both VEGFR2 and neuropilin-1, suggesting that dendritic cells in the CNS are capable of signaling through VEGF receptors (**Figures 1A,B**). This also suggests that neuropilin-1 expression is induced following inflammation. Notably, we do not see upregulation of VEGFR2 or neuropilin-1 expression on CD11c$^-$ immune cells.

We next evaluated VEGFR expression on antigen presenting cells (APCs) in the brain in unvaccinated C57BL/6 mice harboring established GL261 gliomas. We primarily focused on dendritic cells owing to its dominant role in mounting anti-glioma response (9). We determined that dendritic cells isolated from the brain of these animals expressed VEGFR2 at higher levels than CD45+, CD11c$^-$ blood derived cell types (**Figure 1C**). Furthermore, the proportion and absolute counts of isolated dendritic cells that express VEGFR2 is significantly higher in glioma bearing mice (**Figures 1D,E**). We further assessed the expression levels of VEGFRs on other brain-infiltrating and resident immune cells as well. We found the VEGFR2 levels on CD45^{int} CD11b$^+$ microglial cells remained unchanged in comparison to non-tumor bearing littermates (data not shown). Similarly, the expression level on other CD45+, CD11c- immune cell types was unremarkable (**Figures 1C–E**).

Dendritic Cells Are More Activated, and CD8 T Cells Are Less Exhausted, Following Anti-angiogenic Therapy

After demonstrating that dendritic cells express VEGFRs, we next sought to determine the impact of this expression on anti-glioma immune responses. To accomplish this, we implanted GL261-quad cassette gliomas in C57BL/6 mice. Two weeks following tumor implantation, we imaged animals using bioluminescence imaging to remove animals from the study that did not bear tumors. We treated only tumor-bearing animals with VEGF-Trap intravenously. A second cohort of animals was treated with PBS as a control. Two weeks following treatment, or 4 weeks post-tumor implantation, brains were harvested and processed for flow cytometric analysis.

Dendritic cells isolated from the brains of tumor bearing animals were assessed for expression of known activation markers, including CD80 (B7-1), CD86 (B7-2), and I-A^b major histocompatibility complex (MHC) class II. We found that following VEGF-Trap treatment, a higher proportion of dendritic cells expressed each of these markers, as compared with PBS treatment (**Figures 2A–D**). These markers are required for T cell activation, and increase in each of these markers suggests that VEGF-Trap treatment results in dendritic cells that are better capable of stimulating an anti-tumor immune response.

We next assessed the impact VEGF-Trap treatment had on brain infiltrating, tumor antigen-specific CD8 T cells. To accomplish this, we measured expression of the exhaustion markers PD-1 and Tim-3 (14). We assessed proportion of cells expressing these markers on both total CD8 T cells and on K^b: OVA-specific CD8 T cells, as the GL261-quad cassette cell line expresses OVA peptide (SIINFEKL) as a model tumor antigen (10). We determined that fewer CD8 T cells infiltrating the brain following VEGF-Trap treatment had high expression of PD-1 and Tim-3 (**Figures 2E–H**). Therefore, a reduced proportion of CD8 T cells are exhausted as a result of VEGF-Trap treatment. Furthermore, tumor antigen-specific CD8 T cells, defined as being Kb:OVA Tetramer$^+$, are also less exhausted than tumor antigen-specific CD8 cells isolated from PBS treated animals (**Figures 2I,J**). These findings suggest that VEGF-Trap treatment results in a tumor-specific CD8 T cell

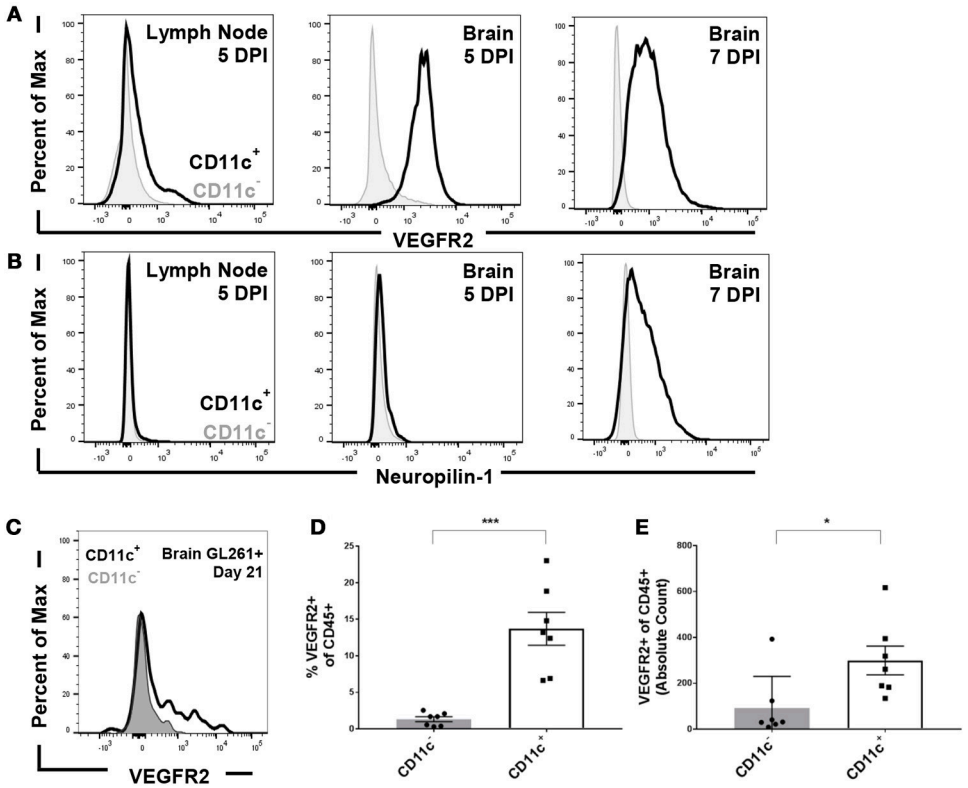

FIGURE 1 | Dendritic cells express VEGFR2 and neuropilin-1 in the brain during picornavirus infection and 21 day established GL261 glioma. Dendritic cells were isolated from the cervical lymph node and brain five and seven DPI. Dendritic cells were gated as $CD45^{hi}$ and $CD11c^{+}$. **(A)** Dendritic cells in the lymph node and brain express VEGFR2, with a majority of dendritic cells expressing VEGFR2 seven DPI in the brain. **(B)** Brain-infiltrating dendritic cells express neuropilin-1, a co-receptor for VEGF, in the brain seven DPI with TMEV. **(C)** Representative flow plot showing expression of VEGFR2 in both $CD11c^{+}$ and $CD11c^{-}$ cells isolated from the brain of unvaccinated animal bearing GL261 glioma. **(D,E)** In untreated mice with 21 day established GL261 gliomas, we observed CD11c+ dendritic cells in the brain express higher levels of VEGFR2 ($N = 7$). Data presented as mean with error bars representing standard error of the mean (SEM). $^{*}p \leq 0.05$ and $^{***}p \leq 0.001$ by Mann–Whitney *U*-Test.

response that is more capable of carrying out their cytotoxic effector function.

DISCUSSION

Here we demonstrate that VEGF-Trap treatment, as one example of anti-angiogenic therapy, results in a treatment response beyond vasculature normalization. In addition to the previously demonstrated effects observed by this treatment in the GL261 glioma model, we observe a significant change in dendritic cell maturation status and in CD8 T cell exhaustion. These findings are of great importance as immunotherapies are developed for CNS cancers.

Dendritic cell maturation is key for effective antigen presentation of tumor antigens. This is true for both generation of an endogenous immune response as well as in the context of vaccination. We demonstrate that dendritic cells isolated from the lymph nodes of VEGF-Trap treated animals exhibit enhanced expression of costimulatory molecules such as CD80, CD86, and MHC class II. Therefore, dendritic cells from VEGF-Trap treated animals have the capacity to be better antigen presenting cells. Likewise, CD8 T cells isolated from VEGF-Trap treated animals have a demonstrable decrease in exhaustion markers. CD8 T cell exhaustion has been shown to be mediated by the immune suppressive tumor microenvironment, and VEGF is likely one way this is accomplished (14). Much like through the use of checkpoint blockade therapy, if the signals that result in CD8 T cell exhaustion can be prevented through VEGF blockade, the CD8 T cells that infiltrate the tumor will be better able to kill tumor cells. Furthermore, these findings are not limited to just cancers of the CNS. Anti-angiogenic therapy is used in colorectal cancer and breast cancer treatment (15, 16). Likewise, immunotherapies are being tested in both of these types of cancer (17, 18). Therefore, our findings may be extrapolated to other combination strategies involving an immune therapy and anti-angiogenic therapy.

Here we show that anti-angiogenic therapy is not only a useful strategy to improve quality of life for patients diagnosed with GBM, but it may be a tractable approach to enhance immunotherapies. This study also builds upon our previous publication in which it was determined that combination

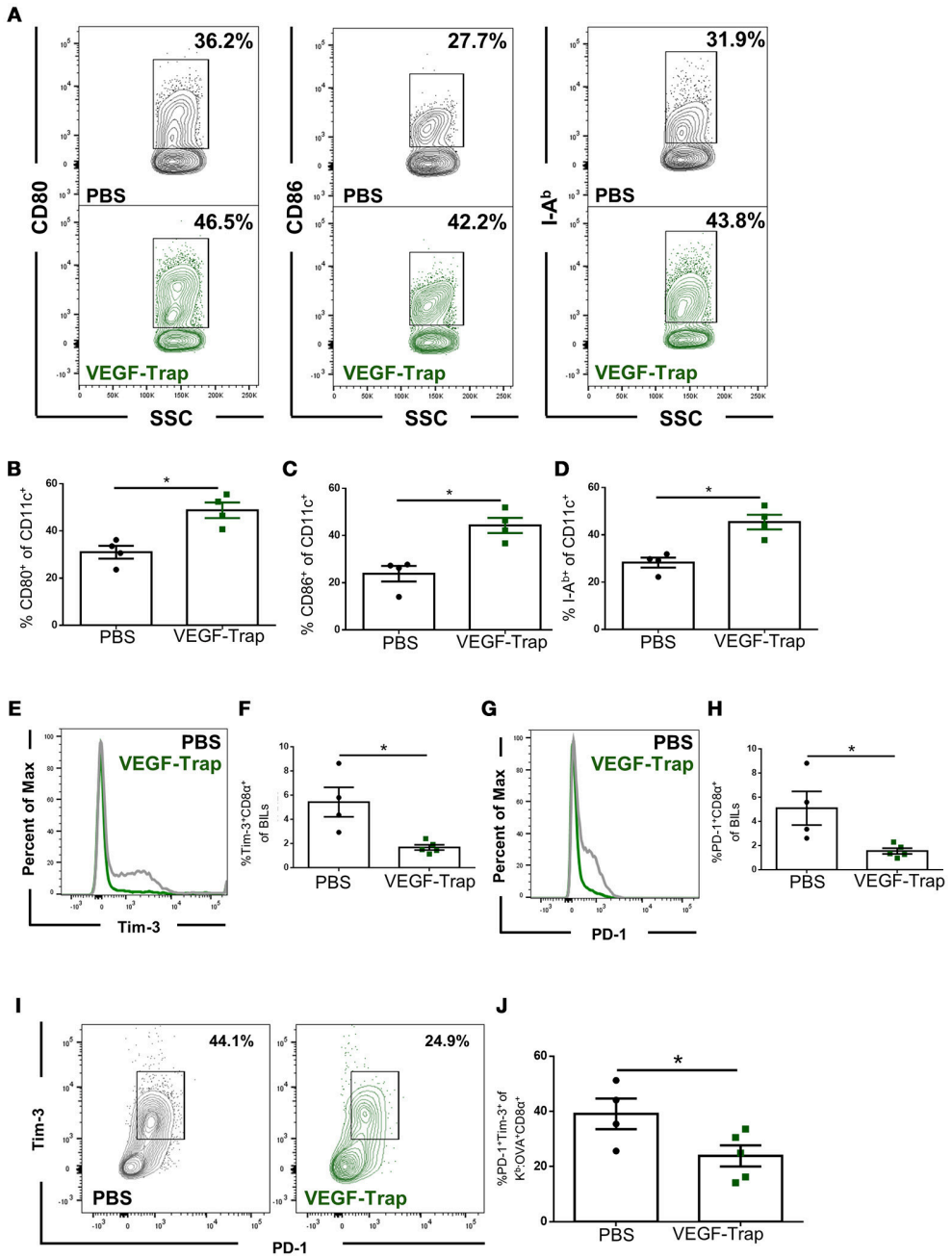

FIGURE 2 | CD8 T cells and dendritic cells isolated from the brain of VEGF-Trap treated GL261-quad cassette bearing mice express a more functional phenotype. GL261-quad cassette bearing animals were treated with PBS ($N = 4$) or VEGF-Trap ($N = 5$) 2 weeks post-tumor implantation. Animals were sacrificed 30 days after tumor implantation and brain infiltrating leukocytes (BILs) were assessed by flow cytometry. **(A)** Representative images of CD11c$^+$ cells isolated from the brain assessing expression of costimulatory markers. VEGF-Trap treatment results in increased expression of CD80 **(B)**, CD86 **(C)**, and I-A^b MHC Class II **(D)**. Representative flow plots **(E)** and quantification **(F)** show reduction in the proportion of Tim-3$^+$ CD8 T cells in the CNS of GL261-quad cassette bearing animals. Representative flow plots **(G)** and quantification **(H)** show a reduction in the proportion of CD8 T cells expressing PD-1 in the brain. A reduction in PD-1$^+$Tim-3$^+$ double positive CD8 T cells was also observed **(I,J)**. Error bars represent mean ± SEM. *$p < 0.05$. Side Scatter (SSC) was included as a measure of granularity.

therapy of picornavirus vaccination plus antiangiogenic treatment extended that lifespan of mice harboring GL261 gliomas (5). Therefore, we contend that we have identified another candidate for the family of checkpoint blockade treatments. VEGF blockade should be considered in pre-clinical models of immunotherapies to dually normalize the vasculature and enhance tumor antigen-specific CD8 T cell responses.

AUTHOR CONTRIBUTIONS

CM, RK, KA, RI, KP, and AJ: Conceptualization; CM, RK, KA, and AJ: Formal analysis; AJ: Funding acquisition; CM, RK, KA, FJ, and AJ: Investigation; CM, RK, and AJ: Methodology; AJ: Project administration; JA, RI, and KP: Resources; AJ: Supervision; CM: Visualization; writing–original draft; CM, RK, KA, and AJ: Writing-reviewing and editing.

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Re-purposing Chloroquine for Glioblastoma: Potential Merits and Confounding Variables

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There is a growing evidence that antimalarial chloroquine could be re-purposed for cancer treatment. A dozen of clinical trials have been initiated within the past 10 years to test the potential of chloroquine as an adjuvant treatment for therapy–refractory cancers including glioblastoma, one of the most aggressive human cancers. While there is considerable evidence for the efficacy and safety of chloroquine the mechanisms underlying the tumor suppressive actions of this drug remain elusive. Up until recently, inhibition of the late stage of autophagy was thought to be the major mechanism of chloroquine-mediated cancer cells death. However, recent research provided compelling evidence that autophagy-inhibiting activities of chloroquine are dispensable for its ability to suppress tumor cells growth. These unexpected findings necessitate a further elucidation of the molecular mechanisms that are essential for anti-cancer activities of CHQ. This review discusses the versatile actions of chloroquine in cancer cells with particular focus on glioma cells.

Keywords: glioblastoma, chloroquine, radio-sensitization, autophagy, glioma stem-like cells

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INTRODUCTION

Glioblastoma (GB) is one of the most lethal human cancers (1). Despite its rarity, GB is among the top priorities in clinical oncology due to its extremely aggressive pattern, high mortality rate and unsatisfactory efficacy of current treatments. An eventual mortality rate close to 100%, 5–years survival rate of <10%, and a median survival of only 15 months remain unimproved since the establishment of standard frontline therapy for GB in 2005 (2, 3). The current standard of care for GB is based on the "one-treatment-for-all" principle and consists of a surgical resection as complete as feasible, followed by combined treatment with hypofractionated radiation therapy and non-selective chemotherapy with DNA alkylating agent temozolomide (TMZ) followed by six cycles of chemotherapy alone (3). However, the clinical effectiveness of TMZ is rather moderate (survival benefit of 2 months compared with radiotherapy alone) and restricted to a subset of GBs (∼50%) lacking methyl-guanine-methyl-transferase (MGMT), an enzyme that removes the alkyl group from TMZ-induced O6-methylguanine DNA adducts (4). GBs re-grow inevitably after (or under) radio-chemotherapy. For recurrent GBs, there is no generally accepted standard therapy. None of the experimentally tested therapeutic options led to significant survival benefit (5). Post-treatment recurrence due to intrinsic and acquired resistance to cytotoxic treatments pose the major challenge to effective treatments of GB. The hallmark of GB's genetic landscape is the co-occurrence of multiple defects in key cancer-related pathways that use distinct mechanisms yet have

partially overlapping functions. RTK, pRb and p53 have been identified as core pathways impaired nearly universally in the majority of GBs (6). Multiplicity of genetic aberrations affecting different pathways in conjunction with the functional redundancy of affected pathways poses a challenge for mono-targeted therapies for GB. Adding a further level of complexity, there is considerable heterogeneity of cell types constituting GBs.

Development of multi-targeted therapeutic approaches using a combination of drugs or a drug with a broad spectrum of targets might provide the solution to overcome intrinsic and acquired resistance of GBs to cytotoxic treatments.

CHLOROQUINE: A CONVOLUTED PATH FROM MALARIA TO CANCER TREATMENT

Chloroquine (CHQ) is a well-known antimalarial that has recently attracted considerable attention for its anti-neoplastic activites. Application of CHQ for cancer treatment is an example of drug re-purposing, a strategy for identifying new therapeutic indications for drugs that have initially been developed for different medical applications (7). Synthetized at I.G. Farbenindustrie Bayer A.G. Laboratories (Elberfeld, Germany) in 1934, CHQ has been the drug of choice for malaria treatment for several decades till its role as anti–malarial has diminished due to the emergence of CHQ–resistant strains of the malaria parasite. One of the early encounters of anti-neoplastic effects of CHQ have been made during an anti-malaria trial launched by WHO in North Africa in the 1970's. It was noticed that the incidence of Burkitt's lymphoma dropped profoundly in the CHQ-treated population during the trial but returned to the basal level after the trial has been discontinued (8). This unexpected observation has remained unfollowed until a series of experimental studies reported on anti–neoplastic effect of CHQ in different types of cancer cells (9). In particular, the potential of CHQ to sensitize neoplastic cells to radiation and some other types of chemotherapy has been emerging as an approach to target treatment-refractory cancers including GBs. Currently, 17 clinical studies have been initiated to test the effects of CHQ as adjuvant treatment for different types of cancer including GB (**Table 1**) (12). Interest to CHQ as an adjuvant treatment for GB was sparked by the initial observation that addition of CHQ to standard therapy leads to a significant prolongation of survival in patients with GB (17) (10). After the initial demonstration that CHQ potentiates therapeutic effects of standard therapy in a double-blinded clinical trial (Phase III) involving a cohort of 30 patients with newly diagnosed GB, (10) further encouraging results have been reported in a case study with 5 patients suffering from recGB treated with CHQ and re-irradiation (18). These observations are coherent with the results from experimental studies indicating that chloroquine can potentiate cytotoxicity of TMZ and ionizing radiation in glioma cells (19–22).

CHQ (7-chloro-4-(4-diethylamino-1-methylbutylamino)-quinoline) is a small, lipophilic, amphiphilic and weakly basic tertiary amine with pK_as of 8.4 and 10.2 (12, 23). At the physiological pH of 7.4, CHQ is unprotonated and highly membrane-penetrating (12). Once inside the cell, CHQ accumulates in acidic compartments and becomes protonated. As a consequence, it raises the intra–organellar pH and affects the activity of endosomes, lysosomes, autophagosomes, and autophagolysosomes (23). Owing to its lysosomotropic properties, CHQ accumulates primarily in the lysosome, where the increase of the lysosomal pH leads to a blockage of the lysosome-autophagosome fusion, a critical event during the late stage of autophagy (24). Good solubility and rapid absorption are attractive pharmacological properties of CHQ. It is rapidly absorbed when administered orally, but sub cutaneous, intra muscular, and rectal administrations are likewise possible (25).

CHQ can elicit an array of distinct biological responses in the CNS, depending on the dose and cell type. The lowest threshold of CHQ concentrations to induce neuronal death *in vitro* is around 20 μM (26, 27). Similar values for cytotoxic concentrations of CHQ were found in normal astrocytes (28) or neoplastic cells derived from astrocytic tumors (29, 30). However, at concentrations of 10 μM or lower, CHQ elicits neuroprotective effects in the context of oxidative damage (31). Thus, various functional outcomes can be elicited by CHQ depending on the cell type, particular pathophysiological condition, dose of the drug and treatment context. While there is an abundance of information about safety and tolerability profiles of CHQ in the context of non–cancer pathologies, CHQ application for cancer treatment will require establishing tolerability ranges in cancer patients and at cancer-relevant doses. This consideration is of special importance in the context of brain tumors, which are protected by the blood brain barrier. A phase I/II trial addressing the effects and feasibility of escalating CHQ doses for GB treatment found that CHQ doses used for treating rheumatoid arthritis may not be sufficient to effectively inhibit autophagy when used in combination with TMZ and radiation in patients with GB (32).

MOLECULAR MECHANISMS OF ANTI-NEOPLASTIC ACTIVITIES OF CHLOROQUINE

The mechanisms of radio- or chemo sensitization mediated by CHQ in glioma cells are not entirely understood. Modulation of the autophagic response is by far the most intensively investigated mechanism of CHQ in non-neoplastic and cancer cells. Until recently, the generally accepted view was that inhibition of autophagy is the major route of cancer cell death induced by CHQ (33). Indeed, several lines of experimental evidence suggest the importance of autophagic inhibition as the underlying mechanisms of radio-sensitization by CHQ. Knock down of beclin-1 or pharmacological inhibition of autophagy by 3-methyladenine or interference with autophagy-promoting signaling mediated through the PI3K/Akt (20) or EGFR signaling (34) have been shown to impair the radio/chemo-sensitizing ability of CHQ in glioma cells. However, the seemingly well delineated causative relationship between CHQ effects on autophagy and tumor suppression has recently been

TABLE 1 | Summary of clinical trials testing chloroquine in GBs.

Study ID	Phase	Patient group		Treatment	Outcomes
		Age	Diagnosis		
NCT00224978	III	18–65	First/second recurrent or relapsed GB (WHO stage = IV) in one hemisphere	Carmustine + radiotherapy + placebo vs. Carmustine + radiotherapy + chloroquine	- Increase OS from 11 to 24 months - No statistical significance - Well tolerated (10, 11)
NCT03243461	III	3–18	Untreated pediatric high-grade glioma (WHO stage ≥ III)	Temozolomide + radiotherapy + valproic acid vs. Temozolomide + radiotherapy + chloroquine	*Estimated study start: Feb. 2018* (12, 13)
NCT02432417	II	18–70	Newly diagnosed IDH wild-type GB (WHO stage = IV)	Radiotherapy + chloroquine	*Estimated study start: Jan. 2020* (12, 14)
NCT02378532	I	≥ 18	Newly diagnosed GB (WHO stage = IV) and confirmed MGMT and EGFRvIII status	Temozolomide + Radiotherapy + chloroquine	*Currently recruiting* (12, 15)
NCT01727531		≥18	Solid primary tumor and at least one brain metastasis	Whole-brain radiotherapy + chloroquine	*No results published* (16)

challenged by some very surprising findings coming from the pharmaceutical oncology field. Nearly simultaneously, research teams from AstraZeneka, Novartis and Pfizer have provided compelling evidence that tumor–suppressing effects of CHQ are independent from its autophagy-inhibiting activities (35, 36). Intriguingly, CHQ-induced cell death was found to be related with the inhibition of cholesterol biosynthesis by autophagy-related pathways but not with autophagy inhibition *per se* (36). These findings prompt to hypothesize that modulation of the cell metabolism might be one of the mechanisms underlying the anti-neoplastic efficacy of CHQ, which affects a range of metabolic processes including the amino acid metabolism, (37) glucose metabolism (38) and mitochondrial metabolism (39). Interestingly, CHQ potently inhibits glyconeogenesis, (40) which is a compensatory mechanism supporting the survival of cancer cells bearing mutations in the isocitrate dehydrogenase (*IDH*) gene. *IDH1/2* genes code for metabolic enzymes that interconvert isocitrate and α-ketoglutarate. Loss of catalytic activity caused by point mutations in *IDH1/2* genes leads to a decrease in α-ketoglutarate and increased production of *D*–2–hydroxyglutarate (41, 42). In glial tumors, *IDH1/2* mutational status is regarded as one of the most important diagnostic and prognostic biomarkers (43, 44). Point mutations in *IDH1/2* associate with longer survival and are found in about 80% of anaplastic astrocytoma (WHO Grade III) and secondary GBs (GBs that progress from lower grade gliomas), but only rarely (< 10%) in primary GBs (GBs that occur without precursor lesions). Although the relationship between *IDH1/2* mutational status and sensitivity to CHQ in gliomas remains to be established, the recently proposed hypothesis that *IDH1/2* mutations might be predictive of the efficacy of CHQ in gliomas seems plausible (42). Recently launched clinical studies aiming to validate the association between *IDH1/2*-mutated molecular subtype and sensitivity to CHQ will test this hypothesis (45).

FUNCTIONAL PLEIOTROPY OF CHLOROQUINE: THE BALANCE OF GOOD AND EVIL

The diversity of CHQ effects reflects the functional pleiotropy of its molecular targets, which include multi-functional factors as transcription factor NF-κB, (46) or DNA damage-inducible factors like the ataxia telangiectasia mutated (ATM) kinase (47) and its downstream target tumor suppressor p53 (48). A broad versatility of responses that can be mediated by CHQ can be exemplified by its effects on p53 whose functional status is an important factor determining the ultimate outcome from CHQ treatment in cancer cells. This, in fact, is not surprising considering the nodal position of p53 in several regulatory hubs that govern diverse cellular responses to different types of stress (49, 50). The ability to trigger distinct effects such as cell survival or cell death is the key fundamental of p53 function as the "guardian of the genome" (51). Amidst a great multitude of factors influencing the choice between pro-survival and death-promoting activities of p53, (52) the ability to repair DNA damages is essential for promoting cell survival after cell injury. Activation of p53 signaling upon DNA damage can lead to a transient arrest of the cell cycle, enabling DNA repair, or cell death, if the extent of DNA damage exceeds the repair capacity of the cell. Whereas the ability of CHQ to induce p53-dependent apoptosis has been well-documented (22, 27, 29), the mechanism of p53 activation by CHQ remains elusive. In the canonical DNA damage response (DDR), activation of the ATM/Chk1/p53 signaling is the initial event in a signaling cascade triggered by DNA-double strand breaks (53). However, CHQ does not cause direct DNA damage. It has been proposed that topological perturbations in the chromatin structure caused by CHQ intercalation into the DNA helix (54–57) may be sensed by ATM leading to its activation by autophosphorylation

(47). Alternatively (or in addition) to its direct effects on DNA topology, CHQ can cause DNA breakage through an indirect mechanism involving mitochondrial damage (58). Considering that both ATM and p53 are sensitive to oxidative stress, (59, 60) these findings indicate that activation of the ATM-p53 signaling by CHQ might be triggered by oxidative DNA damage. Interestingly, while activating key mediators of DDR, CHQ has an intrinsic repair-inhibiting activity manifest in different types of normal and neoplastic cells *in vitro* (30, 58) and *in vivo* (61). Although the exact mechanisms of CHQ–mediated inhibition of DNA repair remain unknown, they are likely to reflect the causative relationship between impaired autophagy and deficient DNA repair (62). It is tempting to hypothesize that conflicting signals generated through the dual ability of CHQ to activate key mediators of DDR and to suppress DNA repair, play a role in shifting the balance in favor of cell death. Potentially conflicting signals can also emanate from the p53 transcriptional response induced by CHQ. p53 activation leads to transcriptional up-regulation of Bax1, which is indispensable for CHQ–induced apoptosis, (27) but also induces a battery of genes that promote cell survival through the activation of autophagic response (52).

The concurrent activation of cell death and pro-survival pathways through the modulation of autophagy might represent yet another death-survival axis regulated by CHQ: On the one hand, CHQ can activate cell death through the lysosome-initiated apoptosis via cathepsin signaling (63, 64). On the other hand, CHQ leads to the accumulation of a multifunctional protein chaperone p62 (also known as sequestome-1, SQSTM-1), whose expression is associated with increased cell proliferation, tumor growth and cytotoxic resistance in different types of human cancers (65). In gliomas, p62 expression correlates with the tumor grade and shorter survival (66, 67). As p62 is an autophagy adaptor targeted for degradation through autophagic clearance, autophagy inhibition by CHQ leads to the increase of the p62 protein levels (68). One of the mechanisms underlying pro-tumor activities of p62 relies on its ability to activate NF-κB, a key pathway regulating cell survival and proliferation. Augmented NF-κB signaling is linked to poor prognosis and treatment resistance in gliomas (69, 70). Moreover, there is evidence that activation of the p62/NF-κB signaling by CHQ may be further amplified through a positive feedback loop whereby CHQ-induced p62 activates NF-κB, which in turn activates the expression of p62 (71). Thus, inhibition of autophagy by CHQ can activate not only the lysosome-mitochondria death pathway, (63, 64) but also survival–promoting signaling mediated through the p62/NF-κB feedback loop (71). Considering that ATM is essential for the function of both p53 and NF-kB proteins,

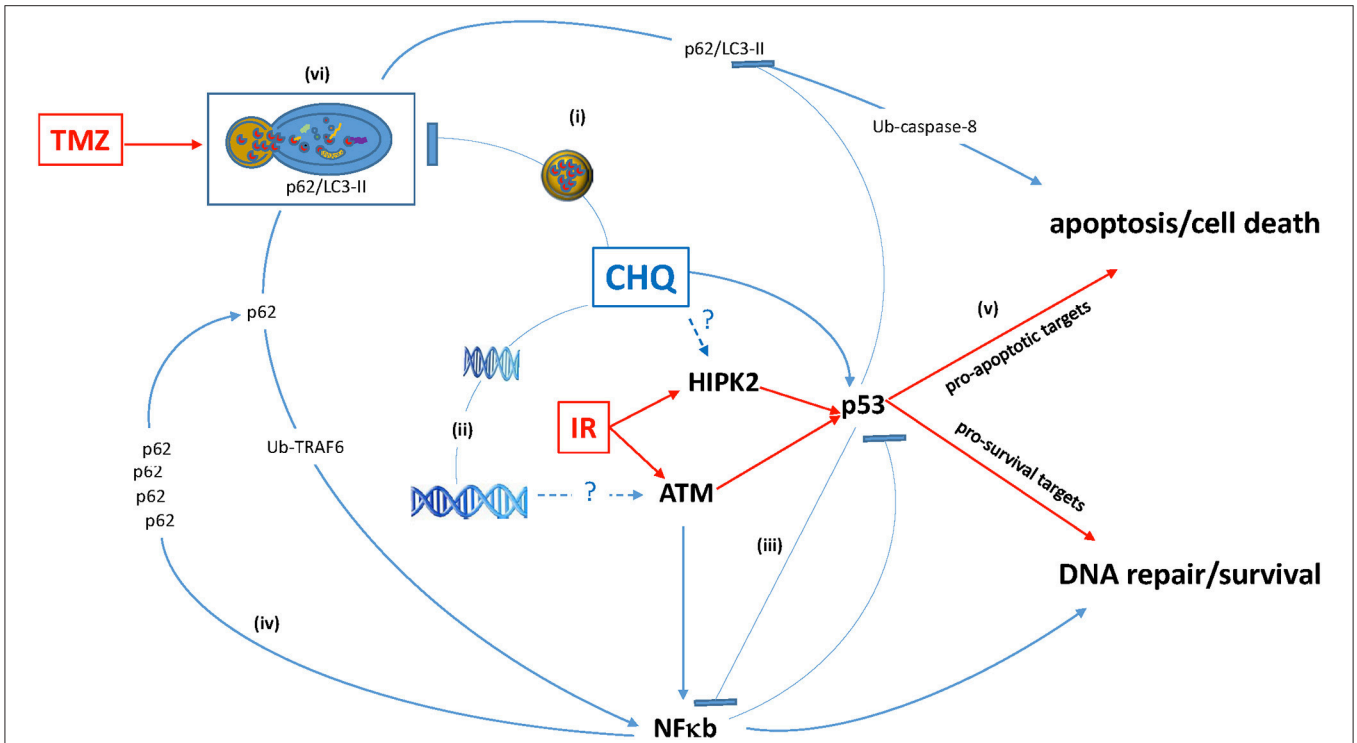

FIGURE 1 | Antagonistic pleiotropy of multifunctional hub proteins modulated by CHQ. (i) CHQ accumulation in the lysosome inhibits the lysosome-autophagosome fusion and impairs degradation of proteins including the ubiquitin (Ub)-binding protein p62 and its binding partner pro-apoptotic LC3-II. (ii) CHQ intercalates into the DNA helix and cause relaxation of chromatin structure, which may be the mechanism of ClQ-mediated activation of a DNA damage-inducible kinase ATM. (iii) CHQ modulates activities of pleiotropic transcription factors p53 and NF-κB and may influence cross-talk between these pathways. (iv) p62 functional duality and positive p62 / NF-κB feedback loop. (v) Augmentation of pro-apoptotic activities of p53 may be a possible mechanism of CHQ-mediated radiosensitization. (vi) Autophagy inhibition by ClQ may counteract autophagic activation by TMZ and thereby sensitize glioma cells to chemotherapy.

which often act in an antagonistic way in the regulation of cell survival, (72) and that CHQ modulates activities of all three factors (**Figure 1**), it is conceivable that p53 status is an important factor in determining cell fate in response to CHQ treatment.

The dichotomy of cellular responses elicited by CHQ is also manifest in its inhibitory effects on the inflammatory response which might be particularly important considering the tumor microenvironment. Normalization of the tumor vasculature has been implicated as a potential mechanism underlying the ability of CHQ to increase the efficacy of chemotherapeutic drugs, by facilitating their delivery to the tumor mass (73, 74). Indeed, there is evidence that CHQ normalizes the tumor vasculature through the reduction of vessel density, improvement of cell alignment, formation of tight junctions and promotion of quiescent phenotype of endothelial cells (73, 74). However, CHQ has also been shown to have pro-inflammatory effects in some types of cells. Within the CNS, CHQ inhibits pro–inflammatory cytokines in microglial cells, but not in astrocytes, in which it induces inflammatory cytokines through the activation of NF-κB signaling (46). Considering that GBs are tumors of astrocytic origin, their responses to CHQ may resemble those observed in astrocytes.

Thus, the ultimate outcome of CHQ treatment is likely to be determined by the intricate balance between activities of pleiotropic pathways involved in the regulation of autophagy, DDR and apoptosis/cell death (**Figure 1**).

CHLOROQUINE AS POTENTIAL ANTI-CANCER DRUG: UNSOLVED QUESTIONS AND CONFOUNDING ISSUES

Despite recent advances in the understanding of molecular mechanisms of anti-tumor effects of CHQ, a number of issues remain unsolved. One confounding factor is that experimental models used for investigating the effects of CHQ may not fully recapitulate distinctive characteristics of treatment-resistant GB. The current paradigm of therapeutic resistance in GB is centered on so-called glioma stem-like cells (GSCs). GSCs are considered the most clinically relevant type of glioma cells driving GBs propagation before and after therapy (75). It has been shown that GSCs possess an augmented DNA damage response (DDR), (76) which renders them capable of surviving cytotoxic treatments that are otherwise effective in killing non-stem glioma cells (76–78). In conjunction with augmented DDR, radiation-induced activation of anti-death and autophagic responses make important contributions to GSCs ability to escape from the cytotoxic effect of radiation (79, 80). Most of the existing studies addressing the effects of CHQ in glioma cells have used conventional serum-dependent cell lines that lack stemness properties and/or poorly recapitulate characteristic features of human GBs. For example, the human glioma cell line U87MG, which has been widely used as an experimental model for investigating biological responses mediated by CHQ (21, 22, 29, 30, 81, 82) does not reproduce certain characteristic traits of GBs such as an invasive tumor phenotype, intra-tumoral heterogeneity and high degree of intrinsic radio-resistance. Considering that GSCs are fundamentally distinct from non-stem glioma cells, it is conceivable that their responses to CHQ might also differ from those operating in the latter ones. Further underscoring this notion, activation of the p53 signaling by CHQ seems to lead to different outcomes in GSCs or non–stem glioma cells. In non-stem glioma cells with wtp53, p53-dependent apoptosis is a profound response to high concentrations of CHQ ($\geq$20 μM) either applied alone or combined with other treatments (22, 29, 30). In contrast, GSCs with functional p53 do not activate apoptosis, but undergo predominantly a G_1 arrest in response to similar CHQ concentrations (20).

Furthermore, the threshold of CHQ concentrations required for inducing cell death in the experimental setting ($\sim$20 μM) is considerably higher than clinically acceptable doses of CHQ ($\sim$5 μM). Therefore, the potential therapeutic value of clinically acceptable doses of CHQ for GB treatment requires further validation. Clarifying this question is particularly important considering the results of a phase I/II trial addressing the feasibility of dose escalation for CHQ treatment of GB (32). It was found that CHQ doses used for treating rheumatoid arthritis may not be sufficient to effectively inhibit autophagy when used in combination with TMZ and radiation in patients with GB (32). Likewise, CHQ potential in sensitizing glioma cells to radiation, observed under experimental conditions (single treatment with 10 Gy) (20) needs to be reproduced under clinically relevant conditions, applying hypofractionated radiation (multiple fractions of 2.0–2.5 Gy).

CONCLUSION

The chemo- and radio-sensitizing effects of CHQ observed under experimental conditions warrant further explorations of CHQ potential as an adjuvant treatment for GB. In order to better define the potential benefits of using this drug as an adjuvant treatment for GB, the remarkable diversity of outcomes that can be elicited by CHQ need to be considered.

AUTHOR CONTRIBUTIONS

PW: literature analysis, manuscript writing and preparation. SK: manuscript revision and preparation. EK: concept, literature analysis, manuscript writing, preparation and final approval.

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A Critical Overview of Targeted Therapies for Glioblastoma

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Over the past century, treatment of malignant tumors of the brain has remained a challenge. Refinements in neurosurgical techniques, discovery of powerful chemotherapeutic agents, advances in radiotherapy, applications of biotechnology, and improvements in methods of targeted delivery have led to some extension of length of survival of glioblastoma patients. Refinements in surgery are mentioned because most of the patients with glioblastoma undergo surgery and many of the other innovative therapies are combined with surgery. However, cure of glioblastoma has remained elusive because it requires complete destruction of the tumor. Radical surgical ablation is not possible in the brain and even a small residual tumor leads to rapid recurrence that eventually kills the patient. Blood-brain barrier (BBB) comprising brain endothelial cells lining the cerebral microvasculature, limits delivery of drugs to the brain. Even though opening of the BBB in tumor core occurs locally, BBB limits systemic chemotherapy especially at the tumor periphery, where tumor cells invade normal brain structure comprising intact BBB. Comprehensive approaches are necessary to gain maximally from promising targeted therapies. Common methods used for critical evaluation of targeted therapies for glioblastoma include: (1) novel methods for targeted delivery of chemotherapy; (2) strategies for delivery through BBB and blood-tumor barriers; (3) innovations in radiotherapy for selective destruction of tumor; (4) techniques for local destruction of tumor; (5) tumor growth inhibitors; (6) immunotherapy; and (7) cell/gene therapies. Suggestions for improvements in glioblastoma therapy include: (1) controlled targeted delivery of anticancer therapy to glioblastoma through the BBB using nanoparticles and monoclonal antibodies; (2) direct introduction of genetically modified bacteria that selectively destroy cancer cells but spare the normal brain into the remaining tumor after resection; (3) use of better animal models for preclinical testing; and (4) personalized/precision medicine approaches to therapy in clinical trials and translation into practice of neurosurgery and neurooncology. Advances in these techniques suggest optimism for the future management of glioblastoma.

Keywords: brain cancer, glioblastoma, personalized therapy, targeted delivery, malignant glioma, oncolysis, cancer immunotherapy, gene therapy

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INTRODUCTION

Glioblastoma is the most lethal primary brain tumor. Since the first surgical resection of a malignant astrocytoma was done in 1884, attempts to cure primary cancer of the brain by resection have been made since the start of modern neurosurgery more than a century ago. Since my first encounter with a case of glioblastoma at the start of my neurosurgical career

60 years ago, I have seen improvements in surgical technique, radiotherapy, and chemotherapy increase the median length of survival of patients only from 6 to 15 months. While standard of care for newly diagnosed glioblastoma include maximal resection, followed by radiation therapy given concomitantly with temozolomide followed by adjuvant TMZ chemotherapy, median time to progression is 6 months and overall median survival 15 months. Almost all tumors recur with more aggressive form of tumors and there is no standard of care for recurrent GBM. During the past two decades, applications of biotechnology and several innovative approaches have been tested both in the laboratory and clinical trials; their impact on survival is negligible because glioblastoma remains incurable. Currently, most of the projects for advancing therapy for glioblastoma listed in **Table 1** are focused on targeted delivery to the tumor and aim to selectively eradicate it without damaging the surrounding brain. Nanobiotechnology plays an important role in targeted delivery of therapy to glioblastoma and will be discussed in a separate section. Various innovative therapies will be critically evaluated including examples from the 1269 clinical trials listed at the US government web site[1]. To start with I will review refinements in surgery including combination with other innovations.

Although several innovations in treatment of glioblastoma have been introduced during the past three decades, evaluation of their efficacy is mostly limited to observation of progression free survival and overall survival. While CT and MRI measure tumor size, metabolic, and other changes at molecular level in response to treatment can be better indicators of response in the absence of an initial reduction of size. Conventional preclinical studies evaluating experimental drugs in cell lines *in vitro* do not recapitulate conditions of *in vivo* tumor microenvironment; even clinical trials conducted in mixed population are not adequate to realize impact of an experimental drug.

REFINEMENTS IN SURGERY

There have been considerable refinements in surgical techniques. In the pre-brain imaging (CT and MRI) era, preoperative diagnosis with pneumoencephalography (which showed mostly the location and mass displacement and cerebral angiography (crude vasculature patterns and avascular areas of necrosis) raised suspicion of malignancy, which had to be confirmed by histological examination. Compared to modern refinement, neurosurgery of glioblastomas 60 years ago was crude as compared to meticulous dissection of benign brain tumors because it was considered a palliative procedure to relieve intracranial pressure and prolong life for a few months with resignation to the fact that the patient was going to die.

Apart from providing adequate sample for histological analysis and removal of a mass to reduce raised intracranial pressure, excision of a tumor provides a cavity for application of local therapies for destruction of residual tumor mass and prevention of recurrence. Maximal removal that is consistent with neurological preservation is usually carried out and has been shown to prolong survival but does not reduce mortality. Radical

TABLE 1 | Innovative therapies for glioblastoma.

New chemotherapeutic agents
Innovations for the delivery of anticancer drugs
• Intraoperative polymer implants in residual tumor bed containing anticancer drugs
• Magnetic cationic microsphere delivery system
• Stereotactic implantation of microspheres containing anticancer drugs
• Lipid-coated microbubbles as a delivery vehicle
Strategies to overcome the blood-tumor barrier for delivery of chemotherapy
• Intra-arterial chemotherapy
• Nanotechnology-based controlled delivery through blood-brain barrier
Chemotherapy sensitization
• Hyperbaric oxygen
• Photodynamic therapy for chemosensitization
Innovations of radiotherapy
• Boron neutron capture therapy
• Brachytherapy: implantation of interstitial radiation-emitting seeds into the tumor
• Enhancing effect of radiotherapy by hyperbaric oxygen
• Radiosurgery: enhancing effects of ionizing radiation
Inhibition of tumor growth
• Receptor tyrosine kinases as a signal blocker to hinder the growth of gliomas
• Telomerase inhibition
• Antiangiogenesis therapy
• Polyinosinic-polycytidylic acid given intramuscularly
• Thalidomide, systemic administration
• Targeting epidermal growth factor receptor-mediated metabolic pathway
Local destruction of tumor
• Genetical modified bacteria for tumor-specific lysis
• Hyperthermia
• Interleukin-4 fusion toxin injection
• Intraoperative photodynamic therapy
• Oncolysis by genetically modified viruses
• Tumor treating fields
Immune therapy
• Brain tumor vaccines
• Immune checkpoint blockade
• Monoclonal antibodies
• Radiolabeled antibodies injected directly into the tumor
• Recombinant interleukin-2 and lymphokine activated killer cells
• Recombinant immunotoxins specific for epidermal growth factor receptor
Cell therapy
• CAR-T cell therapy
• Encapsulated cells engineered to produce therapeutic molecules
• Glioma stem cell therapy
• Grafting of stem cells producing therapeutic molecules, such as IL-4 gene
Gene therapy
• Apoptosis-inducible FADD/MORTI gene transfer
• Deoxycytidine cDNA as sensitizer for cytotoxic effect of cytosine arabinoside
• Direct intratumoral injection of genetically modified neurotrophic viruses
• E. coli gpt gene delivery to sensitize glioma cells to prodrug 6-thioxanthine
• Insertion of drug sensitivity genes
• Suicide gene therapy: herpes simplex virus-thymidine kinase
• Viral vectors containing radiation-inducible promoters
Gene suppression
• Antisense
• RNAi

extirpation of the tumor is often aimed at but is not possible due to infiltration of the tumor into the surrounding brain.

Refinements in brain imaging techniques have contributed considerably in improving planning of surgical procedure. Intraoperative imaging, particularly MRI and use of 5-aminolevulinic acid helps in defining the margins of glioblastoma and for maximizing the extent of resection. According to a systematic review of randomized clinical trials, the impact of image-guided surgery on survival and quality of life are uncertain (1).

Techniques such as cortical mapping, fluorescence-guided surgery, and intraoperative mass spectrometry are routinely used in the operating room for brain tumor resection. Optical coherence tomography, still in experimental stage, may fill the need for a non-invasive approach for real-time distinction between tumor and normal brain. Postoperative imaging provides a useful baseline for size of residual tumor and further evaluation of response to adjunctive therapies.

One of the major refinements in neurosurgical techniques was the introduction of operating microscope, which had a remarkable impact on improving cerebrovascular surgery. It provides better visualization of distinction between the tumor and the normal brain to avoid damage to normal structures. Other refinements in tools for removing tumor tissue include ultrasonic aspiration to minimize trauma and laser vaporization to reduce bleeding and destroy cells in tumor bed by thermal effect. The FDA-approved NeuroBlate® System (Monteris Medical) is a minimally invasive robotic laser thermotherapy tool for glioblastoma that is being studied in the prospective multicenter clinical trial #NCT02392078 due for completion in 2020. The NeuroBlate System is used with MRI to provide a real-time image of a patient's brain for guiding the surgeon. This device is meant for glioblastomas that are not suitable for routine surgery due to their location. The aim is improvement in quality of life of the patient rather than prolonging survival.

Combination of Surgery With Other Innovations

Surgery is supplemented with innovations in chemotherapy and radiotherapy that will be described separately. In addition to systemic chemotherapy and postoperative cranial radiotherapy, surgery provides an opportunity to apply some therapies during the procedure. Examples are implantation of chemotherapeutic agents and photodynamic therapy.

Implantation of Wafers for Local Delivery of Chemotherapeutic Agents

Gliadel wafers consist of biodegradable polymer containing the chemotherapeutic agent BCNU (bis(bis-chloroethylnitrosourea), which are implanted during surgery at the tumor site diffuse into the surrounding tissue and provide therapeutic dose of BCNU locally. A systematic review of phase III trials has shown an increased overall survival from sequentially combining Gliadel wafers with radiotherapy and systemic temozolomide (2).

Photodynamic Therapy

Photodynamic therapy (PDT) is based on release of singlet oxygen with toxic effects on the tumor that occurs when a photosensitizer at the tumor site is exposed to laser light of a certain wavelength. PDT for cancer using talaporfin sodium with laser is approved in Japan. Progression-free survival of 1 year and an overall survival of 2 years and 8 months was shown in an open clinical trial of intraoperative PDT in glioblastoma (3). A prospective study on patients with glioblastoma used fluorescence from talaporfin sodium exposed to laser with wavelength of 600 nm for intraoperative diagnosis of malignant glioma, which was followed by PDT at laser wavelength of 400 nm (4). According to the authors, fluorescence from malignant tissues was at least partially due to the involvement of microvascular structures. A pilot clinical trial, the INDYGO (INtraoperative photoDYnamic Therapy for GliOblastomas) is planned as an addition to the standard of care of glioblastoma as a requirement prior to a randomized clinical trial (5). In this clinical trial, PDT treatment following fluorescence-guided surgical resection will involve delivery of 5-aminolevulinic acid (ALA) processed by the cells to generate a photosensitizer protoporphyrin IX (PIX).

ROLE OF IMPROVEMENTS IN DIAGNOSTIC TECHNOLOGIES

Improvements in diagnostic technologies have played an important role in understanding the genetics and molecular biology of glioblastoma as a basis for developing therapeutics and evaluating their efficacy. Molecular diagnostics is used for discovery of biomarkers of brain tumors and some biomarkers can serve as diagnostics as well as targets for developing therapies, which explains the overlap between the two (6).

Molecular Diagnostics

Molecular analyses of genetic alterations in astrocytomas have been carried out to identify pathways leading to glioblastoma. Molecular diagnostics is an important basis for developing personalized therapy of glioblastomas. The most frequent single-gene alterations are mutation of the p53 tumor suppressor gene, amplification of the epidermal growth factor receptor (EGFR), homozygous deletion of the CDKN2a gene, and mutation of the PTEN (phosphatase and tensin homology) gene on chromosome chromosome 10q23. While most frequent chromosomal alterations are loss of chromosomes 10 and 9p and the gain of chromosomes 7 and 19 and were discovered by studies involving high-resolution comparative genomic hybridization (7), The assays to assess these genetic or chromosomal alterations have emerged as molecular biomarkers of glioblastoma as well.

Magnetic resonance spectroscopy (MRS) mediated detection of oncometabolite 2-hydroxyglutarate (2HG) produced in tumors harboring mutation in the isocitrate dehydrogenase1 (IDH1). A prospective imaging study showed positive correlation between 2HG concentrations tumor cellularity, which differ significantly among high- vs. low-grade gliomas (8). These data

provide rationale for adding 2HG MRS into clinical practice IDH-mutated gliomas.

Cell-free DNA shed by cancer cells is a rich source of tumor-specific biomarkers, but DNA from CNS tumors cannot usually be detected in the blood. However, using patient-specific mutations as biomarkers, detectable levels of CSF tumor DNA were identified in 74% of brain tumors such as glioblastomas that abutted on CSF spaces (9).

Alterations in EGFR, PDGFRA, PTEN, TP53, NF1, CDKN2A/B, and TERT promoter mutations are commonly found in primary glioblastoma, while the prevalence of IDH mutations is high in grades II and III astrocytomas, secondary glioblastoma, oligodendrogliomas, and oligoastrocytomas (10). Survival benefit from surgical resection varies according to IDH1 genotype in glioblastomas, as better prognosis is observed in the IDH1 mutant subgroup following maximal surgical resection (11). Amplification of receptor kinases such as EGFR and PDGFRA (platelet-derived growth factor receptor alpha) are relevant to the prognosis of glioblastoma, and both may coexist in a tumor. Inhibition of both EGFR and PDGFR is essential for eliminating kinase pathway activity in glioblastomas with mixed cell types (12). In view of the cytogenetic heterogeneity of glioblastoma, stratification for prognosis should take into consideration cytogenetic alterations involving chromosomes 7, 9, and 10 which are the most frequent alterations (13).

There is a considerable interest in next-generation sequencing-based technology as it allows comprehensive mapping of genetic alterations such as single nucleotide polymorphism (SNP), fusions, and copy number variations and the epigenetic landscape of DNA methylation in brain tumors. A customized next-generation sequencing gene panel involving 0 genes commonly altered in brain tumors have been developed for the detection of Sahm et al. (14). With the emergence of numerous therapeutic targets, this approach will be important for making decisions about treatment, and classification of brain tumors based on genetic information.

There is a need for a practical method to delineate glioblastomas from adjacent normal brain tissue during surgery. Several intraoperative imaging techniques have been developed for determining the resection margin in brain tumors; these include neuronavigation, MRI, ultrasound, Raman spectroscopy, and optical fluorescence imaging. Combined with discovery of contrast agents, both MRI and optical fluorescence imaging have improved resectability of brain tumors (15). Fluorescence-guided surgery uses preoperative oral 5-aminolevulinic acid (5-ALA) for intraoperative visualization of glioblastoma tissue and enables the neurosurgeon to differentiate tumor from normal brain for achieving a more extensive resection of the tumor as compared to that possible with use of conventional operating room light (16).

Biomarkers

There are several biomarkers of glioblastoma and those relevant to management are listed in **Table 2**. Some biomarkers can be used to control response to therapy, diagnosis, and prognosis.

ALDH1A3, an enzyme plays important role in alcohol metabolism and lipid peroxidation is a specific biomarker for glioma stem-like cells (GSCs), and cells with high expression of ALDH1A3 expression are shown to be highly tumorigenic (18). In samples of glioblastoma from patients, high expression levels of ALDH1A3 were associated with a more rapid fatal course than were tumors with low levels. Small molecule inhibitor of ALDH have been developed, GAI1, an ALDH inhibitor abated glioma sphere forming ability of GSCs in cell culture and the xenograft growth of glioblastoma cells. Thus, GA11 is can be promising therapy or sensitizing strategy for glioblastoma, and clinical trials to test therapeutic potential of GAI1 have been planned (18).

Promoter hypermethylation of *MGMT* (O6-methylguanine-DNA methyltransferase) gene have been associated with response to alkylating chemotherapy (19) is an established biomarker for the alkylation therapy of glioblastoma. The PredictMDx test is a

TABLE 2 | Classification of biomarkers of glioblastoma that are relevant to management.

Cytogenetic biomarkers
• BRAF mutation
• EGF, latrophilin and 7 transmembrane domain-containing 1 on chromosome 1 (ELTD1)
• EGFRvIII deletion
• Loss of heterozygosity (LOH) on chromosomes 1p, 19q, 17p, and 10q
• Loss of p16 tumor suppressor gene pathway
• Loss of p53 tumor suppressor gene
• MAGE-E1, a glioma-specific member of MAGE (melanoma-associated antigen) family
• Mdm2 amplification in 15% of malignant gliomas
• PTEN deletion or mutation
• RBI wild type, no mutation
Methylation profiling of brain tumors
• 06-methylguanine-DNA methyltransferase (MGMT) promoter methylation (17)
• Detection of methylation-dependent DNA sequence variation: methylSNP
• Methylation of TMS1, an intracellular signaling molecule
Protein biomarkers
• ALDH1A3 in glioma-like stem cells in glioblastoma
• CSF protein profiling: N-myc oncoprotein, caldesmon, attractin
• Receptor protein tyrosine phosphatase
• Serum protein fingerprinting: circulating exosomes containing mRNA, miRNA, and angiogenic proteins
Metabolite biomarkers detected by magnetic resonance spectroscopy (MRS)
• Choline
• Lactate
• N-acetylaspartate (diminished)
MicroRNAs (miRNAs)
Prognostic biomarkers
•14-3-3zeta positive expression
• Human telomerase reverse transcriptase (hTERT) transcripts; survival is worse in high hTERT expressors
• Isocitrate dehydrogenase-1 (IDH1) mutation status
Biomarkers of response to therapy
• Biomarkers to predict response to EGFR inhibitors
• MRI as biomarker for response of brain tumor to therapy

commercially available epigenetic assay for testing methylation status of the *MGMT* gene and has impact on decision making especially for the patients with newly diagnosed glioblastoma (20). *MGMT* promoter methylation is also a prognostic biomarker for combination therapies such as temozolomide combined with carmustine (BCNU) wafer implantation (21) and is being tested in phase II-III clinical trials (NCT02152982) of targeted therapy involving temozolomide and PARP inhibitor veliparib (22).

Advanced data mining and a novel bioinformatics were used with associative analysis to accurately identify ELTD1 (epidermal growth factor, latrophilin, and 7 transmembrane domain-containing 1 on chromosome 1) as a biomarker of gliomas in preclinical and clinical diagnosis (23). A clinical study showed that expression of 14-3-3zeta observed in ∼74.5% of patients with glioblastoma and is associated with a lower overall survival rate and median survival time than patients with no14-3-3zeta expression (24). This study shows that 14-3-3zeta positive expression is an independent prognostic biomarker of glioblastoma.

Among various biomarkers, only isocitrate dehydrogenase (IDH) mutations (prognostic), MGMT promoter methylation, and 1p/19q co-deletion (predictive) are being routinely used in clinic for glioma patients in the USA as well as the UK. More biomarkers are being tested in clinical trials, and it will be important in the future to distinguish prognostic biomarkers from predictive biomarkers to enable personalized therapeutic choices with least toxicity and better outcomes for patients with malignant gliomas (25).

Examples of biomarkers that are also targets for therapy of glioblastoma are:

- EGFRvIII amplification is targeted by EGFR vaccine rindopepimut.
- KIT amplification or mutation is target is targeted by KIT inhibitor imatinib
- PDGFRA amplification is targeted by PDGFR inhibitor dasatinib
- PTEN deletion or mutation is targeted by AKT inhibitor or mTOR inhibitor voxtalisib
- MDM2 amplification is targeted by a MDM2 inhibitor such as AMG232
- TP53 wild-type is targeted by a MDM2 inhibitor such as AMG232
- RB1wild-type is targeted by CDK4/6 inhibitor ribociclib

MicroRNAs

MicroRNAs (miRNAs) are small (about 22 nucleotide long) non-coding RNA that regulate gene expression by preventing mRNA translation by base pairing on complimentary sequences of RNA, have been implicated in various pathological processes in the human body. Using glioblastoma tissues and cell lines several groups of miRNAs have been identified whose expression is significantly altered in glioblastoma. Dysregulation of miRNA regulated genes is considered one of the key mechanisms in pathogenesis of glioblastoma, and several miRNAs involved in tumor initiation and progression have been described (26). Therefore, miRNAs are not only excellent diagnostic biomarkers, but also serve as targets for molecular therapies. Targeting miRNA(s) could alter multiple genes simultaneously and may prove more effective than targeted focused at targeting single gene or pathway (27). miRNAs, by playing a role in glioma stem cells (GSCs), may predispose to development of resistance to temozolomide (TMZ) therapy of glioblastoma, and dysregulation of GSCs pathways by targeting miRNA could provide an effective strategy against TMZ-resistant glioblastoma (28).

Specific to glioblastoma, the plasma levels of miR-21, miR-128, and miR-342-3p are found elevated and can distinguish glioblastoma patients from healthy controls or other types of brain tumors (29). Interestingly, the plasma levels of these 3 miRNAs dropped in glioblastoma patients drop following treatment by surgery plus chemo-radiation indicating that diagnostic tests can be developed to assess disease progression or recurrence.

Pronounced reduction of miR-218 in patients with highly hypoxic and necrotic glioblastoma contributes to temozolomide resistance as demonstrated in transplanted glioblastoma in mice, whereas tumor growth is significantly reduced with increase in survival in response to temozolomide in mice with high miR-218 (30). According to these authors, miR-218 downregulates expression of certain components in receptor tyrosine kinase pathway, and hypoxia-inducible factor 2α. Understanding the molecular basis of miR-218-mediated chemoresistance would be useful for the development of targeted therapies.

Molecular-targeted therapy based on miRNA expression in cancer stem cells can facilitate personalized and effective treatment strategies for glioblastoma in the future (27).

INNOVATIONS IN CHEMOTHERAPY

Although temozolomide chemotherapy is the standard treatment for newly diagnosed glioblastoma, which is generally well tolerated with lower toxicity than nitrosoureas. However, combination to temozolomide with other anticancer agents have also been investigated.

Innovations in Delivery of Chemotherapy

To avoid systemic toxicity of chemotherapy, various methods have been used to limit application to the tumor such as implants in tumor cavity following surgical excision, targeted delivery to glioblastoma following systemic administration, and selective delivery of higher concentrations to the tumor, e.g., by intraarterial chemotherapy. Delivery of monoclonal antibodies (MAbs) in glioblastoma will be discussed in the following section on immune therapy.

Drug Formulations for Improved Delivery to Brain Tumors

Delivery of anticancer agents is limited by their inability to reach therapeutic levels in brain tumors with maximally tolerated dose regimens. Drug targeting by conjugating with protein such as transferrin has been extensively studied, as a targeting molecule transferrin helps the transport of drug to glioblastoma which contains abundant transferrin receptors on the surface.

Transferrin-bearing therapeutic agents can be targeted to their site of action on brain tumors (31).

Thermosensitive liposomes can encapsulate drugs to release them at the target site in the tumor in response to hyperthermia without exposing the surrounding normal brain to toxicity. Increase in efficacy of doxorubicin by release from a thermosensitive liposomal nanocarrier as compared to non-thermosensitive liposomes has been demonstrated in a mouse model of human glioblastoma (32).

Angiopep-2 (An2) for BBB transcytosis and anti-CD133 MAb for specific delivery to glioma stem cells have been incorporated in a dual-targeting immunoliposome encapsulating temozolomide (33). A significant reduction in size of implanted glioblastoma in the rat was shown after intravenous administrations of the dual targeting system, indication a potential use in treatment of patients with glioblastoma.

Convection-Enhanced Delivery

Convection-enhanced delivery (CED) involves direct delivery of a therapeutic agent to the brain by injection or a catheter propelling the agent through interstitial spaces under a pressure gradient rather than passive diffusion. It has been used for both chemotherapy drugs and for delivery of macromolecules of some biological therapies for glioblastoma.

A prospective, dose-escalation phase Ib study of the topoisomerase-I inhibitor, topotecan, given by CED in patients with recurrent glioblastoma resulted in significant efficacy as assessed by radiographic changes while using doses that were well tolerated by the normal brain (34). Overall survival was prolonged in this study with minimal drug toxicity, which helped to determine the maximum tolerated dose for further phase II/III studies.

Modification of BBB for Delivery of Chemotherapeutic Agents

Several chemotherapeutic agents used for glioblastoma such as nitrosoureas, temozolomide can cross the intact BBB, but larger molecules such as MAbs may not do so. BBB permeability may be increased in glioblastoma, but this is not a reliable factor in assessing delivery of a therapeutic for brain tumors. Several strategies for drug delivery across the BBB have been described; some of these involve circumventing the BBB, whereas other are directed at osmotic opening of the BBB (35). Disruption of BBB allows uncontrolled passage of the drug into the brain surrounding the tumor rather than into the tumor itself, which may produce neurotoxic effects. Controlled passage through the BBB with targeted delivery to the tumor, as described in the section on nanobiotechnology-based delivery is safer and more effective.

Intra-Arterial Chemotherapy

Intraarterial delivery of chemotherapy to the brain provides a many-fold delivery peak drug concentration in the tumor as compared to the same drug dose given systemically because of damage to the blood-brain barrier and neovasculature in the tumor. However, randomized trials on patients with glioblastoma have not shown a survival advantage with intraarterial BCNU as compared to intravenous administration. The limitations of this technique are significant vascular and neurologic toxicity that can lead to visual loss, stroke, and leukoencephalopathy. Although toxicity of intra-arterial chemotherapy could be reduced by using carboplatin- and methotrexate-based regimens, further clinical studies are needed to determine its utility in the treatment of glioblastoma.

An animal experimental study has demonstrated the feasibility of direct delivery to brain and glioma tissue of cationic liposome after intraarterial injection via an intracarotid route during transient cerebral hypoperfusion (36). This may represent an effective method for delivering antiglioma agents to glioblastoma in humans with a drawback, e.g., cationic liposomes accumulate at higher concentrations in the peritumoral brain than in the tumor core and are retained for a longer time.

IMMUNE THERAPY

Immune therapy of cancer is progressing rapidly and has been applied to glioblastoma as well. Two types of immune therapies, monoclonal antibodies and vaccines, will be considered in this section. Immune gene therapy will be discussed under the section on gene therapy.

Monoclonal Antibodies

Monoclonal antibodies (MAbs) are used for glioblastoma treatment because of their high specificity and affinity for biological targets to improve immunotherapy and for antiangiogenic action by targeting growth factor receptors such as VEGFR, EGFR, and PDGFR (37). MAbs overlap with vaccines, another strategy for immunotherapy of cancer. Bevacizumab is the only approved MAb used for the treatment of glioblastoma. Several MAbs are under investigation.

Bevacizumab

Bevacizumab is a MAb that binds to VEGF and inhibits the growth of tumor blood vessels. It is approved for the treatment of several cancers including glioblastoma for a decade. A systemic review of literature shows that use of bevacizumab prolongs OS either alone or in combination with a cytotoxic agent by about 4 months in recurrent glioblastoma but not in primary glioblastoma (38).

MAbs Targeting EGFR

The best-known example of an anti-EGFR MAb is cetuximab, which is approved for the treatment of other cancers but not for recurrent glioblastoma as phase II clinical trials failed to show its efficacy. MAbs with TK inhibitors targeting EGFR, a tyrosine kinase (TK), which is a receptor for therapeutic agents for glioblastoma such as T cells, oncolytic viruses, and nanoparticles is being investigated (39). Another, anti-EGFR MAb, nimotuzumab, was developed up to phase III clinical trials. It showed some efficacy in combination with other methods of treatment but was not developed further. There is a need for the development of a suitable MAb for targeting EGFR.

Delivery of MAbs for Treatment of Glioblastoma

Due to their large size, MAbs do not cross the BBB easily and nanobiotechnology-based strategies are required. These are described under the section on nanobiotechnology-based drug delivery.

Immune Checkpoint Blockade

Immune checkpoint blockage is achieved by use of T cell inhibitory molecules such as anti-programmed cell death 1 (PD-1) antibody, which was first approved for the treatment of malignant melanoma. In contrast to use of cytotoxic T lymphocytes (CTL) against cancers, immune checkpoint blockade terminates immune response in a way that may activate exhausted CTL to destroy cancer (40). This approach is being investigated for several cancers including glioblastoma, but concern for adverse effects such as autoimmune diseases and prohibitive cost are drawbacks for wider applications.

Rationale for clinical trials of immune checkpoint blockade for newly diagnosed and recurrent glioblastoma is provided by results of preclinical studies (41). Furthermore, the concept of combination therapy involving with vaccine and immune checkpoint inhibitors is a promising strategy for treatment of patients with glioblastoma (42). An open-label pilot study will assess the safety, feasibility, and immunogenicity of a personalized neoantigen-based vaccine for enhancing CTL response again tumor cells plus adjuvant poly-ICLC combined with immune checkpoint inhibitors in patients with newly diagnosed, unmethylated glioblastoma (ClinicalTrials.gov Identifier: NCT03422094).

Vaccines for Glioblastoma

The earlier vaccines for glioblastoma were crude preparation made from patients own tumor tissue and results were disappointing. As of August 2018, 88 clinical trials of vaccines for glioblastoma are listed on the US Government web site for clinical trials[1]. Numerous trials of vaccines employing various strategies against glioblastoma are being conducted from phase I to phase III. Although some have shown promising results, none has come close to curing it. New biotechnologies have enabled better and more effective vaccines for glioblastoma. Some of the more promising of these will be discussed here.

DCVax

DCVax, a dendritic cell (DC)-based personalized cancer vaccine cancer type with purified tumor-specific antigens or tumor cell extracts derived from tumor at the time of resection. DCVax-Brain is approved in Switzerland for the treatment of glioblastoma. In the US, in a phase I trial of autologous DC vaccine, expression level of tumor-associated antigens on glioblastomas cells or glioblastoma stem cell population correlated with prolonged overall survival and progression-free survival (43). The ongoing open label clinical trial #NCT02146066, using already manufactured autologous tumor lysate-pulsed DC vaccine (DCVax-L, is studying patients who were not eligible for enrollment under protocol 020221 due to evidence of disease progression or post chemo-radiation pseudo-progression or lack of availability of adequate vaccine doses.

Vaccination with autologous tumor lysate-pulsed DCs in conjunction with toll-like receptor agonists administered as adjuvant therapy was tolerated well both in newly diagnosed and recurrent glioblastoma patients. Interestingly, mesenchymal gene expression profile, which is mostly defined by inflammation-associated gene signature, may identifies a subgroup of glioblastoma patients likely to respond to immune-based therapy (44).

Pre-conditioning with a potent recall antigen has been a viable strategy to enhance anti-tumor immune response. Experimental studies have shown that pre-conditioning the vaccine site with a potent recall antigen such as tetanus/diphtheria toxoid improves the efficacy of tumor-antigen-specific DCs (45). In a randomized trial on patients with glioblastoma, pre-conditioning with DCs or tetanus/diphtheria toxoid prior to vaccination with DCs pulsed with tumor specific cytomegalovirus phosphoprotein 65 RNA, a tumor-specific target had enhanced DC migration bilaterally and significantly improved survival (45).

One of the ongoing phase II clinical trials, # NCT02808364, is evaluating safety and efficacy of "Personalized Cellular Vaccine for Recurrent Glioblastoma" (PerCellVac2) in recurrent glioblastoma. This trial specifically uses immunization with autologous tumor cells, antigen pulsed DCsor allogeneic peripheral blood mononuclear cells. Results of this trial will be useful for determining the future course of action for cell-based immunotherapy in glioblastoma.

A randomized phase III trial NCT00045968 is evaluating long-term effects of addition of an autologous DCVax®-L vaccine to standard therapy for newly diagnosed glioblastoma, i.e., after surgery and chemoradiotherapy, patients receive temozolomide plus DCVax-L or temozolomide alone. Because of the cross-over trial design, nearly 90% of the patients have received DCVax-L so far and the vaccine has improved the survival rates of some patients and those with median overall survival of 40.5 months are being analyzed further (46). The trial is ongoing to enable continued study of glioblastoma patients who are living beyond the expected length of survival.

Epidermal Growth Factor Receptor Variant III as a Vaccine Target for Glioblastoma

The epidermal growth factor receptor variant III (EGFRvIII) is an important vaccine target because its expression is tumor specific and has a promising role in immunotherapy of glioblastoma. Phase II clinical trials in patients with newly diagnosed glioblastoma showed that EGFRvIII-specific vaccine therapy improves progression free and overall survival (47). In a separate phase II study, rindopepimut (CDX-110, EGFRvIII peptide sequence conjugated to keyhole limpet hemocyanin) combined with standard adjuvant temozolomide chemotherapy improved progression-free and overall survival of glioblastoma patients (48). A pivotal, double-blind, randomized, phase II trial # NCT01498328 on bevacizumab-resistant patients with recurrent glioblastoma combining bevacizumab with rindopepimut was completed in 2017 but results have not been published.

Heat Shock Protein-Based Vaccine

Heat-shock proteins (HSPs) has been used to deliver a variety of tumor antigens to antigen presenting cells (APC) for immune stimulation. HSPPC-96 vaccine, based on internalization of HSP-96 by binding of to the CD91 receptor on DCs resulting in cleaved tumor peptides on major histocompatibility complex classes I and II, has been studied in patients with recurrent glioblastoma in a phase II, multicenter, clinical trial (49). A personalized polyvalent vaccine is obtained by purifying HSP-96 protein complexes from a patient's own tumor and administered for treatment. Results showed that the vaccine was safe but lymphopenia prior to treatment was a concern as it may reduce efficacy. An ongoing 3-arm randomized phase II clinical trial #NCT01814813 is comparing HSPPC-96 vaccine to vaccine in combination with bevacizumab and bevacizumab alone following surgical resection of glioblastoma.

Recombinant Poliovirus

Recombinant nonpathogenic polio-rhinovirus chimera (PVSRIPO) targets the neurotropic poliovirus receptor CD155, which is abundantly expressed on glioblastoma cells, and penetrates these cells to cause lysis and release of tumor antigens as well as molecules recognized by cells of the natural immune response (50). This results in an influx of macrophages, monocytes, and DCs into the tumor to scavenge cellular debris, and released molecules present a pattern for natural killer (NK) cells and tumor antigens to induce effector T cells to kill more cells. This is an example of overlap of viral immune therapy and viral oncolysis of malignant tumors.

A phase II clinical trial confirmed the absence of neurovirulent potential following intratumoral infusion of PVSRIPO in patients with recurrent glioblastoma and observed that survival rate of treated patients was higher at 24 and 36 months than the rate among historical controls (51). Patients in the trial received polio vaccine before treatment to ensure poliovirus immunity at the time of instillation of PVSRIPO into the tumor and reduce the risk of spread of the virus beyond the tumor. A question that has been raised is if viral immunity may reduce the efficacy of the vaccine. There is a potential risk of restoration of replication competence of virus *in vivo*.

NANOBIOTECHNOLOGY-BASED INNOVATIONS FOR TARGETED DELIVERY OF THERAPY FOR GLIOBLASTOMA

Nanobiotechnology-based strategies for drug delivery to cancer are described in detail elsewhere (52). Some of these can be applied to glioblastoma and examples are given here. Nanoformulations of anticancer drugs enable targeted and efficient delivery to tumors through the blood-brain barrier (BBB) with lesser dosage of anticancer drugs than conventional formulations and reduce toxicity of chemotherapy.

Micelles for Delivery of Chemotherapy to Brain Tumors

Micelles can be used as carriers of anticancer drugs such as temozolomide (TMZ) to enhance delivery to glioblastoma. In an experimental study on a mouse model of implanted glioblastoma, pH-responsive micelles containing distearoyl phosphoethanolamine-PEG-2000-amine, N-palmitoyl homocysteine, and platelet-derived growth factor (PDGF) peptide as well as Dylight 680 fluorophore on the surface for targeting were used for delivery of TMZ (53). This resulted in specific uptake and accumulation of TMZ in tumors with increased destruction of tumor cells compared to delivery with untargeted micelles. Thus, micelle-based drug carrier systems have a potential for targeted delivery of anticancer drugs to glioblastoma to reduce their systemic toxicity.

Nanoparticles for Delivery of Drugs to Glioblastoma Across BBB

Nanoparticles are promising tool for targeted delivery of oncology drugs. Nanoparticles made of poly(butyl cyanoacrylate) (PBCA) or PLGA coated with polysorbate 80 or poloxamer 188 have been shown to transport antitumor drug doxorubicin across the BBB (54). In an orthotopic model of glioblastoma in rats, these particles loaded with doxorubicin, significantly improved survival with complete tumor remission observed in 20–40% animals (54). This nanoparticle approach of drug delivery reduced dose-limiting cardiotoxicity and the testicular toxicity of dauxorubicin. The mechanism of nanoparticle aided delivery of dauxorubucin across the BBB remains unclear. However, it is likely that certain plasma apolipoproteins adsorbed by nanoparticles may interact with receptors on brain capillary endothelium to suppress drug exclusion activity (55).

Iron oxide nanoparticles are particles with superparamagnetic properties, also known as superparamagnetic iron oxide nanoparticles (SPION) have been useful in MRI to locate brain tumors with precision and to target chemotherapeutic drugs into the tumors. Folic acid (FA) has been used as the targeting agent combined with polyethylene glycol (PEG) serving to improve biocompatibility and cellular uptake of nanoparticles. These nanoparticles has applicability beyond MRI mediated tumor detection because a variety of small molecules to target receptor tyrosine kinases on tumor or chemotherapy drugs, can be attached to these nanoparticles to facilitate delivery and efficacy.

A biodegradable and nontoxic biopolymer is a universal delivery nanoplatform for design of nanomedicines for intravenous treatment of for malignant brain tumors. A polymeric conjugate of a MAb targeting the brain-tumor barrier for crossing it and attached to an antisense oligonucleotide that inhibits tumor angiogenesis by specifically blocking the synthesis of a tumor neovascular trimer protein, laminin-411, is released into the target tumor cell cytoplasm via pH-activated trileucine (56). This is a promising strategy for treatment of glioblastoma that should be tested in clinical trials.

Nanoparticle Aided Delivery of Chemotherapy

A concept of targeted drug delivery to glioblastoma across the BBB is shown in **Figure 1**. Several techniques have been shown to improve drug transport across BBB, many of these techniques are designed to disrupt BBB, which compromises the ability of brain microvasculature to prevent entry of harmful toxins in to the brain. Nanoparticle-based delivery of anticancer drugs

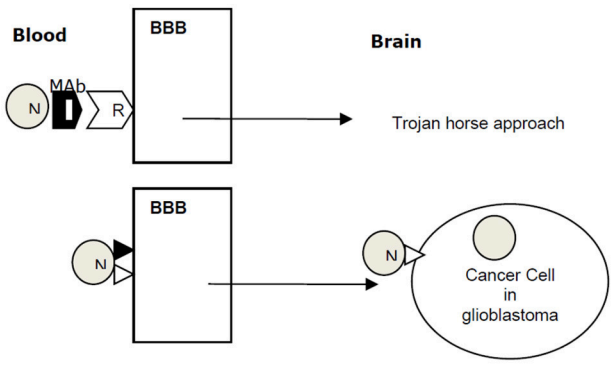

FIGURE 1 | A concept of targeted drug delivery to glioblastoma across the BBB. Nanoparticle (N) combined with a monoclonal antibody (MAb) for receptor (R) crosses the blood brain barrier (BBB) into brain by Trojan horse approach. N with a ligand targeting BBB traverses the BBB by receptor-mediated transcytosis. Ligand ▶ docks on a cancer cell receptor N▷ and delivers anticancer payload to the cancer cell in glioblastoma.

across the BBB is a promising approach. Polymer and lipid nanoparticles are frequently used as nanocarriers for anticancer drugs. A phase I clinical trial of nanoliposomal irinotecan in recurrent glioblastoma has been completed (NCT00734682).

Controlled Delivery of BCNU by Poly-Lactic Acid Nanoparticles

Biodegradable poly-lactic acid (PLA) nanoparticles conjugated with transferrin, an iron-transporting serum glycoprotein were loaded with BCNU for targeted delivery to transferrin receptor expressing glioma cells (57). Efficacy evaluation in C6 tumor-bearing rats, BCNU-loaded PLA nanoparticles showed superior cytotoxicity, and led to prolonged survival animals of as compared to conventional BCNU therapy (57).

Targeted Minicells for Nanoscale Delivery of Chemotherapy to Glioblastoma

Minicells are achromosomal cells formed in certain mutants of rod-shaped bacteria, are products of aberrant cell division, and contain RNA and protein, but little or no chromosomal DNA (58). Designing minicells loaded with anti-cancer drugs and coated with antibodies to target tumor cell specific receptors is relatively new technique. Targeted minicells measuring 400 nm enter cancer cells via receptor-mediated endocytosis and can be loaded with therapeutically significant concentrations of chemotherapeutics. As proof of principle, clinical applicability of minicells was shown in dogs with late stage spontaneous brain cancer, where targeted minicells loaded with doxorubicin were safely administered achieving clinical activity in terms of tumor regression (58). This study demonstrated promising results and potential clinical application of minicells for drug delivery for treatment of patients with glioblastoma. Based on this, a phase I clinical evaluation of EGFR-targeted, doxorubicin-loaded minicells (EGFR(V)-EDV-Dox) was performed in human patients with recurrent glioblastoma in Australia and another phase I trial # NCT02766699 is currently under progress in the USA.

Targeting MAbs Attached to the Surface of the Nanocarrier

The surface of nanocarriers is functionalized with targeting MAbs as ligands to promote drug delivery to tumors with corresponding receptors. One problem with this approach is that when nanocarrier is exposed to biological fluids such as plasma, its surface is covered with various biomolecules forming a protein corona, which masks the targeting ability of the nanoparticle. A pre-adsorption process has been used to attach targeting MAbs to the surface of the nanocarrier, a capsule containing the anticancer drug, which prevents the formation of biomolecular corona and pre-adsorbed MAbs remain functional (59). The authors concluded that this is an efficient method for attaching targeting MAbs to the surface of nanocarriers.

INNOVATIONS IN RADIOTHERAPY

Radiotherapy is usually combined with chemotherapy following surgery in different sequential combinations. There is no convincing evidence that addition of radiotherapy to chemotherapy increases survival in glioblastoma. Traditionally radiation therapy following surgery was whole cranium radiation, which exposed the normal brain to radiation with adverse effects such as cognitive impairment. Current practice is to use "focal" or "limited-field" radiation therapy covering 2–3 cm around the tumor, interstitial brachytherapy, and fractionated radiotherapy.

Fractionated Radiotherapy

Fractionated radiotherapy means splitting the total radiation dose into several fractions that are given over several weeks in combination with chemotherapy. The rationale for using a fraction of the total radiation dose enables normal cells surrounding the tumor to recover between treatments. Adverse effects of full dose radiation in a single session are reduced by fractionated therapy over several weeks. In case of glioblastoma, 30 fractions of radiotherapy dose of 60 Gy with adjuvant temozolomide is the current standard of treatment (60). This approach is included in the American Society for Radiation Oncology Guideline, which is endorsed by the American Society of Clinical Oncology (61). Fractionated radiotherapy can be combined with immunotherapy for glioblastoma. Preclinical evidence indicates that hypofractionated radiotherapy of glioblastoma can prime the immune system to enhance the effect of immune therapy (62).

Brachytherapy

Interstitial brachytherapy involves placement of radioactive isotopes such as ^{125}I seeds in the tumor cavity after resection to deliver high dose radiotherapy to the residual tumor with minimal radiation exposure in the surrounding brain tissue as compared to external-beam radiation. Nevertheless, radiation may leak into the surrounding healthy brain and may accumulate in other organs via systemic circulation. Three-dimensional iridium-192 (^{192}Ir) high-dose-rate brachytherapy

(Ir Knife) allows intratarget dose escalation with superior conformity, which compares favorably with linear accelerator-based stereotactic radiosurgery with a steeper dose fall off at margin of the tumor (63). In non-operable glioblastomas, low-dose ^{125}I brachytherapy may be administered by a stereotactic device. Stereotactic ^{125}I brachytherapy in combination with temozolomide chemotherapy was shown to be effective for treatment of thalamic glioblastoma (64).

Innovations in brachytherapy under investigation include other isotopes with prolonged delivery of higher doses of radiation, and combination of radioactive isotopes with MAbs as ligands for receptors expressed on the surface of tumor cells for selective delivery of radiation therapy. Radiolabeling can further enhance the assessment of effects of therapy by brain imaging. For example, ^{32}p (phosphorus-32)-chromic phosphate-polylactide-co-glycolide (32P-CP-PLGA) for controlled release of the isotope, combined with radiotracer ^{68}Ga-3PRGD$_2$, which targets integrin αvβ3 receptors or the tumor as well as neovasculature, has shown promising results of brachytherapy in a rat model of transplanted glioblastoma (65).

Heavy Particle Radiation

Proton beam radiation therapy due to its "Bragg peak effect," reduces exposure to the surrounding brain, i.e. steeper dose "drop-off" relative to photon radiation therapy. In principle, proton beam therapy can be considered safer than traditional photon radiation therapy; however, it is uncertain if proton beam radiation has any better antitumor effects than photon radiation therapy.

Gamma Knife radiosurgery (GKRS) is a type of non-invasive stereotactic radiosurgery for delivery of a high dose of radiation to a tumor. GKRS is suitable for recurrent glioblastoma as it spares the poorly demarcated healthy brain tissue surrounding the tumor from radiation-induced necrosis (66). Drawbacks of GKRS are that it does not reach areas of the tumor that are not well detected by MRI, and radiation-induced edema has been reported in nearly 30% of patients who received high radiation doses (67). Concomitant administration of bevacizumab was shown to prolong patient survival, while also significantly reduced adverse effects of radiation (67).

Boron Neutron Capture Therapy

In boron neutron capture therapy (BNCT), another form of heavy particle radiation, irradiation of the glioblastoma with low-energy neutrons is followed by the boron emits an alpha-particle that deposits high energy over a short distance systemic administration of a nonradioactive boron isotope. Monte Carlo modeling for BNCT of glioblastoma has shown that it increases the efficacy in destruction of tumor, but extension of the CTV margin may not increase the outcome of treatment significantly (68).

Enhancing Effect of Radiotherapy by Hyperbaric Oxygen

Hyperbaric oxygen (HBO) is known to enhance the effect of radiotherapy by counteracting hypoxia within tumors, a fundamental feature of cancer cells that limits efficacy of radiation therapy (69). HBO is also used for treatment of radiation induced necrosis of normal tissues around tumor treated with high-dose radiation therapy. In a study on glioblastoma cells that were exposed to HBO at 1.3 ATA (atmospheric pressure absolute) showed that HBO treatment reverses radiation-induced enhanced mobility of tumor cells (70). In the first report of a clinical pilot study, results of radiotherapy combined with HBO in glioblastoma were compared with those of radiotherapy without HBO and all patients receiving HBO with radiotherapy showed more than 50% regression of the tumor (71). Several studies have reported that radiotherapy immediately after HBO therapy is safe and effective in patients with glioblastoma and may protect normal brain tissues from radiation injury (72).

LOCAL DESTRUCTION OF TUMOR

Oncolysis by Genetically Modified Viruses

Viral oncolysis is a targeted therapy and oncolytic viruses are genetically modified to specifically lyse tumor cells. They are replication-selective rather than replication-defective viral vectors. Oncolytic viruses differ from viral vectors in that they increase in number in tumor cells and lyse the cells directly, not by transducing specific genes. Studies both in a mouse glioma model as well as on glioma stem-like cells from patients suggest that the efficacy of viral oncolysis HSV type 1 may be enhanced when used in combination with inhibitors of histone deacetylases or other proteins that modulate cellular trafficking of these therapeutic viruses (73). Most of the oncolytic viruses currently in clinical trials are derivatives of adenovirus or herpes simplex virus (HSV) type I. T-Vec (talimogene laherparepvec), an oncolytic HSV-1 armed with GM-CSF, has been approved as the first oncolytic virus therapy in the USA and Europe. A phase II clinical trial of G47Δ, a third-generation oncolytic HSV-1 designated as breakthrough therapy, is ongoing in glioblastoma patients in Japan (74).

Oncolytic virotherapy is administered into the residual tumor after resection of glioblastoma and may not penetrate all tumor cells. Some of the precautions that need to be observed during clinical introduction of oncolytic therapy include the following (75):

- Oncolytic virus delivery should be completely optimal and safe.
- Viruses should be monitored for replication or dissemination beyond the tumor.
- Reaction of immune system of the patient to oncolytic viruses should be closely monitored.

Genetically Modified Bacteria for Tumor-Specific Lysis

Genetically modified bacteria can be used for selective destruction of glioblastoma. Genetically altered strains of bacteria such as Salmonella have been used as bacterial vectors for preferential delivery of anticancer drugs to solid tumors. Historically, bacteria have been used as oncolytic agents for

malignant brain tumors. The most promising approach to glioblastoma was the use of genetically engineered bacteria that selectively destroys tumor cells while sparing the normal brain tissue (76). Genetically altered bacteria can be maintained in the confines of the brain tumor, where they thrive and unable to survive if they escape into brain or other tissues. Once the tumor destruction is completely, a bactericidal drug can eliminate bacteria. Refinements in gene editing can bring this concept closer to application for treatment of glioblastoma in patients.

Hyperthermia

Hyperthermia, i.e., raising the tumor tissue temperature to 41–46°C damages tumor cells by changes such as protein misfolding and disruption of signaling pathways leading to apoptosis. Hyperthermia can be induced by microwaves, infrared irradiation, ultrasound, and magnetic nanoparticles. In addition to destroying tumor cells, hyperthermia can also be used for targeted delivery of drugs to glioblastoma by thermosensitive release from drug carriers guided to enter the tumor. Gold nanoparticles can be used both as contrast agent for MRI and for photothermal therapy, e.g., gold nanorods have been used for thermal ablation of glioblastoma (77).

Tumor Treating Fields

Tumor treating fields (TTF), a non-invasive wearable technology, is based on low-intensity alternating electric fields, which disrupt mitosis and inhibit tumor growth. TTF is approved for glioblastoma by the FDA in US. A systematic review of preclinical studies and clinical trials of TTF for glioblastoma has shown that TTF was as effective as chemotherapy but the combination of both prolonged overall survival and progression-free survival of glioblastoma without systemic adverse effects (78). Combinations of TTF with radiation therapy, targeted chemotherapy as well as immunotherapy remain to be explored.

GENE THERAPY

No gene therapy for glioblastoma has been approved in the US or Europe yet, but 77 clinical trials are listed on US Government web site[1] as of August 2018. Gene therapy may be used in conjunction with surgery or as an alternative to chemotherapy/radiotherapy or in cases of recurrences following excision as well as chemotherapy-resistant tumors. With improvement in diagnostic imaging and methods of delivery, it feasible that small tumors detected very early may be treated noninvasively or by minimally invasive gene therapy procedures.

The first clinical trial of the herpes simplex virus, thymidine kinase, and ganciclovir gene therapy for glioblastoma was conducted a quarter of century ago (79). However, by the end of twentieth century, this gene therapy approach was discontinued from further development after completion of phase III clinical trials as it failed to show efficacy in patients (80). The results in patients were less striking than in experimental animals. Initially there was reduction in the size of the tumor, but the tumors resumed the growth and the OS was not extended. Various innovations in gene therapy have been employed since then. The number of current preclinical research projects worldwide to find a cure for glioblastoma exceeds 100.

A classification of gene therapy approaches to glioblastoma is shown in **Table 3**. Discussion in the article does not follow or include all the technologies or follow the same order due to overlap with other approaches. Some technologies relevant to the topic of this article will be described here.

TABLE 3 | Strategies for gene therapy of glioblastoma.

Viral vector mediated insertion of drug sensitivity genes
Direct intratumoral injection of genetically modified neurotrophic viruses
Insertion of drug sensitivity genes
Suicide gene therapy: HSV thymidine kinase
Use of *E. coli* gpt gene to sensitize glioma cells to prodrug 6-thioxanthine
Use of viral vectors containing radiation inducible promoters
Regulated toxin gene therapy
Baculovirus as diphtheria toxin gene vector
Transfer of apoptosis inducible FADD/MORTI gene
Gene transfer into brain tumors by using targeted adenoviral (Ad) vectors
Single chain antibody combined with Ad vector and targeted to EGFR receptor
Gene transfer into brain surrounding tumors by using targeted adeno-associated viral (AAV) vectors
Transduction of normal cells in the brain with an AAV vector encoding interferon-β (IFN-β)
Selective oncolysis by genetically engineered microorganisms: bacteria and viruses
Immunogene therapy
Cell-based gene therapy
Mesenchymal stem cells engineered to produce therapeutic molecules
Neural stem cells engineered to produce therapeutic molecules
Grafting of stem cells producing therapeutic molecules such as IL-4 gene
Growth factor manipulation
Apoptosis induced by introduction of gene for nerve growth factor receptor TrkA
Inhibition of epidermal growth factor receptor associated tyrosine kinase receptor
Antiangiogenesis approaches directed against tumor blood vessels
Oncogene antagonism: anti MYC oncogene MAD therapy
Insertion of tumor suppressor genes
Transfer of wild type p53 or p27
Retinoblastoma gene transfer
Antisense therapy
Blocking of action of transforming growth factor β2 by triplex forming oligonucleotides
Episome-based antisense cDNA transcription of insulin like growth factor 1
Antisense vascular endothelial growth factor.
Oligodenucleotides targeted to tumor necrosis factor
RNA interference (RNAi)-based approaches
siRNA directed against EGFR and its variants
siRNA directed against PI3K/Akt signaling pathways
Telomerase inhibition by RNAi

Choice of a Viral Vector for Gene Therapy of Glioblastomas

Three types of viral vectors have been found to be efficient for delivery of gene therapeutics in most of the clinical trials: (1) retrovirus, (2) herpes virus, and (3) adenovirus. An adenoviral vector has the following advantages for treating glioblastoma:

- It can be easily produced at high titers.
- It can be directly injected as purified particles into brain tumors without the need for virus producing cells.
- It does not integrate into the host genome. This avoids the risk of insertional mutagenesis.
- Gene delivery by an adenovirus is independent of the host cell cycle. This feature allows higher transduction rates than with retroviruses because only a small number of glioma cells are replicating at any given time.

Disadvantages of adenoviral vectors are as follows:

- Actual release *in vivo* cannot be quantified.
- Antigenicity of viral proteins; this might limit repeated treatments. This problem has been seen in gene therapy trials for cystic fibrosis, but it is uncertain if this problem would occur in the brain, as it is an immunologically privileged site.
- Unlike retrovirus vectors, they infect all cells of the brain. This lack of discrimination could result in significant toxicity to the normal surrounding brain. This can be remedied by use of tissue-specific promoters. One example is glial fibrillary acidic protein, a gene that is found in glial cells. Transfer of a vector containing a cytotoxicity gene under the control of a glial fibrillary acidic protein promoter should result in expression only within the cells that normally synthesize glial fibrillary acidic protein.

Glioblastoma expresses high levels of type 2 somatostatin receptors, which can be targeted for improving transduction efficiency in these tumors. An adenoviral vector was designed based on the introduction of the full-length somatostatin somatotropin release-inhibiting factor sequence into it, and it was shown that low doses of this vector were sufficient for infecting high-grade human glioblastoma cells with marked enhancement of gene expression (81).

Results of an open-label, randomized, phase III trial of locally applied adenovirus-mediated gene therapy with herpes-simplex-virus thymidine kinase (sitimagene ceradenovec) followed by intravenous ganciclovir in patients with newly diagnosed glioblastoma after resection can increase time to death or reintervention, but not overall survival (82). Further clinical trials are in progress, and it will be the large randomized phase III controlled clinical trials that will provide evaluation of the success of gene therapy for the treatment of glioblastoma (83).

Antiangiogenic Gene Therapy

Gene therapy strategies, developed to disrupt normal function of vascular endothelial growth factor receptors, have been successful in experimental models to suppress tumor angiogenesis and growth (84). Some of the antiangiogenic gene therapies are described here briefly.

Targeting Endothelial Vasculature in Brain Tumors

VB-111 (ofranergene obadenovec), a genetically modified adenovirus, selectively targets endothelial cells in neovasculature of tumors (85). VB-111 inhibits vascular density in mouse models bearing glioma xenografts, which justifies its clinical development as a treatment for glioblastoma (86). A phase III trial #NCT02511405 for recurrent glioblastoma is studying VB-111in combination with bevacizumab as well as without it.

Combined Antiangiogenesis Approach

Systemic adenoviral delivery and sustained production of endostatin and soluble vascular endothelial growth factor receptor-2 can slow glial tumor growth in animal models by both reducing cell proliferation and increasing tumor apoptosis (87).

Gene Therapy for Reducing Adverse Effects of Chemotherapy

Raising the dose of chemotherapy to improve clinical efficacy is limited by toxicity, particularly myelosuppression, and neurotoxicity. Gene therapy using mutant MGMT (P140K) gene-modified hematopoietic stem cells can reduce the toxic effects of chemotherapy on hematopoietic cells, and autologous P140K-modified hematopoietic stem cells transplantation has been used in glioblastoma patients with poor prognosis prior to administration of multiple cycles of chemotherapy, resulting in increase of survival without adverse effects (88).

Glioblastoma frequently develops resistance to temozolomide, which can be overcome by adding O6-benzylguanine (O6BG), but the combination produces myelosuppression. Results of a prospective clinical trial have shown that gene therapy P140K-modified hematopoietic stem cells to confer O6BG resistance improves chemotherapy tolerance and outcome in these patients (89).

Nanobiotechnology for Improving Delivery of Gene Therapy

Nanobiotechnology, particularly use of nanoparticles, has improved drug delivery in cancer, and this technology can be applied to gene therapy of glioblastoma (90). A nanoparticle preparation using low molecular weight polyethylenimine, modified with myristic acid, and complexed with DNA, has been used successfully for targeted delivery of gene therapy for glioblastoma (91).

RNA INTERFERENCE THERAPY OF GLIOBLASTOMA

Nearly 40–50% of glioblastoma tumors show alterations (amplification, truncation, or mutations) of epithelial growth factor receptor (EGFR) resulting in an uncontrolled multiplication and expression of gene encoding normal EGFR or truncated form called EGFRvIII. Because of the delivery problems with commonly used pharmacologic inhibitors of EGFR, RNA interference (RNAi) would be an ideal approach to target EGFRvIII to destroy brain cancer cells and spare healthy cells. Similarly, small-interfering RNA (siRNA)-based downregulation of DNA repair protein MGMT in tumor cells can enhance the chemosensitivity of malignant gliomas against

temozolomide (92). The siRNA applied to glioblastoma cells *in vitro* was shown to reduce gene expression of EGFR and β-catenin and significantly inhibit their migratory as well as invasive ability (93). This is potentially an effective therapy for human glioblastoma and warrants further study *in vivo*. Knockdown of DNA repair protein apurinic endonuclease 1 by nanoparticle-based delivery of a siRNA has been demonstrated to increase sensitivity to radiotherapy in a genetic mouse model of glioblastoma resulting in prolonged survival (94).

CELL THERAPY OF GLIOBLASTOMA

Stem Cell Therapy for Glioblastoma

Implanted neural stem cells (NSCs) are known to migrate to glioblastomas and distribute inside the tumor in experimental animals, indicating their potential use as delivery vehicles for targeted therapeutics including gene therapy. The mechanism of the attraction of NSCs to the tumor has not been elucidated. Various proposed mechanisms that drive NSC migration include multiple factors such as chemoattractant molecules released by the tumor cells and inflammatory or degenerative changes in tumor microenvironment (95).

Intratumoral injection of interleukin (IL)-12 secreting NSCs in mice bearing intracranial gliomas significantly prolongs survival and leads to long-term antitumor immunity. Tumoricidal potency of IL-12 with the extensive tumor tracking capability of NSCs result was thought to render exceptional therapeutic benefit. Genetically engineered NSCs have been shown to specifically target glioblastoma cells after traveling through brain parenchyma and hinder tumor growth through local activation of cyclophosphamide-activating enzyme cytochrome p450 2B6 (96). Significance of the NSC-based gene therapy for brain tumor is that it exploits the tumor-tropism of these cells to mediate effective, tumor-selective therapy for brain cancer (97).

Human embryonic stem cell (hESC)-derived engineered mesenchymal stem cells (MSCs) have been shown to inhibit tumor growth and prolong survival after they were injected directly into the glioblastoma xenografts in the brains of mice who received concomitant prodrug ganciclovir (98). A preclinical study using MSCs engineered to express cytosine deaminase has shown that that stem cell-based gene therapy may be effective against glioblastoma stem cells (99). The authors proposed starting clinical studies in human patients based on encouraging results of preclinical studies of stem cell-based gene therapy for glioblastoma.

Cancer stem cells (CSCs), which are a type of tumor cells with self-renewal ability and high tumorigenicity, which contribute to the high rates of recurrence after treatment as well as development of resistance to treatment in glioblastoma patients. Therapeutic strategies for CSCs include inhibition of CSC-specific pathways and receptors by agents that increase sensitivity of CSCs to chemotherapy and radiotherapy, virotherapy, and gene therapy (100). A subset of CSCs in glioblastoma is marked by cell surface expression of CD133, a glycosylated pentaspan transmembrane protein. CD133-LV (lentiviral) is a novel tool for the selective genetic manipulation of CSCs in glioblastoma that can be used to precisely study the role of CSCs in tumor biology and therapy resistance (101).

Aptamers that specifically bind to tumor-initiating cells were identified by adopted Cell-Systematic Evolution of Ligands by Exponential Enrichment (Cell-SELEX) technique (102). These aptamers selectively bind on and internalize into cells that self-renew, proliferate, and initiate tumors. Because they can be modified to deliver payloads, aptamers could selectively target or facilitate imaging of tumor-initiating cells to improve therapeutic outcomes in individual patients.

CAR-T Cell Therapy of Glioblastoma

Chimeric antigen receptors (CAR)-T cells combine the antigen binding site of a MAb with the signal activating machinery of a T cell, freeing antigen recognition from MHC restriction and thus breaking one of the barriers to more widespread application of cell therapy. CAR-T technology uses retroviral or lentiviral vectors to engineer CARs which graft an arbitrary specificity onto an immune effector cell such as a T cell. These modified T cells are then transferred to the patient. Targeting with CAR-T cells is like that with MAbs with additional advantages of active passage to tumor sites, *in vivo* expansion as well as long duration of action, and possibility of gene transfer for counteracting tumor immune evasion (103).

Regression of glioblastoma has been reported following multiple infusions of CAR-T cells targeting the tumor-associated antigen IL-13 receptor alpha 2 (IL13Rα2) in a patient with recurrent multifocal glioblastoma and no toxic effects were observed into the resected tumor cavity followed by infusions into the ventricular system (104). Intravenous delivery of a single dose of autologous CAR-T cells targeting EGFRvIII mutation in patients with recurrent glioblastoma has been shown to be feasible and safe, without evidence of off-tumor toxicity or cytokine release syndrome (105).

Cell Therapy for Chemobrain

Chemotherapy for glioblastoma can produce "chemobrain" with severe cognitive dysfunction that can persist after the cessation of treatment. Pathomechanism is chemotherapeutic agent-induced inflammation in the hippocampus, which is involved in learning and memory. This inflammation can destroy neurons and other cell types in the brain. A study in rodent model of chemobrain, cognitive impairments due to chronic cyclophosphamide treatment resolved after intrahippocampal transplantation of human NSCs, which triggered the secretion of neurotrophic growth factors for regeneration and reduction of inflammation (106). A clinical trial to analyze the safety of such approaches is feasible.

COMBINATION OF INNOVATIVE WITH CONVENTIONAL THERAPIES

Combination of Gene Therapy With Chemotherapy

An example of combination of gene therapy and chemotherapy is clinical trial # NCT02414165 titled "Toca 511 & Toca FC vs. Standard of Care in Patients With Recurrent High Grade

Glioma." This is a randomized, open-label phase II/III trial of combination treatment using retroviral vector Vocimagene amiretrorepvec (Toca 511), a replicating virus that only infects actively dividing tumor cells to deliver the gene for enzyme, cytosine deaminase (CD), and sustained release 5-fluorocytosine (Toca FC), the prodrug of the chemotherapy 5-fluorouracil. Once inside tumor cells, CD converts the prodrug to 5-fluorouracil to, which destroys them as well as immunosuppressive myeloid cells, enhancing the patient's immune system to recognize and attack the cancer cells. As of August 2018, the trial is still ongoing but analysis of a subset of phase I (selected to proceed to phase III) showed that durable response rate of 21.7% in patients with recurrent glioblastoma who were treated with a gene therapy combination were alive 33.9+ to 52.2+ months after treatment (107).

Combination of Gene Therapy and Car-T Cell Therapy for Glioblastoma

Regulatory T cells (Tregs), tumor associated macrophages (TAMs) and myeloid derived suppressor cells (MDCSs) are immunosuppressive cells in microenvironment of glioblastoma, and they inhibit cytotoxic T cells anticancer functions, thus reducing the efficacy of immunotherapy. Tumor cells in glioblastoma acquire new mutations after treatment and make it resistant to further treatment, which requires combination of gene therapy for glioblastoma with enhancement of the immune system's ability to fight it, e.g., immune checkpoint blockade combined with gene therapy prevents cancer cells from hijacking the host immune system (108). This study also supports the concept that heterogeneity of the glioblastoma should be taken into consideration of a personalized approach to therapy.

ANIMAL MODELS FOR TESTING INNOVATIVE THERAPIES FOR GLIOBLASTOMA

Translation of preclinical research into clinical application is limited by lack of a suitable animal model of glioblastoma. The most commonly used model in the past has been a subcutaneous xenograft of human glioblastoma cell line in an immunodeficient rodent model. A limiting factor in intracranial xenografts is the short survival. Malignant brain tumors induced by injection of chemicals and viruses are not suitable for glioblastoma studies. The most appropriate rodent model is transgenic as genetic engineering allows selective introduction of mutations relevant to human glioblastomas. Advances in genome-wide sequencing will enable creation of mouse models of glioblastoma to reproduce gene mutation patterns of human glioblastoma that are suitable for preclinical testing of personalized therapies (109).

Spontaneously occurring glioblastoma in the dog resembles glioblastoma of human and immune system of dogs is also somewhat like that of humans. CT-guided stereotactic techniques for drug delivery and tumor biopsy from tumors in the brain have been developed for use in dogs. Thus, dogs with spontaneous brain cancers offer a scientifically and ethically attractive system for preclinical evaluation of novel interventions for glioblastoma (110). Clinical trials of glioblastoma therapies in dogs have already been conducted and the findings are expected to benefit human patients (111, 112).

PERSONALIZED/PRECISION APPROACHES

Personalized medicine, also referred to as precision medicine is a type of medical care in which treatment is customized for an individual patient taking into consideration the genetic and environmental factors that influence response to therapy. Advancements in genomic/proteomic technologies have been crucial in development of personalized medicine, but other technologies such as metabolomics and adoptive immunotherapy also have contributed significantly to this effort. Personalized medicine is perfectly integrating technological advancements such as nanomedicine for understanding pathobiology of glioblastoma and management of disease in clinic (113).

Brain Cancer Chip for Personalized Drug Screening

Selection of an anticancer therapeutic best suited for an individual's glioblastoma is challenging. Conventional 2D cell cultures used in current drug discovery do not reflect the tumor and its 3D environments. A novel 3D brain cancer chip composed of photo-polymerizable poly(ethylene) glycol diacrylate (PEGDA) hydrogel, uses cultured glioblastoma cells that form 3D cancer tissues are is promising technology for drug screening (114). PEGDA hydrogel is permeable to water and biomolecules, so it enables "smart release" of chemicals carried on the chip for studying drug response in the surrounding 3D environment. Realistic cell–cell/cell–matrix interactions are like *in vivo* environment for drug screening. This chip was used to test combined treatment with two FDA-approved anticancer drugs—pitavastatin and irinotecan. High-throughput drug screening and massive parallel testing of drug response was possible using only a tiny sample from the tumor biopsy to determine the drug combinations and their dosages likely to be effective for personalized therapy of glioblastoma.

Countering Drug Resistance in Glioblastoma

A major obstacle to effective treatment is *de novo* or acquired resistance to standard-care therapies as well as for targeted therapies, EGFR, or mTOR inhibitors. Despite its nearly universal activation of mammalian target of rapamycin (mTOR) signaling in glioblastoma, tumors are resistant to mTOR-targeted therapy (115). Molecular analysis of glioblastoma cell lines, patient-derived cell cultures and clinical samples from phase I clinical trials suggest that the expression of promyelocytic leukemia (PML) gene may be responsible for resistance to cytotoxicity of mTOR inhibition (115). Consistent with this hypothesis, blockade of mTOR signaling by inhibitors of mTOR or EGFR promote nuclear PML expression in glioblastoma cells. Furthermore, studies using genetic ablation or overexpression techniques also demonstrate that PML abates cytotoxic effects

of mTOR or EGFR inhibition; while inhibitors of PML restored sensitivity to mTOR kinase inhibitor tested *in vivo*. These results indicated role for PML in mTOR and EGFR inhibitor resistance and provide a strong rationale for testing this combination in clinical trials.

Because intratumor heterogeneity in glioblastoma is an important factor that contributes toward treatment failure and drug resistance. Genomic analysis has been used to uncover intratumor heterogeneity in terms of copy number alterations in EGFR and CDKN2A/B/p14ARF as early events, and aberrations in PDGFRA and PTEN as later events during progression of cancer (116). Striking findings of this study revealed patient-specific patterns of cancer evolution that can be useful for designing more effective personalized therapy.

Enhanced DNA repair enables cells to survive the genotoxic effects of chemotherapy. Akt3, found in glioblastoma, enhances tumor progression by activating DNA repair pathways, leading to enhanced survival of human glioblastoma cells following radiation or temozolomide treatment (117). This finding has potential applications as blockade of Akt3 may help prevent or alleviate DNA repair-mediated therapeutic resistance.

Genomic Analysis as a Guide to Personalized Therapy of Glioblastoma

Gene expression profiling combined with mutation analysis plays an important role in the development of rational targeted therapies for glioblastoma. Application of these approaches is limited by spatial and temporal heterogeneity of glioblastoma. An analysis of data from patients with glioblastoma showed that samples from the same tumor are likely to have same genomic and expression signatures, whereas geographically separated, multifocal tumors or recurrent tumors often represent clonal variability (118). In this study, patient-derived glioma cells showed that therapeutic response correlated with genetic similarity while drug-response was heterogeneous in multifocal tumors enriched with PIK3CA mutations suggesting that an integrated genomic analysis of biopsies from multiple foci in the tumor can guide targeted therapeutic interventions for patients with glioblastoma.

Induced Neural Stem Cells for Personalized Therapy of Glioblastoma

Induced neural stem cells (iNSCs) derived from a patient's skin cells are ideal for personalized cell therapy of glioblastoma. Genetically engineered iNSCs with tumoricidal gene products retain the capacity to differentiate while induced apoptotic response in co-cultured human glioblastoma cells. These iNSCs also demonstrate unique capability to reach distant tumor sites in the murine models of glioblastoma to deliver anticancer molecule TRAIL (TNF-α-related apoptosis-inducing ligand) with resulting increase in survival (119). The use of iNSCs could lead to highly selective delivery of therapeutics to brain tumors, reducing systemic toxicity and improving efficacy. iNSCs may effectively bypass the BBB and attack glioblastoma (120). Considerable preclinical work needs to be done before moving iNSCs into clinical trials.

CONCLUDING REMARKS

Introduction of new technologies has improved the outlook for cure of glioblastoma although much remains to be done. Prolongation of survival time does not necessarily translate into eventual prospects of cure. Reduction of size of tumor alone is not enough as recurrences and rapid progression of the tumor eventually kill the patient. Improvements in targeted delivery of therapies is important for efficient and safe destruction of the tumor. MAbs with ligands for receptors on tumor surface have been used for targeted delivery of therapies. Although several receptors have been identified on glioblastoma cells, they may occur in other organs. A future challenge is discovery of receptors that are unique to glioblastoma and a combination of receptors may be needed for selective delivery to the tumor.

Nanobiotechnology has provided important diagnostic and therapeutic tools and enables more efficient delivery to the tumor across biological barriers. The aim is complete removal of the tumor as even a miniscule amount of residual tumor can lead to fatal recurrence.

Immunotherapy is another promising approach and adjuvants have been found useful to enhance response to vaccines, and this approach has been evaluated inclinical trials of various vaccine therapies using autologous tumor antigens or tumor-associated/specific antigen peptide in patients with glioblastoma. Choice of an appropriate target and vaccination strategy combined with an immune modulator to increase the body's ability to mount an immune response against the tumor could lead to more durable responses in patients with glioblastoma. However, control of immune response is still inexact as overstimulation may have a destructive effect on normal tissues. Several clinical trials are currently being planned to test such immunomodulators. One clinical trial will test the combination of vaccination with immune checkpoint blockade There is a trend for immunotherapy to be integrated into the multimodal treatment including radiotherapy and chemotherapy for patients with glioblastoma as the actions of the individual treatment modalities may fortify each other (121). Future clinical trials of brain tumor vaccines may incorporate this strategy.

Because, there are limitations on extensive use and repetition of current treatments: surgery, chemotherapy and radiation, the ideal therapeutic agent should be one that can continue to act until the tumor is eradicated. Among novel biotechnologies, gene therapy is usually a one-time treatment and cannot be repeated for residual and recurrent tumor. Among genetically modified microorganisms, bacteria appear to be more promising than viruses. Genetically modifies bacteria that selectively destroy glioblastoma while sparing normal brain tissue can be more precise than any surgical tool and can be left in until the tumor destruction is complete. The bacteria can then be killed with an antibiotic.

Multimodal therapy of glioblastoma will be needed as no single method is adequate. Surgery will remain as an essential part of this multimodal approach unless a non-invasive or minimally invasive tool is developed to replace it.

There are interindividual difference in response to the growth of glioblastoma and response to treatment as well as

variations in genomics of each tumor. Therefore, personalized approach to management of glioblastoma is important. Genetic and transcriptomic profiling of patient tumors by Next Gen Sequencing helps classify patients in to molecular subgroups for personalized approach of treatment. To this end, re-analysis of same genetic, and transcriptome data can help identify genetic or epigenetic alterations and discovery of new targets, that can be helpful in designing new drugs or nanoformulations that can be more efficient in drug delivery and efficacy (122).

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

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MINI REVIEW
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Epigenetic Targeting of Glioblastoma

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Glioblastoma is one of the first tumors where the biological changes accompanying a single epigenetic modification, the methylation of the *MGMT* gene, were found to be of clinical relevance. The exploration of the epigenomic landscape of glioblastoma has allowed to identify patients carrying a diffuse hypermethylation at gene promoters and with better outcome. Epigenetic and genetic data have led to the definition of major subgroups of glioma and were the basis of the current WHO classification of CNS tumors and of a novel classification based solely on DNA methylation data that shows a remarkable diagnostic precision.The reversibility of epigenetic modifications is considered a therapeutic opportunity in many tumors also because these alterations have been mechanistically linked to the biological characteristics of glioblastoma. Several alterations like *IDH1/2* mutations that interfere with "epigenetic modifier" enzymes, the mutations of the histone 3 variants H3.1 and H3.3 that alter the global H3K27me3 levels and the altered expression of histone methyltransferases and demethylases are considered potentially druggable targets in glioma and molecules targeting these alterations are being tested in preclinical and clinical trials. The recent advances on the knowledge of the players of the "epigenetic orchestra" and of their mutual interactions are indicating new paths that may eventually open new therapeutic options for this invariably lethal cancer.

Keywords: glioblastoma, epigenetics, therapy, DNA methylation, histone code

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INTRODUCTION

Glioblastoma (Glioblastoma Multiforme, GBM) is a rare tumor (Orphanet 360) that, being responsible for 4% of all tumor deaths and with a 5-years survival of 2%, is one of the deadliest human tumors (1) with the median survival ranging from 14 to 24–30 months depending from the molecular subtype of the tumor (2).

GBM, like other tumors, harbors many genetic alterations that interfere with cancer-related pathways (3), however clinical trials targeting molecular alterations in this tumor were largely unsuccessful so far (4–6). In the last 30 years, the only significant improvement in OS occurred with the introduction of Temozolomide (TMZ) in addition to surgery and radiotherapy (7, 8). GBM patients are stratified into two categories according to the methylation status of the O-6-methylguanine-DNA methyltransferase gene (*MGMT)* that repairs the DNA damages induced by TMZ and the patients whose tumor contains methylated *MGMT* have an overall survival of 21.7 months compared to the 12.7 months of those carrying unmethylated *MGMT* (9).

Epigenetic modifications are considered a key mechanism in GBM development (10). Epigenetic inheritance is mediated by the four deeply interconnected layers shown in **Figure 1**:

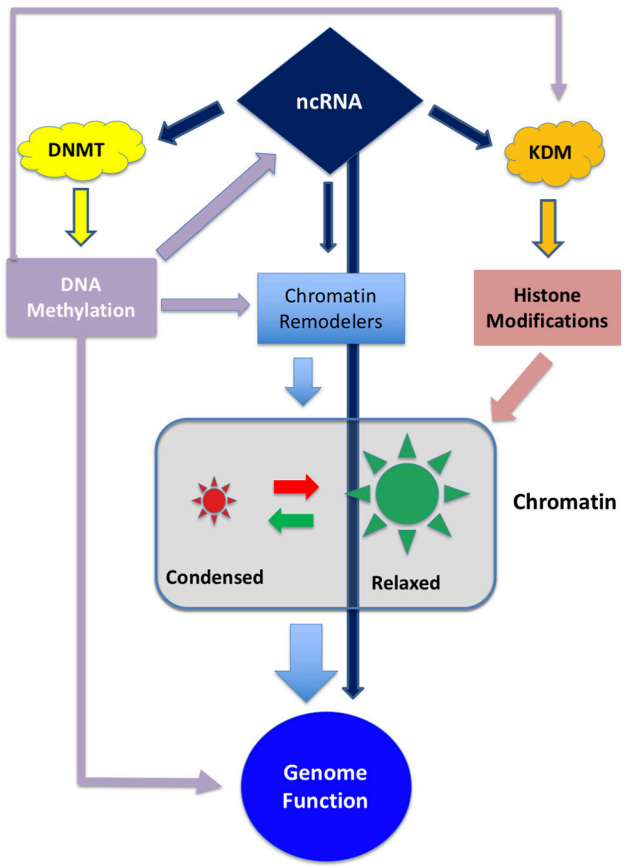

FIGURE 1 | Schematic representation of the interplay between the different epigenetic layers. ncRNA can directly influence genome activity by interfering with transcripts or, indirectly, by degrading transcripts involved in DNA methylation, histone modification or chromatin remodeling. On the other hand ncRNA can be epigenetically inactivated by DNA methylation (11, 12). DNA methylation can directly interfere with gene expression and, indirectly, can regulate the expression of chromatin and histone modifiers.

1- DNA methylation
2- Histone modifications
3- Chromatin remodeling
4- Non-coding RNA

These layers are controlled by a set of enzymes that act as "writers," "readers," and "erasers" that modify their target by adding, removing or regulating the interactions between proteins and DNA. Both DNA methylation and histone modification, along with chaperon molecules, participate to chromatin remodeling thus conferring an exquisite plasticity to the genetic apparatus (13, 14).

The latest WHO classification defines subgroups of glioma integrating genetic and epigenetic criteria (10, 15–18) (**Figure 2**) and a novel classification of CNS tumors based on DNA methylation data, shows a remarkable diagnostic precision being able to correctly modify the primary diagnosis in 12% of the cases (19). A large number of intrinsically reversible cancer-related modifications that are attractive targets of therapy was unveiled and the present review provides an overview of the most recent preclinical and clinical attempts to defy GBM through epigenetic reprogramming.

TARGETING EPIGENETIC ALTERATIONS IN GLIOBLASTOMA

Manipulating the epigenome has been lengthy considered a therapeutic opportunity in cancer. The epigenetic landscape of GBM was thoroughly explored, and many epigenetic modifications were mechanistically linked to the biological characteristics of this tumor and some of them were considered as therapeutic targets. At the moment, only the molecules acting on DNA methylation and histone methylation/chromatin remodeling were tested in clinical trials. Manipulation of ncRNA expression is restricted to pre-clinical studies and is not discussed in the present review.

Layer 1: DNA Methylation

Methylation of cytosine at C-5 within CpG doublets, is mediated by a set of DNA Methyltransferases that are responsible mainly but not exclusively of maintenance (DNMT1) and *de novo* DNA methylation (DNMT3a and 3b) to preserve genomic integrity (20). The human genome contains approximately 3 X 10^7 CpG doublets and although methylation at single doublets may, in principle, have functional consequences (21, 22), the biologically-relevant DNA methylation is that occurring at CpG clusters (CpG islands) in gene promoter regions and inversely correlates with gene transcription (23, 24). Intragenic CpG clusters are generally hypermethylated to prevent spurious initiation particularly at internal promoters.

In GBM DNA methylation is tightly linked to the response to TMZ treatment. The alkylating agent TMZ, the first-line chemotherapy for GB, methylates guanine in position N^7 and O^6 and Adenine in position N^3. O^6-methylguanine adducts lead to strand breaks, triggering p53-mediated apoptosis through the Fas/CD95/Apo-1 receptor in p53wt cells or through the mitochondrial pathway in p53mut tumors (25). The action of TMZ is counteracted by the MMR system and by the product of the *MGMT* gene that repairs the O^6 adducts that limit the activity of the drug (26). To mimic the effects of *MGMT* methylation, synthetic inhibitors of MGMT entered human trials (9, 27). However, several studies revealed that the MGMT inhibitors O^6-benzylguanine and PaTrim-2 (Lomeguatrib) did not improve the response rate to TMZ and increased the adverse effects of chemotherapy (27–30).

Inducing TMZ sensitivity in *MGMT*-unmethylated tumors with other molecules (Resvetrol, oncolytic viruses or by MGMT depletion) was tested only in preclinical models with alternate success (31). The correlation between *MGMT* methylation and MGMT protein expression is controversial and the lack of correlation seen in recent studies likely depends on the early method utilized for methylation analysis (32–34).

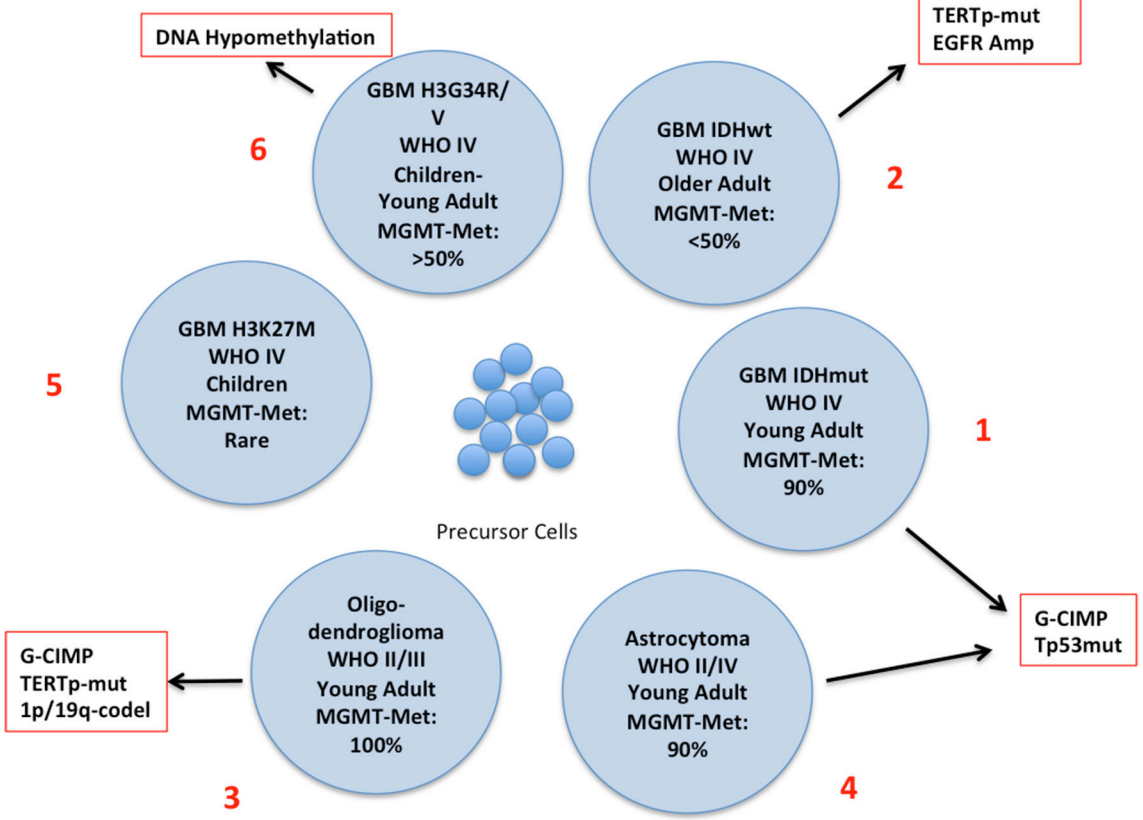

FIGURE 2 | Schematic representation of the different subtypes of glioma with the principal molecular and epigenetic characteristics. In this chart are represented the possible carcinogenic evolutions of the precursor cells. The major genetic and epigenetic alterations are reported along with the clinical characteristics of each subtype.

Layer 1: Methylator Phenotype and *IDH1/2* Mutations

The discovery of mutations of the Isocitrate Dehydrogenase (*IDH*) genes and of the DNA hypermethylation signature (*G*lioma *C*p*G* *I*sland *M*ethylator Phenotype: G-CIMP) has led to the definition of a distinct GBM subtype characterized by younger age and improved survival (2, 17, 18, 35) (**Figure 2**, N. 1). *IDH* mutations are rare in GBM developing in older patients who usually carry EGFR and PTEN alterations (primary GBM), (**Figure 2**, N. 2), but are present in a large proportion of low-grade glioma and, along with TP53 mutations, in high-grade glioma that evolved from low–grade tumors (secondary glioblastoma) (35) **Figure 2**, N. 3 and 4). IDH genes can be mutated at two mutually exclusive sites, R132 (*IDH1*) or R172 (*IDH2*) and these mutations have important metabolic consequences and are driving alterations in gliomagenesis. The product of *IDH* converts Isocitrate into αKetoglutarate (αKG) which is involved in a variety of cellular processes (**Supplementary Figure 1**). IDH mutants produce 2-Hydroxy Glutarate [2-HG] that is a competitive inhibitor of αKG-dependent dioxygenases including the histone demethylases JHDM1 and KDM4 and the DNA demethylase TET2. Thus, IDH mutations, that are not restricted to brain tumors, result in extensive epigenetic dysregulation including DNA and histone hypermethylation (36, 37) and altered cell differentiation (38). Other IDH mutations were occasionally found but only few of them produce 2-HG (39).

Strategies to target IDH-mutant tumors can be designed to either inactivate the functions of IDH mutants or to block the effects of 2-HG. The treatment with hypomethylating agents of mice xenografted with IDHmut GBM cells resulted in delayed tumor growth and improved survival (40, 41). Along this line phase I and II clinical trials were started to test two formulations of 5-azacytidine (NCT02223052) and the combination decitabine/immunotherapy (NCT02332889) in GBM and other solid tumors.

Normalizing the 2-HG concentration could reverse DNA hypermethylation and release the block of differentiation in IDH-mutated cells. Several inhibitors of mutated IDH1/2 were synthesized and showed to be effective in *in vitro* models (42–44); this finding was the starting point for a large series of clinical trials to assess the safety and bioavailability of the molecules under investigation in a variety of tumors, mainly AML, MDS and glioma (**Supplementary Table I**). Preliminary data on the

clinical efficacy of IDH inhibitors showed promising results in hematological malignancies opening the way for stringent randomized trials (45–47). As of June 2018, no public data are yet available for glioma patients.

Mutated IDH1/2 can be functionally considered as highly specific tumor-associated neoantigens that could be targeted by immunotherapy; a vaccine targeting mutant IDH1 showed antitumor activity in a glioma animal model opening the possibility of new experimental therapies (48).

Layer 2: Histone Modifications

Histones are subject to modifications that could either repress or activate transcription (**Supplementary Figure 2**). More than 100 enzymes act in concert to assemble a "code" of histone modifications that define the transcriptional properties of a given gene (49) determining drug response (50) and the development of cancer and other diseases (10, 51–53).

The rapid acquisition of drug resistance is a major cause of treatment failure in GBM (54) and could be explained by the development of epigenetically poised cells that undergo chromatin remodeling and display transient drug resistance (55–57).

Histone Acetylation

The addition of acetyl groups to certain lysines of H3 and H4 weakens the interaction between the core histones and DNA favoring the accessibility of the transcription apparatus. Deacetylation removes the acetyl groups provoking chromatin condensation and gene inactivation (49, 58). Acetylation and deacetylation are dynamic processes mediated by histone acetyltransferases (HAT) and histone deacetylases (HDAC) that maintain the balanced state of acetylation. Gain of HDAC expression has been found in many tumors, including GBM, and inhibitors of HDAC (HDACi) have been extensively explored for GBM therapy. HDACi have a large spectrum of antitumor activity and six HDACi have been approved by FDA: Vorinostat (11 studies concluded and 3 ongoing), Romidepsin (one study concluded), Belinostat (one study ongoing), Panobinostat (2 studies terminated before completion), Valproic acid (two studies terminated before completion and two recruiting) and Entimostat (no studies yet) (59) (https://clinicaltrials.gov, June 2018). Preclinical studies have shown that HDACi are very effective against GB cells, but the results of the clinical trials were largely disappointing. In adult patients Vorinostat was utilized as single agent and in combination with standard or biological therapies and in one study (NCT00238303) prolonged disease stabilization in a small subset of patients when used as single agent (60) but its addition to the standard radio/chemotherapy did not improve survival (61). Phase I/II trials with Romidepsin, with Panobinostat and anti-VEGF, or with Vorinostat and the proteasome inhibitor Bortezomib were either ineffective or toxic and were discontinued (62–64). Panobinostat however, is now being tested as a radiosensitizing molecule with promising results (65). Along this line phase II studies demonstrated that the addition of valproic acid to the standard radio/chemotherapy or to radiotherapy alone improved survival (66, 67). Randomized trials are necessary to confirm this finding.

Histone Methylation

Histone methylation was discovered along with histone acetylation (58) but its function remained obscure for many years because methylation does not change the DNA/protein interactions and it seemed an irreversible modification. With the discovery of LSD1 (KDM1), the first histone demethylase, it became clear that histone methylation is a reversible process (68) mediated by approximately 30 enzymes subdivided into distinct classes, and linked to a variety of physiological and pathological conditions including cancer, cardiovascular diseases, abnormal immune response and neurological disorders (51, 69). Histone methylation involves certain lysine and arginine of H3 and H4 and can either activate or repress transcription (**Supplementary Figure 2**).

In glioblastoma, histone methylation has distinct implications in pediatric and adult patients. Histone variant H3.3 (*H3F3A*) marks active chromatin domains and in pediatric tumors can be mutated at two sites: lysine 27 (K27M) and glycine 34 (G34R/V) (70, 71) (**Figure 2**, N. 5 and 6). In pediatric glioma the K27M mutation is restricted mostly to midline tumors whereas G34R/V is prevalent in hemispheric gliomas. K27M decreases methylation at K27 leading to transcriptional activation. G34R/V is associated with the redistribution of the activation mark H3K36 methylation and results in the upregulation of the oncogene *MYCN* (72) whose exogenous overexpression initiates glioma formation during development (73). *H3F3A*-K27M also inhibits the PRC2-EZH2 axis (2, 74), that acts as a histone methyltransferase, leading to the generalized loss of H3K27 methylation and to the CpG hypomethylator phenotype (CHOP) whose consequence is the aberrant activation of gene expression (75).

The methylation of H3K27 is regulated by PRC2-EZH2 methylases and by the UTX (KDM6A) and KDM6B demethylases; the effect of the K27M mutation could be reversed by inhibiting H3K27 demethylation. In an experimental model of diffuse intrinsic pontine glioma (DPIG), the small molecule GSK J4 was utilized to inhibit the activity of KDM6B (76). It was found that GSK J4 passes the Blood Brain Barrier and prolongs survival of mice xenografted with H3K27 tumors but not that of mice carrying WT H3.3 or G34R/V tumors. Although GSK J4 has proven to be effective in *in vivo* tumor models as single agent or synergically with HDACi (76–78), as of June 2018, clinical trials employing this or similar molecules have not been launched yet.

Targeting EZH2 is another mechanism to modulate histone methylation and to reverse tumor growth (79). Several FDA-approved EZH2 inhibitors are available (Tazemetostat, CPI-1205, GSK2816126) and others are in advanced pre-clinical testing. More than 20 trials that include EZH2 inhibition are reported in the Clinical Trial database mostly aimed at hematological disorders. As of June 2018, most studies are still ongoing and recruiting. However, studies with Tazemetostat

(NCT03213665; NCT03217253) were suspended because of adverse events and one study with GSK2816126 (NCT02082977), was interrupted because of insufficient evidences of clinical response.

Mutations of H3.3-*H3FA* are uncommon in adult GBM where H3.3 can be functionally inactivated by the *MLL5* gene that is overexpressed in GBM stem cultures (80). Finally, it was found that GSK J4, like in pediatric GBM, has strong suppressive effects on cell viability and self-renewal properties (80, 81).

Several histone demethylases are constitutively or transiently overexpressed in adult GBM. LSD1 (KDM1) is FAD-monoamine oxidase that demethylates several lysine of H3 (K4, K9, K27, and K36). KDM1 interacts with non-histone substrates and inhibits p53 activity by demethylating K370me1 and by inhibiting the interaction with the coactivator 53BP1 (82). Inhibitors of KDM1 derive mainly from MAO inhibitors utilized in the clinical practice and are strong suppressors of tumor cell proliferation *in vitro* and in animal models (83). Most KDMi are non-selective for KDM1 and have additional irreversible activity on MAO. As of June 2018, three molecules were approved by FDA for clinical utilization (GSK2879552, IMG-7289 and INCB059872) in addition to the antidepressants Tranylcypromine and Phenelzine whose antitumor activity is being explored in phase I trials. Some of these trials were prematurely concluded because of toxicity and low efficacy while others are still ongoing.

In GBM, recurrence occurs from residual cells at the margin of resection that rapidly acquire radio- and chemo-resistance during treatment and cannot be efficiently counteracted by other drugs.

The induction of drug resistance is accompanied by the overexpression of several *KDM* genes. Indeed it was shown that upon treatment, a restricted population of slow-cycling cells undergo epigenetic, thus reversible, changes that result in drug resistance and sustained tumor growth (55, 56). A key effector of this mechanism is the H3K4 demethylase KDM5A gene whose exogenous expression or inactivation mimics drug resistance and sensitivity in different tumors including GBM (55, 56, 84, 85). Overall many pre-clinical and clinical evidences indicate that the entire KDM5 family, as well as other KDMs are emerging targets in cancer therapy (69, 86–89).

The pan-KDM inhibitor JIB 04 is maximally active against KDM5A but, at lower efficacy, inactivates also other KDMs found overexpressed in TMZ-resistant GBM cells (85) and has a strong antitumor effect (90). JIB 04 was utilized in a model of acquired TMZ resistance and shown to ablate TMZ-resistant cells, to synergize with TMZ at clinically-relevant concentrations and finally, in a pilot experiment, to have promising activity *in vivo* (91). Similar effects were obtained with CPI-455, a selective inhibitor of KDM5 (92) but at a concentration difficult to reach *in vivo* (91, 92). Similarly, NSCLC cells that acquired resistance to taxane/platinum combinations became sensitive to JIB 04 and GSK J4 that reverted, at least in part, the transcriptional program of resistant cells to that of drug-naïve cells and synergize with standard chemotherapy as JIB 04 and Temozolomide (93). As of June 2018, none of these promising molecules is being tested in clinical trials.

Layer 3: Chromatin Remodeling

Changing chromatin conformation regulates accessibility to transcription factors, to the DNA replication and repair machineries. The proper chromatin conformation is determined by histones and their modifications and by the chromatin remodeling complexes that include the histone modifiers described in section Layer 2: Histone Modifications and the ATP-dependent chromatin remodeling complexes (SWI/SNF; ISWI; CHD and INO80) (94–96). These complexes include many components that play essential and redundant roles in normal cells and that are variably altered in most human cancers (97). Because of their complexity, chromatin remodelers are very difficult targets for drug discovery and the identification of their synthetic inhibitors is still in its infancy (94). The tumor suppressor SWI/SNF complex was the first chromatin remodeler discovered, is mutated in more than 20% of the tumors (97, 98) and is involved in the maintenance of stemness in glioma cells (99). The effects of SWI/SNF inactivation can be counteracted by inhibitors of the TK pathway and of NF-kB (100, 101); however, as outlined previously, these targeted therapies were unsuccessful in GBM patients. PARP-1 polymerase is involved in chromatin remodeling mechanisms through histone modification and inhibition of the ISWI complex (102). Two PARP inhibitors (Oliparib and Veliparib) were recently licensed by the FDA for ovarian cancer treatment and several other experimental molecules are undergoing extensive testing in humans and in animal models (103). For GBM, the NIH Clinical Trials Database reports seven ongoing or completed trials with Olaparib (104, 105) (NCT01390571, NCT03212274, NCT02974621) Veliparib (NCT02152982, NCT03581292, NCT01514201) and with BSI-201 (NCT00687765). The results of these studies were not yet disclosed.

Targeting of histone chaperon molecules in glioblastoma is just beginning to be explored, however promising results in animal models were obtained by targeting FACT, a nucleosome reorganization protein (106) with CBL0137 in combination with TMZ (107).

CONCLUSIONS

Despite all the progresses in medicine, the median survival of GBM patients has not substantially improved, likely because this tumor rapidly becomes radio- and chemo-resistant and infiltrates the surrounding brain tissue making impossible the complete surgical eradication. To overcome this deadlock many experimental therapies were devised but none of them met the expected results. Epigenetic modifications are gaining strong relevance in glioblastoma because they can be either clinical biomarkers for the optimal stratification and classification of the patients and because they can be also potential drug targets as suggested by many preclinical trials. Molecules with epigenetic effects can potentially modulate the plasticity of the tumor environment in glioma and may drive the changes of the epigenomic environment restoring or rendering more susceptible the tumor cells to standard chemotherapy rather

than be used as a monotherapy. In this respect the timing and the scheduling of the epigenetics and cytotoxic drugs could be crucial for the best clinical result and should be carefully defined on the basis of the chemical, biological and cellular effect of these treatments (91). Certainly, the addition of proteomic and metabolomic approaches to the extensive epigenomic and transcriptomic studies already conducted will have the capacity to unveil the inner mechanisms of glioma biology allowing the design of more effective drugs.

AUTHOR CONTRIBUTIONS

MR conceived the idea of this mini-review article and wrote the first draft. BB, MP, and MR equally participated to the final writing of the article.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2018.00448/full#supplementary-material

Supplementary Figure 1 | IDH1/2 pathways. Metabolic pathways involving IDH1 (cytoplasmic) and IDH2 (mitochondrial). IDH1/2 (wt) converts Isocitrate into αKetoglutarate (αKG) while the mutated forms convert Isocitrate into 2-hydroxyglutarate that competitively inhibits αKG-dependent dioxygenases including the histone demethylases JHDM1 and KDM4 and the DNA demethylase TET2

Supplementary Figure 2 | Schematic representation of the major H3 and H4 modifications and their functional role. In green and red the activating and the repressive modifications, respectively.

Supplementary Table 1 | Clinical trials employing FDA-approved IDH1/2 inhibitors.

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Immune Checkpoints and Innovative Therapies in Glioblastoma

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Targeting the Immune Checkpoint molecules (ICs; CTLA-4, PD-1, PD-L1/2, and others) which provide inhibitory signals to T cells, dramatically improves survival in hard-to-treat tumors. The establishment of an immunosuppressive environment prevents endogenous immune response in glioblastoma; therefore, manipulating the host immune system seems a reasonable strategy also for this tumor. In glioma patients the accumulation of $CD4^+/CD8^+$ T cells and Treg expressing high levels of CTLA-4 and PD-1, or the high expression of PD-L1 in glioma cells correlates with WHO high grade and short survival. Few clinical studies with IC inhibitors (ICis) were completed so far. Notably, the first large-scale randomized trial (NCT 02017717) that compared PD-1 blockade and anti-VEGF, did not show an OS increase in the patients treated with anti-PD-1. Several factors could have contributed to the failure of this trial and must be considered to design further clinical studies. In particular the possibility of targeting at the same time different ICs was pre-clinically tested in an animal model were inhibitors against IDO, CTLA-4 and PD-L1 were combined and showed persistent and significant antitumor effects in glioma-bearing mice. It is reasonable to hypothesize that the immunological characterization of the tumor in terms of type and level of expressed IC molecules on the tumor and TIL may be useful to design the optimal ICi combination for a given subset of tumor to overcome the immunosuppressive milieu of glioblastoma and to efficiently target a tumor with such high cellular complexity.

Keywords: glioblastoma, therapy, immune checkpoint, CTLA-4, PD-1, PD-L1

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INTRODUCTION

Since the discovery in 2005 of the clinical utility of Temozolomide in glioblastoma (GBM) patients (1), no other cytotoxic drug was added in the standard treatment protocols. In the meantime our knowledge on the molecular mechanisms deranged in GBM has had impressive advancements and the possibility of targeting these pathways has been extensively exploited in the hope to improve the current standard of care (2–4). Differently from many other tumors, in GBM the promises of molecularly targeted therapies against oncogenic alterations did not meet success in phase I/II and III trials even though they were highly promising in preclinical models; thereafter they have limited clinical utilization (5). The lack of success of targeted therapies and the limited activity of standard cytotoxic treatments in GBM, reside in the cellular complexity and clonal evolution of this tumor (6, 7). Moreover, many molecules that display strong antitumor activity *in vitro* against glioma cells and that are utilized for the therapy of other tumors, are ineffective *in vivo* because they cannot pass through the Blood Brain Barrier (BBB), or because of drug efflux, intrinsic or

rapidly developing drug resistance and, last but not least, the presence of a pool of cancer cells with stemness characteristics (7).

In the recent years, targeting the so-called Immune Checkpoint molecules (ICs) which provide inhibitory signals to T cells, has offered new exciting treatment opportunities in cancer (8). Inhibition of autoreactive $CD8^+$ T-cells through ICs is a physiological mechanism to prevent autoimmunity; on the other end, this mechanism inhibits the immune response against aberrant cancer cells. Differently from conventional cytotoxic or from targeted therapies that are aimed at the cancer cells, the therapies that involve the modulation of ICs attempt to redirect the function of the immune system to elicit cancer cell death. Several checkpoint molecules capable to shut down the response against neo-antigens are present on T cells as well as on tumor cells. These molecules are at the center of regulatory networks that result in immunosuppression. Antibodies against the "classic" IC molecules (CTLA-4, PD-1, PD-L1, and PD-L2) are considered the "first generation" IC inhibitors (ICis) that interfere with the immune escape of tumor cells, followed by second and third generations ICis targeting other immunoregulatory molecules and pathways (9, 10).

Immune checkpoints inhibition dramatically improved survival in hard-to-treat tumors like lung cancer and melanoma so that the therapy with IC inhibitors (ICis) has entered in the standard clinical practice for these tumors whereas clinical trials have been launched for many other tumors (8, 10).

BLOOD-BRAIN BARRIER, IMMUNOLOGICAL MECHANISMS AND IMMUNE CHECKPOINTS INTERPLAY IN GLIOBLASTOMA

For many years the CNS has been considered as an immune-privileged compartment with the BBB responsible to maintain a constant brain microenvironment from metabolic insults and, at the same time, physically blocking or actively favoring the transport of bioactive molecules. During the development of glioma, the integrity of the BBB is preserved up to a tumor size of ∼2 mm^3; above that, the angiogenetic pressure elicited by GBM releases the tight and adherent junctions between the cerebral endothelial cells allowing the passage of molecules up to 12 nm (11, 12). With further tumor growth the BBB becomes freely permeable to larger molecules. Nevertheless, tumor cells in niches at the boundary of the surgically excised tumor remain protected by the BBB reducing the efficacy of the treatment. Beside immune cells (13), several cell types in the brain (microglia, astrocytes) can act as antigen-presenting cells and elicit immune response against the tumor. This mechanism is aided by the permeability of the damaged BBB that enables the passage of tumor antigens outside the brain (14–16). Similarly to other tumors, GBM is associated with significant immunosuppression particularly in the T-cell compartment (17) because of the combined effect of steroid/cytotoxic treatment, the downregulation of MHC-I antigens and of the secretion of immunosuppressive cytokines. Moreover, also in glioma, the correct maintenance of the physiological status of immunological tolerance and response is mediated by the coordinate interplay of many actors, including IC molecules, and different cell types as summarized below and in **Figure 1**.

CTLA-4

CTLA-4 (CD152) was the first immunoregulatory molecule to be targeted for therapeutic purposes utilizing the humanized antibody Ipilimumab approved by FDA and EMA in 2011, initially for melanoma (18), soon followed by Tremelimumab for mesothelioma (19, 20). CTLA-4, expressed on T-cells (activated and regulatory), interacts with its ligands CD80 and CD86 on APCs to inhibit co-stimulators T-cell pathways (21). In GB the expression of CTLA-4 on $CD4^+$ and $CD8^+$ cells is strongly inversely correlated with outcome (22).

PD-1/PD-L1

PD-1 on T cells and its ligand PD-L1 on APC and tumor cells are the most important immunosuppressive molecules so far identified. Their interaction leads to the suppression of early T-cell activation, abolishing their cytotoxic activity and interferes with the production of inflammatory cytokines (23, 24). Two PD-1 suppressive Ab were licensed in 2014 (Nivolumab) and in 2016 (Pembrolizumab) and two anti-PD-L1 Ab: Atezolizumab in 2016 and Avelumab in 2017. Their original indications were rapidly extended to other tumors and many clinical trials with newer molecules are ongoing (8, 25, 26). The expression of PD-L1 on glioma cells has been documented as well as that of PD-1 on tumor infiltrating lymphocytes (TIL). The functional and clinical implications of PD-1/PD-L1 expression in GBM are still unclear. Indeed, no correlation between PD-L1 expression and overall survival was seen in two cohorts (27, 28). On the other hand, in another study, PD-L1 staining and PD-1/PD-L1 expression were associated with decreased survival (29). The absence of standardized experimental parameters, of defined cut-off values and the heterogeneity of the cohorts might explain these contrasting findings. Interestingly PD-L1 expression is directly correlated with WHO grade and within Grade IV tumors, PD-L1 expression is significantly higher in IDH1/2 wt tumors compared to IDH1/2 mutated or hyper methylated GBM (27). Overall, the expression of PD-L1 is linked to well-known negative prognostic indicators in GBM and its effect on survival must be examined in homogeneous cohorts.

Tim-3

Tim 3 is a molecule expressed by CD4+ and CD8+ T cells that, similarly to PD-1, is involved in immune suppression and promotes tumor escape through the exhaustion of T cells (30). A large proportion of TILs in GBM and other tumors is composed by T cells not capable of cytokine secretion and not exerting their physiological function. In GBM the overexpression of TIM-3 is associated with higher malignancy (higher grade, lower

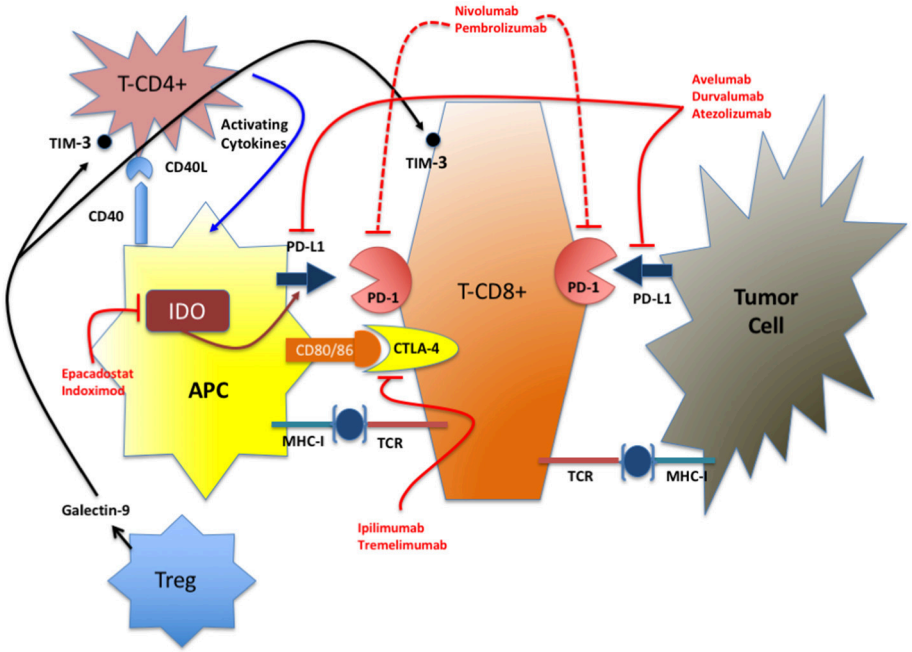

FIGURE 1 | Simplified representation of the IC network. In red are indicated the FDA-approved drugs and the IDO inhibitors in advanced stage of clinical test (phase III). TIM-3 inhibitors are at an early stage of development for clinical use (phase I).

Karnofsky score, and IDHwt) and is thus considered a strong negative prognostic indicator (31, 32).

IDO

Although IDO is not a classical immune checkpoint molecule and lacks receptorial capacity, it is included in this functional class because of its suppressive properties on T-cell activation and NK cell function (33). Similarly to TIM-3, IDO overexpression is linked to poorer outcome in GBM patients (34) and targeting IDO with Epacadostat or Indoximod (35), was a successful experimental strategy in *in vivo* models (36).

GENETIC AND EPIGENETIC FACTORS DETERMINE THE FUNCTIONALITY OF IC MOLECULES

Targeting IC molecules with blocking antibodies alone or in combination with other ICi therapies, or with standard chemotherapy has revolutionized the therapeutic approach to lung cancer and other hard to treat tumors. Nevertheless, along with very favorable response rate, other patients are unresponsive to the therapy or show life-threatening side effects. Predicting the response and the appearance of major side effects during treatment is a major health issue. Limiting certain therapies to patients likely to respond and strict monitoring of patients at risk have several ethical implications and could enable the Public Health Systems to offer the best available therapy to the patients who could really benefit from it. However, reliable biomarkers predicting response or adverse reactions to ICi therapy are not yet available.

Single nucleotide variations (SNVs) of IC genes can affect the expression levels of IC molecules thus altering immune tolerance and leading to increased susceptibility to autoimmune diseases or to reduced immunological response against cancer cells.

A meta-analysis that included 12 studies and more than 5,000 tumor patients and an equal number of healthy controls showed a decreased cancer risk for TT homozygous individuals at polymorphism PD-1.5 (rs2227981) and, for Asian populations, a decreased risk was seen for AG individuals and an increased risk for AA, at PD-1.3 (rs11568821) (37). PD-1.5 allele frequencies and risk of low- and high-grade glioma development were examined in 156 mid-Eastern patients and significantly higher frequency of the PD1.5 C/T and T/T genotype were found in high-grade glioma compared to low-grade tumors and control individuals (38).

Some studies, described below, have examined the clinical impact of IC polymorphisms in tumor and autoimmune disease patients treated with ICi. Years ago, it was shown that SNVs in *CTLA-4* may affect the transcriptional activity of the gene as well as the interaction with CD80 and influence immune response in autoimmune diseases (39, 40). SNV of *CTLA*-4 are implicated in clinical response and survival in melanoma patients (41, 42) and, more recently, SNV-1577G > A and SNV CT60G > A were linked to a better response to Ipilimumab in a 173-patients cohort (43) and SNV-1661A > G to the onset of endocrine adverse events (44).

In NSCLC *CTLA-4*, *PD-1,* and *PD-L1* were examined in two studies that involved more than 400 patients (21, 45). Overall three *PD-L1* SNV (rs2282055G > T; rs4143815C > G, and rs2297136T > C) were significantly associated with a better overall response rate and improved OS and PFS when treated with chemotherapy alone or with Nivolumab as second or third line of treatment after chemotherapy.

Overall these results indicate that genetic variations in IC molecules can be utilized as conventional biomarkers predictive of response to treatment and outcome to optimize patients' treatment. This is particularly important also in view of the availability of new drugs whose efficacy and toxicity may be genetically-dependent and whose utilization requires a "personalized approach" to cancer treatment.

Studies on hematologic disorders like myelodysplastic syndrome (MDS) treated with inhibitors of DNA methyltransferases demonstrated the up-regulation of PD-1 as a consequence of the therapy (46, 47). High expression of PD-1 is a negative prognostic indicator and it has been proposed that the treatment of MDS with hypomethylating agents should be coupled with the blockade of ICs.

The analysis of several solid tumors demonstrated that epigenetic mechanisms regulate the expression of IC molecules and that methylation of PD-1 and PD-L1 promoters is associated with worse outcome (48–51).

The immunological landscape of glioma is influenced by IDH1/2 mutations; indeed, in mutated tumors PD-L1 is significantly diminished supporting the rationale of ICi treatment in IDH1/2wt patients (52). Besides DNA methylation, other epigenetic modifiers may influence IC expression in glioma. Namely, miRNAs may directly impact CTLA-4, PD-1, and PD-L1 expression through a series of miRNA like mir-155 (CTLA-4), mir-138 (CTLA-4 and PD-1), miR-424 (PD-L1 and CD80), mir-28 (PD-1), miR-34a, miR-200 miR-513, and miR-138-5p (PD-L1).

Moreover, the same or other miRNA regulate the expression of cytokines like IFN-γ or transcription factors that are positive or negative regulators of IC generating a redundant and extremely complex network. The interaction between IC molecules, and miRNA have been recently shortly reviewed (53).

IMMUNE CHECKPOINT BLOCKADE IN GBM: PRECLINICAL FINDINGS

Several preclinical trials conducted utilizing two immunocompetent animal models (GL261/C57Bl/6 and SMA560/VM/dk) (54) demonstrated that IC blockade utilizing ICi as single agent or in combination significantly prolongs survival at an extent that depends on the molecule, or their combination. In one study, CTLA-4 blockade alone resulted in 80% of long survivors (55) whereas in two others the percentage of long survivors was 40 and 25% (28, 56). PD-1 blockade alone resulted in 56% long survivors in one study (57) but had no effect in another study unless associated with radiotherapy (15–40% long survivors) (58). PD-L1 blockade was tested in two studies leading to 60% (57) and 25% long survivors (56). Only one study examined the effect of TIM-3 blockade with no effect on survival (59). The effect of the therapy was strongly augmented when different ICis were utilized in combination or with standard therapy. Two studies in murine models demonstrated that the combination of radiation therapy and PD-1 and/or TIM-3 exerted a strong antitumor response over the treatment with a single agent and the maximal activity (100% long survivors) was seen when PD-1 and TIM-3 inhibition were combined with stereotactic radiosurgery (58, 59). Another study described the effects of the concomitant CTLA-4/PD-L1/PD-L2 inhibition that resulted in 75% long survival (56). Finally, disabling the entire IC network (CTLA-4/PD-L1/IDO) (57) or the dual IC blockade (TIM-3/PD-1) coupled with radiosurgery (59), both resulted in the survival of 100% of the treated mice. Importantly in all these treatments it was possible to demonstrate the activation of the immune system within the tumor (cytokines production, TIL activation, etc.) and sustained anti-tumor immune response since regrowth of the tumor was not observed after tumor cell re-challenge.

Overall, these preclinical models support the rationale for disabling many components of the IC network in conjunction with standard therapies for an efficient glioma treatment. Moreover, since the concomitant utilization of several ICis could increase the risk of life threatening adverse effects, the need of identifying molecular markers predicting response and therapy-induced toxicity, as previously mentioned, appears of the utmost importance for the clinical utilization of combined IC treatment.

IMMUNE CHECKPOINT BLOCKADE IN GBM: CLINICAL TRIALS

The successful preclinical trials and the very favorable results obtained with other tumors like NSCLC and melanoma, set the basis for the utilization of ICis in many other tumors including GBM. The survey of the NIH Clinical Trials Database (https://www.clinicaltrials.gov) performed on July 2018, showed 60 registered trials; only two of them were completed (**Table 1**). One of the completed studies (NCT01860638) is a phase II randomized study to test the safety of the combination Bevacizumab/Lomustine as second line treatment followed by Nivolumab as third line. The primary endpoint of the study that enrolled 296 patients and ended in 2017 was OS but the results were not made available to the public. The second completed study was NCT02550249, a phase II study that enrolled 29 patients and had as primary outcome the evaluation of the expression of PD-L1 in tumor cells and lymphocytes upon treatment with Nivolumab. Also, in this case the results are not available.

The ongoing studies (mostly phase I or II) test several ICi molecules as single agents or in various combinations with standard cytotoxic molecules, targeted therapies, or other immunological therapies and are aimed, not only at determining the clinical utility of these molecules, but also

TABLE 1 | Clinical trials with IC inhibitors in glioma (July, 2018).

Target	Clin. Trial ID	Molecule	Disease	Phase	Patients	Status	Year S/E
CTLA-4	NCT03460782	Ipilimumab	Glioblastoma	I	?	?	2018/?
PD-1 + CTLA-4	NCT03430791	Nivolumab + Ipilimumab	Glioblastoma	II	60	Not yet recruiting	2018/2021
	NCT03233152	Ipilimumab + Nivolumab	Glioblastoma	I	6	R	2016/2019
	NCT02017717	Ipilimumab + Nivolumab + Bevacizumab	Glioblastoma	III	626	A-NR Data available (Ref. 59)	2013/2018
	NCT03367715	Ipilimumab + Nivolumab	Glioblastoma *MGMT* Unmeth	II	24	R	2018/2020
	NCT02311920	Ipilimumab + Nivolumab + TMZ	Glioblastoma Gliosarcoma	I	32	A-NR	2015/2018
	NCT03425292	Ipilimumab + Nivolumab + TMZ	Glioblastoma	I	45	R	2018/2020
	NCT03422094	Ipilimumab + Nivolumab + personalized vaccine (NeoVax)	Glioblastoma	I	30	Not yet recruiting	2018/2023
CTLA-4 + PD-L1	NCT02794883	Tremelimumab + Durvalumab	Glioblastoma	II	36	R	2016/2019
PD-1	NCT01952769	Pidilizumab	DPIG	I/II	50	A-NR	2014/2019
	NCT02359565	Pembrolizumab	DPIG and other brain tumors	I	110	R	2015/2020
	NCT02529072	Nivolumab + Dendritic cell vaccine	Glioblastoma	I	7	A-NR	2015/2017
	NCT03576612	Nivolumab + immunostimulator	Glioblastoma	I	36	A-NR	2018/2022
	NCT03557359	Nivolumab	IDHmut GB	II	37	A-NR	2018/2021
	NCT03347097	PD-1 producing pluripotent killer cells	Glioblastoma	I	40	R	2017/2018
	NCT02311582	Pembrolizumab + laser ablation	Glioma	I/II	58	R	2015/2021
	NCT02658981	Nivolumab + anti-LAG-3	Glioblastoma	I	100	R	2016/2020
	NCT02852655	Pembrolizumab	Glioblastoma	NA	35	A-NR	2016/2021
	NCT02335918	Nivolumab + Varilumab	Glioblastoma solid tumors	I/II	175	A-NR	2015/2020
	NCT02526017	Cabiralizumab + Nivolumab	Glioblastoma solid tumors	I	295	A-NR	2015/2019
	NCT03058289	INT230-6 (cytotoxic carrier, intratumor) + Nivolumab	Glioblastoma solid tumors	I/II	60	R	2017/2020
	NCT01860638	Bevacizumab + Lomustine + Nivolumab + TMZ + Radiotherapy	Glioblastoma	III	296	C - No results available	2013/2017
	NCT03014804	Dendridic cell vaccine + Nivolumab	Glioblastoma	II	30	To be started	2018/2020
	NCT03493932	Nivolumab + Anti-LAG-3	Glioblastoma	I	15	R	2018/2021
	NCT02798406	Oncolytic Adenovirus (intratumor) + Nivolumab	Nervous System Tumors	II	48	R	2016/2020
	NCT02937844	Chimeric T cells armed with PD-1 and CD28 to activate T cells and kill PD-L1+ tumor cells	Glioblastoma	I	20	R	2016/2019
	NCT03173950	Nivolumab	Brain tumors not GB	II	180	R	2017/2021
	NCT03170141	CAR-T cells	Glioblastoma	I	20	R by invitation	2017/2020
	NCT03491683	Cemiplimab + immunomodulators INO-5401 and INO-9012	Glioblastoma	I/II	52	R	2018/2021
	NCT02829931	Nivolumab + radiotherapy	Glioblastoma	I	26	S by the Company	2016/2020
	NCT02550249	Nivolumab	Glioblastoma	II	29	C - No results available	2015/2017
	NCT02648633	Nivolumab + Valproic Acid	Glioblastoma	I		WT	2016/2017
	NCT03452579	Nivolumab + Bevacizumab	Glioblastoma	II	90	R	2018/2018

(Continued)

TABLE 1 | Continued

Target	Clin. Trial ID	Molecule	Disease	Phase	Patients	Status	Year S/E
	NCT02667587	Nivolumab + TMZ + radiotherapy	Glioblastoma	III	693	R	2026/2023
	NCT02617589	Nivolumab + TMZ + radiotherapy	Glioblastoma	III	550	R	2016/2019
	NCT03311542	Pembrolizumab	Glioblastoma Melanoma	?	?	?	2017/?
	NCT02313272	Pembrolizumab + Bevacizumab + radiotherapy	Glioblastoma	I	32	A-NR Data Available (Ref: 62)	2015/2019
	NCT02054806	Pembrolizumab	Glioblastoma and many solid tumors	I	477 (26 GB)	A-NR Data Available (Ref: 61)	2014/2018
PD1 + IDO	NCT03491683	Epacadostat + Nivolumab	Glioblastoma	I/II	52	R	2018/2021
PD-L1	NCT02968940	Avelumab + radiotherapy	Glioblastoma IDHmut	II	43	R	2017/2019
	NCT03291314	Avelumab + Axitinib	Glioblastoma	II	52	R	2017/2018
	NCT02866747	Durvalumab + radiotherapy	Glioblastoma	I/II	62	R	2017/2020
	NCT03341806	Avelumab + lasertherapy	Glioblastoma	I	30	R	2018/2020
	NCT02336165	Durvalumab + radiotherapy + Bevacizumab	Glioblastoma	II	159	A-NR Data Available (Ref:63)	2015/2018
	NCT03047473	Avelumab	Glioblastoma	II	30	R	2017/2019
	NCT03174197	Atezolizumab + TMZ	Glioblastoma	I/II	60	R	2017/2021
	NCT03158389	Atezolizumab + targeted therapy with various molecules	Glioblastoma	I/II	350	R	2018/2024
IDO	NCT02052648	Indoximod + radiotherapy + TMZ + Bevacizumab	Glioblastoma	I/II	160	A-NR	2014/2018
	NCT02502708	Indoximod + TMZ + radiotherapy + other cytotoxic drugs	Pediatric brain tumors	I	115	R	2015/2019
	NCT02764151	PF-06840003	Brain tumors	I	17	A-NR	2016/2018

Data taken from https://www.clinicaltrials.gov

R, Recruiting; C, Completed; A-NR, Active Not Recruiting; AC, Accrual completed; NI, Not Indicated; WT, Withdrawn; S, Suspended; Year S/E, Year Start/End.

at determining the safety of the treatment and are expected to be completed starting from 2019 but mostly after year 2020.

The only study with published results is NCT02017717 (CheckMate 143), a large phase III randomized trial that enrolled over 600 patients. This study was the first large trial where the effect of IC inhibitors was stringently tested. Encouraging results were initially obtained in one of the study arms where three patients showed partial response and 8 disease stabilization with the combination Nivolumab+Ipilimumab (60), however when the study was extended, this arm was closed because of the treatment failure (61).

Two other large phase III randomized trials (NCT02617589 and NCT02667587) are testing the effect of Nivolumab on MGMT methylated or unmethylated patients and the results of these studies are expected in 2019 and 2023, respectively.

Phase Ib trial NCT02054806 tested the safety and efficacy of the PD-1 inhibitor Pembrolizumab on a large series of solid tumors. In the GBM arm (26 patients), one partial response and 12 disease stabilization were observed (62).

Phase I trial NCT02313272 tested the effect of the addition of Pembrolizumab to Bevacizumab and radiotherapy. The initial results were encouraging since more than 50% of the patients at 6 months showed partial or complete response (63).

Finally, phase 2 trial NCT02336165, the PD-1 inhibitor Durvalumab was tested in combination with Bevacizumab and radiotherapy and showed partial response or disease stabilization in 60% of the patients after 6 months. Four patients remained progression free (64).

CONCLUSIONS

Targeting ICs has revolutionized the therapeutic approach to certain tumors. There is strong hope that this therapy could be effective also for GBM patients. Indeed, the preclinical trials and the initial results obtained in some phase I/II studies suggested that ICis could offer new therapeutic options to these patients. The results of the first large phase III trials were somehow disappointing and inhibiting PD-1 could not fully restore the host immune response (61).

Nevertheless, the treatment with Nivolumab doubled the response to therapy in 8% of the patients (11.1 months vs. 5.3 with Bevacizumab) (61). Moreover, the high levels of VEGF seen in GBM are strongly immunosuppressive and this effect should be better counteracted. In this respect, targeting multiple IC pathways also in combination with cytotoxic drugs could be

a winning strategy. The results of the two ongoing phase III trials and of the phase I/II trials where combination therapies are explored may provide new weapons against this rapidly and invariably deadly cancer.

AUTHOR CONTRIBUTIONS

MR conceived the idea of this mini-review article and wrote the first draft. BB, MP, RC, AM, and MR equally participated to the final writing of the article.

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REVIEW
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Developments in Blood-Brain Barrier Penetrance and Drug Repurposing for Improved Treatment of Glioblastoma

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Glioblastoma (GBM) is one of the most common, deadly, and difficult-to-treat adult brain tumors. Surgical removal of the tumor, followed by radiotherapy (RT) and temozolomide (TMZ) administration, is the current treatment modality, but this regimen only modestly improves overall patient survival. Invasion of cells into the surrounding healthy brain tissue prevents complete surgical resection and complicates treatment strategies with the goal of preserving neurological function. Despite significant efforts to increase our understanding of GBM, there have been relatively few therapeutic advances since 2005 and even fewer treatments designed to effectively treat recurrent tumors that are resistant to therapy. Thus, while there is a pressing need to move new treatments into the clinic, emerging evidence suggests that key features unique to GBM location and biology, the blood-brain barrier (BBB) and intratumoral molecular heterogeneity, respectively, stand as critical unresolved hurdles to effective therapy. Notably, genomic analyses of GBM tissues has led to the identification of numerous gene alterations that govern cell growth, invasion and survival signaling pathways; however, the drugs that show pre-clinical potential against signaling pathways mediated by these gene alterations cannot achieve effective concentrations at the tumor site. As a result, identifying BBB-penetrating drugs and utilizing new and safer methods to enhance drug delivery past the BBB has become an area of intensive research. Repurposing and combining FDA-approved drugs with evidence of penetration into the central nervous system (CNS) has also seen new interest for the treatment of both primary and recurrent GBM. In this review, we discuss emerging methods to strategically enhance drug delivery to GBM and repurpose currently-approved and previously-studied drugs using rational combination strategies.

Keywords: GBM, glioblastoma, Blood-brain barrier, repurposed drugs, recurrent GBM, pharmacotherapy

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INTRODUCTION

Glioblastoma (GBM) is the most common and deadly primary brain tumor in adults. The World Health Organization (WHO) classifies GBM as a grade IV astrocytoma, which carries a dismal prognosis, resulting in an ∼30% survival rate over 1 year, with ∼3–5% of patients surviving beyond 5 years (1, 2). Upon diagnosis, patients undergo maximal safe surgical resection to

remove the bulk of the tumor, followed by radiotherapy (RT) and concomitant oral chemotherapy using the DNA-alkylating agent temozolomide (TMZ) (3, 4). Unfortunately, GBM cells are invariably left behind following surgery due to their highly invasive nature. The residual, invasive tumor cells contribute to near universal tumor recurrence (5–8). There are few effective treatment options for patients with recurrent GBM that prolong lifespan and the median survival rate remains at ∼8 months (9). Numerous clinical trials aimed at treating recurrent GBM have failed to improve survival due to unexpected toxicity or ineffectiveness related to limited efficacy and/or targeted action against specific signaling networks that drive tumor recurrence.

Chromosomal, mutational, copy number variation, gene-expression, and proteomic analyses have provided a well-defined characterization of the molecular landscape of primary GBM tumors, but much less is known about recurrent tumors (10, 11). Despite the current state of knowledge regarding GBM biology, little progress has been made in the form of new pharmacological agents as stand-alone or adjuvant therapies. GBM is notoriously heterogeneous which limits the therapeutic value of agents that strictly target a single aspect of the disease within the broad pool of redundant pathways and potential targets (12, 13). It is the pronounced molecular and cellular heterogeneity present within these tumors that creates a substantial therapeutic challenge. This biological feature creates the potential for therapy-resistant subpopulations of GBM cells within the tumor to survive and evolve when exposed to single agent therapies and lead to recurrent tumors from these resistant clones which are refractory to available treatments.

Even as new information becomes available regarding recurrent GBM biology, multiple therapeutic delivery challenges remain, and these must be overcome to effectively treat recurrent GBM. As such, the majority of approved cancer drugs do not readily cross the blood-brain barrier (BBB), significantly limiting the options for GBM treatment. Therefore, exploring new avenues to enhance drug delivery into the brain to treat GBM are currently underway. Some techniques include convection-enhanced delivery, high-intensity focused ultrasound, delivery of drug-packaged nanoparticles, and antagonism of efflux pumps (14). In addition to improving the delivery of drugs with poor BBB permeability, there is a greater focus on the development of drugs that are predicted to cross the BBB, as well as repurposed drugs that are known to cross the BBB.

FACTORS LIMITING PHARMACOTHERAPY FOR GBM

Blood-Brain Barrier (BBB)

The BBB is formed by endothelial cells connected by tight junctions and functions to protect the brain from infectious agents and environmental neurotoxicants (15). Astrocytes, pericytes, and perivascular macrophages also contribute to the structure of the BBB, and as maturation occurs, astrocytic end feet line the perivascular space, and pericytes and perivascular macrophages line the basal lamina of the endothelial cells in order to help maintain rigidity (16, 17). Although some molecules are able to passively cross through the endothelial cell monolayer, the expression of efflux pumps, such as P-glycoprotein, actively transports them back into the blood. Because of this efflux system, many drugs display a high brain efflux index (BEI), preventing most cancer drugs from entering normal brain tissue, rendering clinically relevant concentrations of precision-targeted therapeutics unattainable (18). Certain physiochemical properties such as molecular weight, lipophilicity, and charge affect a molecule's ability to permeate the BBB and identification of efficacious drugs that are indicated for the treatment of GBM which meet all of these requirements is difficult. Thus, *in silico* predictive modeling systems have been put in place to examine whether certain pharmacophores have the potential to cross the BBB (19, 20). Despite selecting for drugs that exhibit ideal features for BBB permeability, other factors such as the electrostatically charged and anisotropic brain extracellular space (ECS), which contains a dense network of extracellular matrix (ECM) proteins which can bind drugs and inhibit tissue penetration (21, 22), and the glymphatic system (GLS), which is a conduit for the clearance of many therapeutics from the brain parenchyma into the lymphatic system and blood, are additional barriers that preclude effective drug delivery to and retention in the brain (23–25).

Drug Distribution

For molecules that bypass the BBB, additional challenges are met once at the site of the tumor. GBM displays an invasive phenotype at the rim of the tumor, where cells invade into the brain parenchyma; however, the bulk of the tumor, primary or recurrent, has a high degree of mitotic activity, forming a densely-packed region of cancer cells. Drug distribution is severely limited within the bulk tumor, due to the absence of a functional vascular network. An increased interstitial fluid pressure (IFP) between cells and a limited blood supply results in varied concentrations of chemotherapy being exposed to different regions of the tumor (26, 27). It has been postulated that treatment can drive clonal evolution, either through the selection of clones with drug-resistant molecular profiles or drug-induced genomic alterations, driven by sub-lethal doses of drug (28).

Tumor Hypoxia

Without neovascularization occurring to meet the nutritional demands or bring oxygen toward the center of the tumor, GBM cells use certain mechanisms to survive these harsh conditions. Most notably, as is the case for many solid tumors, ATP production through glycolysis occurs in both oxygenated and oxygen-depleted (hypoxic) conditions. Tumor acidity, potentially due to enhanced glycolysis, has been shown to alter uptake of certain drugs into tumor cells (29). Drugs are able to pass through the membrane more easily when in the ionized form, but are protonated at low pH, making cellular uptake less efficient.

Hypoxia is frequently observed in certain regions of tumors. Hypoxic cells divide slowly and have greater energetic demands, but maintain viability through other cell-survival mechanisms. The transcription factor hypoxia inducible factor 1 (HIF-1) induces a transcriptional program which up-regulates factors that

contribute to angiogenesis and the activation of macroautophagy (autophagy) (30). These mechanisms of cell survival confer a malignant phenotype and are attractive targets for GBM treatment (31, 32). The monoclonal antibody bevacizumab (tradename: Avastin) targets the angiogenic protein vascular endothelial growth factor-A (VEGF-A), and suppresses the formation of nascent vasculature. Bevacizumab has been approved for the treatment of recurrent GBM, but does not have any impact on overall survival (33, 34). Autophagy was initially described as a mechanism of cell death, but new information has revealed this is a stress-response pathway that restores the cell's energy balance when nutrients (or oxygen) are limited. Thus, it has been shown that regions of tumors where autophagy is high often co-localize with regions of hypoxia, and autophagy can promote tumorigenesis (35).

Glioma Stem-Like Cells (GSCs)

The glioma stem-like cell (GSC) subpopulation has recently been associated with invasion and chemoresistance, which is thought to give rise to recurrent tumors. GSC interaction with the tumor microenvironment and the ability to self-renew has been shown to promote survival and has made these cells extremely difficult to target with chemotherapeutics (36). Importantly, GCSs are located in both hypoxic and highly vascularized regions, surrounded by microglial cells which influence the survival and stem-like state of GSCs (37, 38). The underlying molecular biology regarding the origin of GSCs is still a major research interest; however, ongoing studies are underway to identify the transcriptional programs that endow these GCSs with highly invasive or chemoresistant properties.

APPROACHES TO MITIGATE THE BBB FOR DRUG DELIVERY

Although the blood vessels that supply the tumor core are commonly incompletely formed and leaky, especially as the histological grade of the tumor progresses, the components of a healthy BBB are still present in the invasive regions of most GBM tumors and low grade gliomas (15). Even if the core of the tumor is sustained by abnormal vessels with a degree of permeability to drugs, the cells that inevitably migrate away from the core of the tumor and establish secondary tumors in distant locations within the brain are smaller and supplied by normal brain vasculature and thus remain impenetrable to drugs.

Molecules can enter the CNS via free diffusion through the BBB, which is restricted to lipophilic molecules of <400 Da in size. Larger molecules necessary for brain function cross the BBB via active transport by pumps located on the apical endothelial surface (carrier-mediated transport, CMT) or through the endocytic process of receptor-mediated transport (RMT) (39). Although there are numerous clinical trials using systemic and directly added interstitial therapeutics aimed at disrupting or bypassing the BBB, progress remains hindered by concerns about efficacy and safety of combinations of BBB penetrating methods with chemotherapeutics for GBM. The methods detailed here each carry risks and benefits that should be critically evaluated for effective delivery of drugs without compromise of healthy brain parenchyma. The strengths and limitations of each strategy is summarizied in **Table 1**.

TABLE 1 | Strategies to improve BBB penetration for enhanced drug delivery.

Strategy	Pros	Cons
Convection-enhanced delivery	Enhanced distribution Drug combination delivery	Invasive Not targeted Expensive
Focused ultrasound	Targeted Non-invasive	Expensive
Vasoactive peptides	Transient Non-invasive	Poor clinical efficacy
Pharmacological disruption	Transient	Short half-life of antagonists Conflicting clinical trial results
Nanoparticles	Targeted Controlled release	Clinical efficacy not demonstrated
Osmotic agents	Transient	Invasive Non-specific
Peptide masking	Targeted Non-invasive	Low efficiency

BBB, blood-brain barrier.

Convection-Enhanced Delivery—Bypassing the BBB

The first studies of convection-enhanced delivery (CED) took place in the early 1990s at the National Institute of Neurological Disorders, where CED was found to be a reliable method for delivering molecules directly into the brain with varying physical properties (40). CED directly bypasses the BBB, relying on bulk flow to move both solutes and water along a pressure gradient. Catheters are inserted into the brain parenchyma and positive pressure is applied, pushing infusates into the extracellular fluid. Through this method, large molecular weight drugs can enter the CNS in a way that does not induce systemic toxicity. CED also allows for control over the spatial distribution of drugs in the brain, unlike drugs delivered systemically. These benefits make CED an attractive possibility for the treatment of GBM. However, early randomized trials with CED and conventionally delivered standard of care (TMZ and radiation therapy) showed that CED did not significantly increase survival, potentially due to "first generation" delivery techniques (41). Tissue damage can also occur in the instance of reflux of infusate, which must be carefully controlled for by adjusting flow rates and the properties of the cannula (42).

Focused Ultrasound With Microbubbles—Mechanical Disruption of the BBB

Focused ultrasound (FUS) can enable localized, selective permeability of the BBB. Initial work on the safety and efficacy of FUS showed that short, pulsed ultrasound waves disrupted the BBB in animal models, but with considerable collateral damage of healthy brain tissue. The introduction of lipid-encased gas-filled microbubbles lowered the frequency and power thresholds required for FUS to disrupt the BBB, allowing for safer treatments. When FUS is applied transcranially to the desired region of the brain, the intravascular microbubbles oscillate in the acoustic field, which produces mechanical forces against the tight junctions of the endothelial cells that line the vessel wall (43). The bubbles may also collapse and swiftly move fluid that is thought to act as a microjet that forms channels between endothelial cells. Notably, the effects of FUS are reversible, generally lasting 4–6 h. Unlike CED, FUS is not invasive, and can be used along with MRI to visualize BBB disruption and target the FUS effects to specific sites (44). FUS does not represent direct administration of the drug past the BBB, but can allow drugs that are administered using traditional methods (e.g., intravenous or intra-arterial) to cross the disrupted BBB at the FUS-treated site. Preclinical models demonstrate that FUS can make the BBB permeable to chemotherapy drugs including TMZ, doxorubicin, methotrexate, and carmustine in rat models of glioma (43). Clincal trials are ongoing in the US, Canada, and South Korea investigating this approach in patients with malignant gliomas.

Vasoactive Peptides—Chemical Disruption of the BBB

Bradykinin, a nine amino acid peptide, is an inflammatory mediator generated by the kinin-kallikrein system. Bradykinin's physiologic roles include vasodilation, decreasing blood pressure, increasing vascular permeability, and mediating pain sensation. Bradykinin exerts its effects by binding to B2 G-protein coupled receptors, which increases intracellular calcium and activates nitric oxide (NO) synthase. The subsequent increase in NO induces vasodilation and an increase in vascular permeability (14). Studies in the late 80s and early 90s took advantage of this physiology and reported that bradykinin infusion into cerebral vasculature allowed for passage of drugs past the BBB. Sanovich et al. (45) were the first to show that the bradykinin analog RMP-7, also known as labradimil or Cereport, promotes increased BBB permeability. They reported that administration of RMP-7 allowed a tracer molecule to gain access to the CNS via widened gaps in endothelial tight junctions rather than through transcellular mechanisms. This work was extended by the same group who later investigated the systemic effects of RMP-7 in a rodent model of glioma. This study established that RMP-7 exhibits tachyphylaxis with continuous infusion and provided the pharmacokinetic foundation for dosing parameters (46). In a subsequent phase II clinical trial, RMP-7 combined with carboplatin was determined to be no more efficacious than carboplatin alone. RMP-7 also did not change the dose of carboplatin required to reach therapeutic levels and reduce toxicity (47). Given these results, phase III clinical trials with RMP-7 were discontinued.

Pharmacological Disruption of the BBB

Several pharmacological mechanisms of BBB disruption have been uncovered thus far and key agents include adenosine agonists and P-glycoprotein antagonists. Adenosine is an endogenous purine nucleoside that signals through G-protein coupled receptors, including the inhibitory A1 and excitatory A2A receptors. Both neurons and glial cells release adenosine into the CNS, where it serves to regulate the release of neurotransmitters, vasodilation, and local inflammation. Adenosine is thought to allow recruited immune cells to enter the CNS by inducing BBB permeability through the modification of tight junction proteins and cytoskeletal rearrangement. Although adenosine shows promise in preclinical studies (48), its pharmacodynamics may be problematic if administered in the clinic. Adenosine itself has a 10 s half-life and requires adenosine receptors and the surface marker CD73 to be present on the BBB endothelium in sufficient amounts to cause a significant physiological response.

P-glycoprotein is an ATP-dependent drug efflux transporter that comprises the protective role of the BBB. This efflux transporter removes toxicants from endothelial cells, preventing harmful molecules from moving from circulation to the CNS. Fellner et al. (49) showed that the P-glycoprotein inhibitor PSC833 increased taxol accumulation in the mouse brain. Despite success in preclinical investigations, early clinical trials with P-glycoprotein antagonists were disappointing. However, in 2018, de Gooijer et al. found that inhibiting two transport proteins—P-glycoprotein and ABCG2—increased efficacy of TMZ in murine models (50). This study supports the reconsideration of drug efflux pump antagonism as a means of accessing CNS tumors despite earlier negative results.

Nanoparticles

Recently, nanoparticles of a variety of compositions have been investigated for their ability to carry drugs across the BBB [for a focused review, see Hersh et al. (14)]. They are typically administered intravenously and have varying ability to penetrate the BBB and remain in circulation long enough to have an effect (51). Studies of nanoparticles for drug delivery across the BBB must optimize the combination of drug, stabilizer, and composition of the nanoparticle to maximize the stability in circulation, the mechanism by which the cargo gets past the BBB, and the avoidance of uptake by the mononuclear phagocyte system (MPS).

Polymeric nanoparticles encapsulate drugs and cross the BBB via endocytosis. Several combinations of polymers, stabilizers, and drugs have been investigated so far. A 2018 study by Li et al. highlights the potential for combining both previously established and novel approaches for getting drugs across the BBB. This study used polysorbate-80-stabilized poly (D,L-lactide-co-glycolate) (PLGA) polymeric nanoparticles loaded with paclitaxel paired with FUS in mouse models of glioma. They found that using PLGA nanoparticles and FUS in combination to deliver paclitaxel across the BBB disrupted endothelial cell tight

junctions, decreased P-glycoprotein expression, and allowed for greater antitumor efficacy of the paclitaxel (52). Liposomes, like polymeric nanoparticles, can also encapsulate drugs. Liposomes represent an option for both hydrophobic and hydrophilic drug delivery and these drug carriers are relatively easy to prepare and carry little risk of toxicity. However, the MPS readily recognizes and removes liposomes from circulation, so it is necessary for the surface of liposomes to be modified with antibodies targeting RMT proteins (see below), or chemicals that make them smaller and more difficult for the MPS to recognize (51).

In the case of metallic nanoparticles, drugs can be conjugated to the surface, but cannot be contained within the particle itself. One study found that transactivator of transcription (TAT) peptide-modified gold nanoparticles (TAT-Au NP) can cross the BBB and deliver doxorubicin and gadolinium contrast agent to brain tumor tissue in a murine intracranial glioma xenograft model (53).

Unlike artificially synthesized nanoparticles, exosomes represent endogenous cell-derived particles that can potentially be harnessed for drug delivery. They are thought to be more stable than liposomes and they express surface markers for cell-cell communication that make them ideal for manipulation of the RMT system (54).

Osmotic Agents—Mannitol/Arabinose

Low concentrations of mannitol are already used routinely to decrease intracranial pressure following traumatic brain injuries and in brain tumor patients (55), and this technique has been shown to allow a variety of intra-arterially administered agents to cross the BBB, including small molecule drugs, peptides, and viral vectors (56). In osmotic disruption of the BBB, hypertonic arabinose or mannitol solutions are infused into the carotid artery for 30 s. This infusion of hypertonic solution causes endothelial cells to shrink as they lose water to the temporary osmotic gradient. This shrinkage widens the gaps between endothelial cells. This permeability is compounded by vasodilation, which occurs as water leaves cells and subsequent rising intracellular calcium levels modulate the contraction of the endothelial cell cytoskeleton. It is estimated that osmotic disruption of the BBB causes a 10-fold increase in permeability that lasts ∼10 min. Osmotic disruption, although widely applicable, is not selective for specific sites in the brain, introducing the risk of toxicants from the circulatory system gaining access to the CNS. The rebound phenomenon also represents a risk to consider specifically with the use of mannitol in GBM patients. Mannitol can leak through the disrupted BBB in parts of solid tumors and cause a reversal of the osmotic gradient. This rebound phenomenon can increase edema surrounding the tumor and increase intracranial pressure rather than decrease it (57).

Peptide Masking

The underlying principle of peptide masking is to trigger endogenous RMT mechanisms to endocytose cargo into the BBB endothelium. This can be achieved by conjugating drugs with peptides, receptor ligands, or antibodies that initiate RMT pathways. Some examples of receptors on the endothelial surface that are targets for the induction of RMT include transferrin receptor, insulin receptor, low-density lipoprotein receptor (LDLR), diphtheria toxin receptor, and heparin binding epidermal growth factor like growth factor (58). A phase I study of GRN10005, a peptide-drug conjugate that targets LDLR-related protein 1 was found to successfully deliver paclitaxel across the BBB of patients with recurrent glioma (59). Investigators working to design preclinical and clinical studies of peptide masking must evaluate not only the choice of peptide to trigger RMT, but also the choice of peptides that will target the GBM cells themselves. There is a need for peptides that can function both to initiate RMT and target GBM cells once they cross the BBB (58).

REPURPOSING DRUGS WITH BBB-PERMEABILITY FOR GBM TREATMENT

Due to the limitations and the side-effects caused by opening the BBB to augment drug delivery, another strategy to make novel treatment options readily available for GBM patients would be to explore FDA-approved drugs with known BBB penetrance and CNS activity. Since the implementation of the Stupp protocol in 2005, the treatment strategy of removing the primary tumor, followed by RT with concomitant TMZ, has not changed. Therefore, a great deal of effort has been placed on finding drugs that enhance the effects of RT and TMZ, but a greater emphasis should be placed on understanding recurrent tumor biology and the pathways that drive survival, proliferation, and invasion, and finding drugs that have current FDA-approval to inhibit these pathways. The following is a partial list of drugs either with current or former FDA-approval for alternate indications that penetrate the BBB, target established and emerging factors that are required for GBM survival, and have strong pre-clinical/clinical evidence for use against GBM. These drugs and their mechanisms of action in GBM cells are summarized in **Figure 1**.

Metformin

The biguanidine metformin is indicated for the treatment of Type 2 diabetes. It is orally available and acts by decreasing hepatic glucose production through the activation of AMP-activated protein kinase (AMPK). Activated AMPK (phosphorylated at threonine 172 of the alpha subunit) is a known repressor of mTOR activity through phosphorylation and activation of TSC2 (60). Multiple reports have identified overactive mTOR signaling in GBM, and inhibiting the downstream effects of mTOR is a common therapeutic approach (8, 61). Accordingly, metformin has been shown to sensitize glioma cells and glioma stem cells to TMZ both *in vitro* and *in vivo*, and has been used in a Phase 1 clinical trial for GBM (62, 63). Additionally, targeting both oxidative phosphorylation and glycolysis with metformin and 2-DG synergistically inhibits cellular bioenergetics, resulting in a loss of stemness and viability in GBM tumorspheres and offers a survival benefit in an orthotopic xenograft mouse model (64).

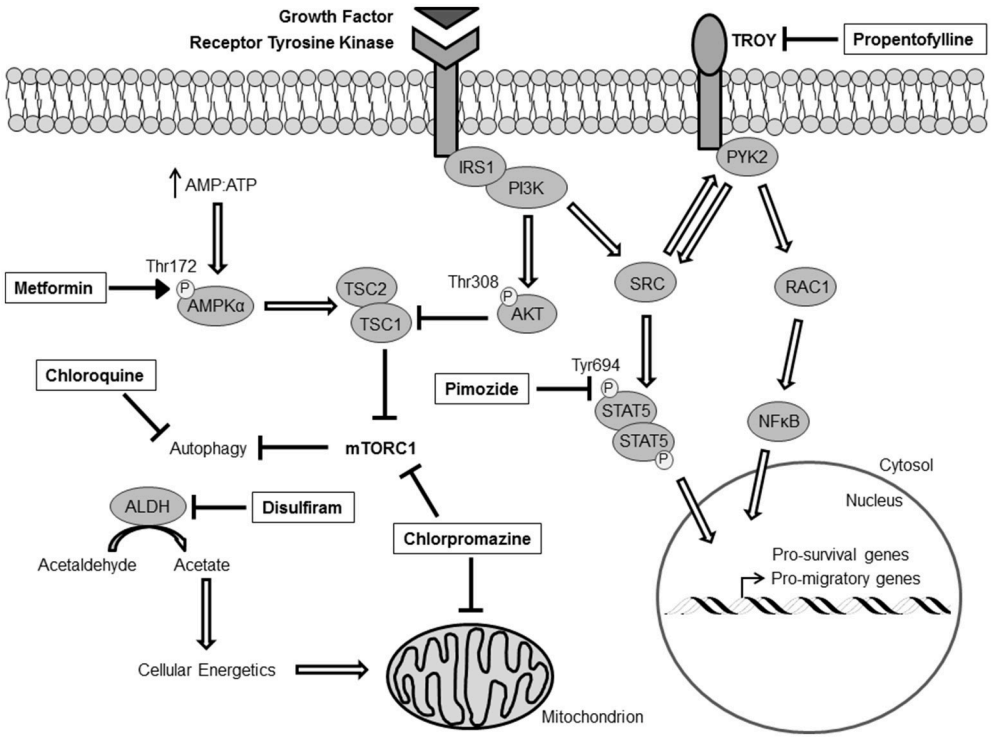

FIGURE 1 | Critical signaling networks in GBM that have druggable targets. All indicated drugs (boxed) exhibit current/former FDA-approval status, established BBB-penetrance, and evidence targeting GBM survival *in vitro* and *in vivo*. IRS1, Insulin receptor substrate 1; PI3K, Phosphatidylinositol-4,5-bisphosphate 3-kinase; PYK2, Protein tyrosine kinase 2; RAC1, Ras-related C3 botulinum toxin substrate 1; NFκB, Nuclear factor kappa-light-chain-enhancer of activated B cells; STAT5, Signal transducer and activator of transcription 5; TSC, Tuberous Sclerosis Complex; AMPK, AMP-activated protein kinase; ALDH, Acetaldehyde dehydrogenase.

Propentofylline

Propentofylline (PPF) is a xanthine derivative and a well-established inhibitor of the phosphodiesterases. This activity of PPF in microglial cells reduces the mechanisms that drive inflammation, which has been thought to contribute to vascular dementia and Alzheimer's disease. After extensive testing, results from a Phase III clinical trial reported that PPF did not provide a benefit for people with dementia or Alzheimer's disease, and was subsequently withdrawn from trials in humans, despite a good safety profile and documented brain accumulation. In the context of cancer, PPF was shown to inhibit the pro-tumorigeneic effects of microglia in a rodent model of glioblastoma (65). PPF was found to target TROY, an orphan receptor in the Tumor Necrosis Factor Receptor (TNFR) superfamily, which is highly expressed on microglia and drives microglial migration toward CNS-1 cells (66). A later study also found that glioma cells express high levels of TROY, which confers an invasive and chemoresistant phenotype (67, 68). Indeed, PPF was able to blunt the invasiveness and survival of GBM cells by decreasing TROY expression (69). Despite its effectiveness on suppressing the pro-tumorigenic functions of microglia in the tumor microenvironment and on GBM cells that overexpress TROY directly, the mechanism by which PPF inhibits TROY expression remains unknown.

Pimozide

There is a significant amount of literature suggesting that antidepressant and antipsychotic drugs should be repurposed for the treatment of GBM because of their established CNS activity (70). Pimozide is an antipsychotic drug of the diphenylbutylpiperidine class that was FDA-approved in 1985. It is currently used for the treatment of psychotic disorders such as tourette's syndrome, schizophrenia, and bi-polar disorder, but more recent data from a drug repurposing screen showed that pimozide had a pronounced effect on prostate cancer and acute myeloid leukemia cells via inhibition of STAT5 signaling (71). A recent report from our group identified overactive STAT5 signaling downstream of the constitutively active EGFR variant III (EGFRvIII), and pimozide treatment was able to decrease the migration and survival of GBM cells alone in a STAT5-dependent manner (72). Additionally, TMZ was shown to be more effective in combination with pimozide. STAT5 was shown to drive the expression of the TNFR family member fibroblast growth factor-inducible 14 (Fn14), a transmembrane protein reported to induce cancer cell invasion and survival, and pimozide was able to decrease the expression of Fn14 in a STAT5-dependent manner. Therefore, additional studies are warranted to observe the effects of pimozide in combination with other anti-cancer therapeutics in tumors that display enhanced STAT5 signaling.

Disulfiram

Disulfiram is a well-known inhibitor of acetaldehyde dehydrogenase (ALDH) and commonly used to treat chronic alcoholism. Recent data has suggested that disulfiram may be effective against GBM. High ALDH1 expression in GBM has been reported, identifying it as a key factor in maintaining brain tumor stem cell capacity (73). Inhibition of ALDH activity with disulfiram results in perturbations of cellular energetics and thus affects migration and viability of GBM cells (74). Additionally, ALDH expression has been implicated in TMZ resistance; however, there is a report identifying disulfiram as an inhibitor of the DNA repair enzyme MGMT by reducing its protein levels, thereby re-sensitizing GBM cells to alkylating agents and augmenting DNA-damage-induced apoptosis (75, 76).

Chloroquine

Chloroquine has been an effective anti-malaria drug for decades. It is known to inhibit the life cycle of the malarial parasites belonging to the *Plasmodium* genus; however, resistance to chloroquine has occurred in different regions of the globe, forcing the production of other anti-malarial drugs with different mechanisms of action. Interestingly, chloroquine has emerged as an attractive anti-cancer therapy due to its effect on the inhibition of lysosome-mediated degradation. The inhibition of lysosomal-mediated degradation also affects the late stage of autophagy, inhibiting the completion of autophagic flux and causing a build-up of cellular cargo and debris that is meant to be broken down. This imbalance in proteostasis forces cells to undergo apoptosis, which is why chloroquine has been shown to be an effective adjuvant cancer therapeutic (77). The use of chloroquine in combination with other cancer drugs with distinct mechanisms of action could be beneficial because of the likelihood that autophagy is induced by other anti-cancer drugs as a cell-survival mechanism.

Pre-clinical studies indicate that inhibition of autophagy with chloroquine can sensitize glioma cells to the cytotoxic effects of TMZ (78, 79). This approach has also been tested in the clinic, and hydroxychloroquine was shown to be more effective with radiation therapy and concurrent and adjuvant TMZ (80). Moreover, a randomized, doubled-blinded, placebo-controlled clinical trial with oral-delivered chloroquine added to conventional therapy was conducted in GBM patients. The addition of chloroquine improved mid-term survival (81). These are encouraging results and large-scale trials are needed to definitively determine if chloroquine should be added as an adjuvant therapy for the treatment of GBM. Unfortunately, one of the limitations to using chloroquine in patients is the fact that high concentrations are needed to achieve the desired lysosomotropic effects, which offers considerable toxicity. As a result, derivatives of chloroquine or other autophagy inhibitors with distinct mechanisms of action and enhanced potency could minimize toxicity in patients and have an overall better outcome in combination with radiation therapy or TMZ.

Chlorpromazine

Chlorpromazine is an antipsychotic medication used primarily to treat schizophrenia and bi-polar disorder. It was the first typical antipsychotic drug discovered in the 1950s and it is still effective today, even with more potent atypical antipsychotics available. A publication from the mid-1990s showed that chlorpromazine, in combination with BCNU, was an extremely effective treatment regimen in a rat orthotopic glioma model (82). The authors attributed the effects of chlorpromazine to the inhibition of calmodulin. More recently, treatment of C6 glioma cells with chlorpromazine caused cell-cycle arrest at the G2/M phase through transcriptional activation of p21(Waf1/Cip1) (83). This transcription appeared to be mediated through the activation of early growth response-1 (EGR-1), which occurred independent of p53. Moreover, chlorpromazine also had an inhibitory effect on PI3K/AKT/mTOR signaling, leading to a form of caspase-independent cell death (84). Lastly, the effect of chlorpromazine was also tested in a model of chemoresistant patient-derived glioma stem cells. It was determined that chlorpromazine inhibited cytochrome c oxidase (CcO, complex IV) activity (85). Previous research from this group also found that the acquisition of chemoresistance coincides with a switch in the expression of CcO subunit 4 isoform 2 (COX4-2) to COX4-1 (86). Taken together, chlorpromazine may be very useful in the clinic against GBM due to its multiple mechanisms of action. Though the targets of chlorpromazine may be non-canonical survival mechanisms for GBM, this fact may call for the use of chlorpromazine as an adjuvant therapy, rather than a specific, front-line therapy.

CONCLUSION

There have been numerous promising developments related to drug penetration through the BBB and the identification of existing drugs that may be repurposed for the treatment of GBM. Coordinated efforts to effectively treat GBM and significantly increase patient survival while minimizing the negative impact of these treatments on brain function will be enhanced by technologies that enable controlled penetration of the BBB and multi-modal treatment of this complex, heterogeneous disease.

AUTHOR CONTRIBUTIONS

BH wrote most of the manuscript and created the figure. MB contributed to the text. JW, AK, GW, JW, JL, and NT edited the manuscript.

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Radiation-Induced Alterations in the Recurrent Glioblastoma Microenvironment: Therapeutic Implications

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Glioblastoma (GBM) is uniformly fatal with a median survival of just over 1 year, despite best available treatment including radiotherapy (RT). Impacts of prior brain RT on recurrent tumors are poorly understood, though increasing evidence suggests RT-induced changes in the brain microenvironment contribute to recurrent GBM aggressiveness. The tumor microenvironment impacts malignant cells directly and indirectly through stromal cells that support tumor growth. Changes in extracellular matrix (ECM), abnormal vasculature, hypoxia, and inflammation have been reported to promote tumor aggressiveness that could be exacerbated by prior RT. Prior radiation may have long-term impacts on microglia and brain-infiltrating monocytes, leading to lasting alterations in cytokine signaling and ECM. Tumor-promoting CNS injury responses are recapitulated in the tumor microenvironment and augmented following prior radiation, impacting cell phenotype, proliferation, and infiltration in the CNS. Since RT is vital to GBM management, but substantially alters the tumor microenvironment, we here review challenges, knowledge gaps, and therapeutic opportunities relevant to targeting pro-tumorigenic features of the GBM microenvironment. We suggest that insights from RT-induced changes in the tumor microenvironment may provide opportunities to target mechanisms, such as cellular senescence, that may promote GBM aggressiveness amplified in previously radiated microenvironment.

Keywords: glioblastoma, radiotherapy, tumor microenvironment, extracellular matrix, recurrence

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INTRODUCTION

Glioblastoma multiforme (GBM) is the most common and lethal adult primary brain malignancy (1, 2). Interactions within the brain extracellular matrix (ECM) facilitate diffuse infiltration, making GBM surgically incurable (3–5). After standard treatment involving maximal safe resection, RT, and chemotherapy, most tumors recur within 18 months. Eighty percent of recurrences occur at the resection margin, wherein highest radiation doses are delivered (6–8). Following recurrence, patients are managed only with palliative care and clinical trials, which rarely achieve prolonged remission of recurrent lesions (9, 10).

Since GBM recurrences typically occur within previously radiated brain parenchyma, understanding altered biology of the radiated microenvironment is critical. We here review evidence suggesting RT has lasting effects on structure and milieu of the GBM microenvironment, facilitating tumor aggressiveness upon recurrence. Such alterations include

hypoxia, innate immune activation, and ECM changes. The extent these changes may impact drug penetration, pharmacokinetics, pharmacodynamics, and facilitate cellular resistance requires further investigation (11–13). Radiation, aging, and DNA damage promote senescence—a cellular state defined by a senescence-associated secretory profile (SASP), characterized by pro-inflammatory cytokine and ECM-degrading enzyme production. Given recent advances in therapies targeting senescence, we hypothesize senolytics may attenuate pro-tumorigenic features of GBM microenvironments.

GLIOBLASTOMA MICROENVIRONMENT

GBM cells coexist with genetically normal stromal cells in a dynamic tumor microenvironment (TME) (14). Within the TME, the extracellular matrix (ECM) provides scaffolding for inter-cellular communication and cell migration. The GBM ECM is notable for altered ECM synthesis/degradation and aberrant cell surface profiles (15). GBM heterogeneity adds to GBM microenvironment complexity, spanning hypoxic, proliferative, and infiltrative regions, superimposed upon genetically and transcriptionally heterogeneous cells (16). With hypoxic and other hyper-perfused tumor regions, redox state gradients, oxygen tensions, and pro-inflammatory cytokines, a dynamic GBM microenvironment is formed, which promotes cell proliferation, and tumor infiltration (3–5).

Tumor heterogeneity is a formidable impediment to identifying effective GBM therapies. Variable distribution of soluble factors throughout tumors creates chemotactic gradients (5). Furthermore, infiltrating cells in surrounding brain tissue differ in type and number of genetic alterations compared to cells harvested from the ischemic core or proliferative zone of the tumor (16). Tumor hypoxia promotes matrix production and remodeling, facilitating cell resilience and crosstalk between ECM and the pro-tumorigenic microenvironment (17).

The GBM ECM Promotes Tumor Growth and Cell Invasion

Distinct from normal brain and other solid tumors, the GBM ECM is comprised of a diverse array of glycoproteins, proteoglycans, and polysaccharides that form specialized structures that signal through cell surface receptors (18). The GBM ECM is mechanically rigid compared to normal brain. Fibrillar proteins, like fibronectin, laminin, and collagen, contribute to rigidity (19) and promote proliferation and migration (20). Upregulation of specific glycoproteins (collagen-IV, fibronectin and vitronectin) and proteoglycans (lecticans), promote surface receptor interactions with other molecules as hyaluronan, CD44, tenascins promoting cell invasion pathways (21–31) and can promote neo-angiogenesis, causing vessel leakiness, which facilitates macrophage entry and microglial activation (32). GBM ECM degradative changes are caused by matrix metalloproteinases (MMPs) as MMP-2, MMP-9, which are essential for cell invasion (33). Increased MMP expression alters cell attachment, allowing GBM cells to spread on myelin pathways, facilitating parenchymal disruption and tumor infiltration (34, 35). Furthermore, immune-active proteoglycans as heparan sulfate proteoglycans (HSPGs) are upregulated in GBM (21, 36) and can act as co-receptors for chemokines, cytokines and growth factors (such as CCL2, IL-1β; tumor necrosis factor-α, TNF-α; transforming growth factor-β, TGF- β), harnessing pathways of progenitor proliferation, cell migration, and axonal pathfinding to facilitate tumor cell proliferation and infiltration (21, 37). Elevated extracellular adenosine in GBM also promotes proliferation, metastasis, microglial phagocytic activity, and adaptive tumor immune responses (38, 39). As critical mediators of tumor growth and cell invasion, various ECM components and associated receptors are hypothesized as therapeutic targets for GBM, including glycoproteins (e.g., tenascin-c; collagen, and its receptor DDR-1, discoidin domain receptor-1) (28, 29, 31) proteoglycans (brevican) (30), and extracellular nucleotides (adenosine triphosphate, ATP) (36, 40–44). Potential therapeutic targets of the radiated brain and GBM microenvironment are enlisted in **Table 1**.

Stromal Cell Populations and Functions in GBM

Various non-neoplastic stromal cells are important in tumor maintenance and recurrence. Resident microglia and infiltrating macrophages are attracted to GBM and comprise approximately 30% of cells in the tumor (77, 78) and with other CNS stromal cells spanning neurons, endothelial cells, astrocytes, and oligodendroglia, create favorable milieu for glioma proliferation and infiltration. Research on impacts of prior radiation on these cells has only just begun.

Resident microglia orchestrate behavior of other immune cells that enter the brain by secreting cytokines and chemokines upregulated in GBM, leading to chronic inflammation. These immune-modulatory factors include cytokines as TNF- α, TGF-β, chemokines as CX3CL1/Fractalkine, CCL2, CCL5 and growth factors as fibroblast growth factor, FGF-2, and granulocyte-monocyte colony stimulating factor, GM-CSF (68, 69, 79–82). Since microglia are difficult to distinguish from bone marrow-derived macrophages, the term "TAM," for tumor associated macrophages, is often a blanket-term, describing all monocytic cells within the tumor, regardless of specific origin (83, 84). TAMs release various growth factors and cytokines in response to GBM-secreted factors or microenvironment-associated factors, facilitating tumor proliferation, survival, and invasion (83, 84). TAMs can express markers for pro-inflammatory/tumor-suppressing M1 or anti-inflammatory/tumor-promoting M2 phenotypes (83, 85). M1 macrophages are mostly found in oxygenated glioma regions and M2-polarized macrophages are increased in hypoxic areas (86, 87). Hypoxia causes recruitment of macrophages and M2 differentiation through Sema3/Nrp1 signaling (88). Colony stimulating factor 1 receptor (CSF1R) is a key regulator of monocyte/macrophage survival and proliferation and is upregulated in GBM and encourages M2 polarization (89, 90). CSF-1 inhibitors have shown to deter glioma recurrence after radiation *in vivo* (91), prevent

TABLE 1 | Radiation-induced alterations to the GBM micro-environment.

Biological process	Consequence	RT-effect	References
EXTRACELLULAR MATRIX COMPOSITION AND BIOSYNTHESIS			
Collagen	Migration and Invasion	+/Up	(38, 45, 46)
Tenascin C	Tumor proliferation, Invasion	+/Up	(28, 29, 47)
Hyaluronin	Invasion	+/Up	(48, 49)
Brevican	Migration, Invasion	+/Up	(25, 30, 50)
Vitronectin	Survival, Migration, Inflammation	+/Up	(26)
MDA-9/Syntenin	Metastasis, tumor progression	+/Up	(51)
LOX	Migration	+/Up	(52)
ECM-GLIOMA CELL (LIGAND-RECEPTOR) INTERACTION			
DDR-1, ICAM-1, α5β1, αvβ3	Migration, Invasion	+/Up	(31, 53–55)
ECM DEGRADATION			
MMPs	Invasion	+/Up	(56–59)
TIMP	Angiogenesis, Metastasis	+/Up	(60, 61)
TUMOR CELL ADAPTATION MECHANISMS			
Oxygen tension: HIF-1	Hypoxia, malignancy	+/Up	(62)
Metabolism: ATP, NAD	Proliferation	+/Up	(63)
Anti-apoptosis: BCL2/BAX	Migration, invasiveness	+/Up	(55)
Redox regulation (ROS/RNS, NOX4)	Senescence, inflammation	+/Up	(64–66)
Angiogenesis (VEGF, Ang)	Angiogenesis	+/Up	(67)
Inflammation (Cytokines, Chemokines, Chemokine receptors)	Tumor proliferation, migration, invasion	+/Up	(68–72)
Glia activation (MHC, CD68, GFAP)	proliferation	+/Up	(70, 73–75)
Neurogenesis (NSC)	Cognitive decline	-/Impaired	(76)

MDA-9, Melanoma differentiation-associated gene-9; LOX, Lysl oxidase; DDR-1, Discoidin domain receptor-1; ICAM-1, Intercellular Adhesion Molecule 1; Integrins α5β1; Integrin, αvβ3; MMPs, Matrix metalloproteinases; TIMP, Tissue inhibitor of matrix metalloproteinase; NSC, Neural stem cell; HIF-1, Hypoxia-inducible factor 1; VEGF, Vascular endothelial growth factor; Ang, Angiotensins; CALR, Calreticulin; HGMB1, High mobility group box 1 protein; NOX4, NADPH oxidase 4; ROS, Reactive oxygen species; RNS, Reactive nitrogen species; MHC, Major histocompatibility complex; ATP, Adenosine triphosphate; GFAP, Glial acidic fibrillary protein; NAD, Nicotinamide adenine dinucleotide; CD, Cluster of differentiation; BCL2, B-cell lymphoma-2; BAX, BCL2 Associated X. Cytokines: Tumor necrosis factor-α, TNF- α; Transforming growth factor-β, TGF-β, IL, Interleukins (IL-6, IL-8, IL-1β). Chemokines: CX3C family (CX3CL1, Fractalkine), CCL family (CCL2, CCR3, CCL7, CCL8, CCL12), CXC family (CXCL4, CXCL12/stromal cell-derived factor 1, SDF1). Chemokine receptors: CC-chemokine receptor family (CCR1, CCR2). RT-effects: +, positive; Up-upregulation/increased; –, negative.

radiation-induced cognitive impairment in preclinical models (92, 93), and are well-tolerated in clinical trials (94).

Neurons can communicate with astrocytes, oligodendrocytes, and microglia via signal molecule release from pre-synaptic terminals. Similarly, tumor growth is stimulated by factors such as electrical activity (95), release of neuroligin-3 (96, 97), neurotransmitters, and neurotrophins (96, 98). Within the TME, glioma cells release microvesicles, transporting miRNAs, mRNAs, angiogenic, and oncogenic factors which promote TAMs, inducing proliferation, infiltration, and immune detection evasion (99).

Astrocytes within the GBM TME exhibit a reactive phenotype, characterized by increased expression of glial acidic fibrillary protein (GFAP) (100). Reactive astrocytes release cytokines, matrix metalloproteinases, stromal cell-derived factor 1 (SDF-1) (101), and upregulate survival genes via gap junction communication with glioma cells, promoting tumor invasiveness and growth (102, 103).

The multiplicity of mechanisms by which the TME promotes glioma growth creates both challenges and opportunities. Although the various pro- tumorigenic activities of TAMs have prompted interest in eliminating them from the TME via CSF1 inhibition (104), the importance of monocytes for innate defense is suggested through increased gliomas in preclinical CSF1 mutants (105) and pharmacologic activation of macrophages to promote glioma phagocytosis (106, 107). Pro-tumorigenic features of the TME such as hypoxia, ECM changes, and neuroinflammation may be augmented following RT. Despite standard clinical use, certain impacts of prior radiation on the TME likely nurture growth of recurrent glioma.

EFFECTS OF RADIATION THERAPY ON THE GBM MICROENVIRONMENT

Although RT remains a first-line GBM therapy, dose-dependent risk of devastating neurologic effects precludes doses sufficient for disease eradication, making recurrence inevitable (12, 108, 109). Over 90% of GBM patients experience recurrence at original lesions and 5% develop multiple lesions after RT (110–112). In contrast to intracranial metastatic tumors, which can be eliminated via localized or whole-brain radiation, glioma stem cells survive RT and eventual recurrence is fostered by radiation-induced changes in the TME (113, 114).

Many radiation-induced changes in the TME have been documented (115, 116). Ionizing radiation (IR) generates

reactive oxygen and nitrogen species (ROS/RNS) that directly damage DNA, proteins, and phospholipid membranes (64). RT is partially dependent upon double-stranded DNA breaks that overwhelms cellular repair mechanisms, triggering apoptosis in proliferating cells (117). GSCs that escape apoptosis persist in a relatively non-proliferative state until recurrence. Changes in irradiated TME include increased oxidative stress, hypoxia, neuroinflammation, altered cell adhesion molecule expression, changes in ECM, stem/progenitor cell death, cellular senescence induction, and impaired neurogenesis (70, 118–121), followed by neo-angiogenesis, vasculogenesis (114), and tumor recurrence. The extent that these radiation-induced impacts may augment aggressiveness of recurrent tumors is a growing research area. However, the extent that radiation-induced senescence may exacerbate pro-tumorigenic microenvironment following RT is an underexplored avenue. We here discuss the key pathophysiologic changes of the irradiated brain and GBM microenvironment, and the central role ECM/cell-matrix interactions play in manifesting these processes. Their collective involvement in the establishment of a reactive TME that is supportive of aggressive tumor growth, is illustrated in **Figure 1**.

Radiation-Induced Cellular Senescence

Senescence is a defensive mechanism in response to stress that arrests cells at risk for malignant transformation. Radiation-induced DNA damage and oxidative stress, cytotoxic exposure, and/or aging, prompts apoptosis, unless upregulation of cyclin-dependent kinase inhibitors such as p16 or p21 allows senescence induction (122). GBM cells harbor a heterogeneous array of mutations, leading to constitutive activation of repair mechanisms that prevent apoptosis in response to RT, despite damage that would otherwise render cells unviable (123). Such mutations may facilitate upregulation of oncogene-induced senescence (124, 125), prompting a baseline level of senescence-associated signaling in GBM that is amplified following radiation in a dose- and time-dependent manner (126, 127).

A hallmark feature of senescent cells is the "senescence-associated secretory phenotype" (SASP), characterized by release of proinflammatory signaling molecules, proteolytic enzymes, and ECM components (128). Together with MMPs, proinflammatory SASP components are thought to create a microenvironment that promotes survival, proliferation, and dissemination of neoplastic cells across brain parenchyma (129, 130). Such adaptations may contribute to increased aggression of recurrent GBM and have been shown in multiple cancers to promote tumor progression and metastatic spread (124). SASP also has paracrine effects, spreading the phenotype to neighboring cells (131). Discovery of senolytic drugs and their therapeutic combinations, such as dasatinib and quercetin (D+Q) has raised hopes that senescent cell-induced diseases may soon be curable (132, 133). Since radiation is among the most reliable experimental strategies to induce senescence, it is reasonable that the previously radiated tumor will be exposed to SASP. Prior work has demonstrated that co- implantation of radiated with non-radiated cells increases tumor aggressiveness. We (134), and others (133, 135–137), have observed increased tumor aggressiveness after implantation of glioma cells into previously radiated hosts. Given the potential for senescent cells to induce tissue dysfunction and inflammation, several studies have addressed the potential of metabolically active senescent cells and SASP factors to exacerbate recurrences of various cancers (124, 138, 139). Though much work remains to establish mechanisms, cellular senescence after radiation likely has important implications for recurrent GBM.

Elevated levels of ROS cause matrix dysfunction through remodeling and fragmentation of collagen and proteoglycans, pronounced protease activity, and altering cytoskeletal contractility (by modulating actin, and tubulin), fueling senescent phenotypes marked by irregular collagen meshworks and ECM degradation (140–142). These phenomena may reduce tension and elasticity of affected tissue, supporting invasion and metastasis. Oxidative stress and associated mitochondrial function can further propagate RT-induced senescence (143–145).

Radiation-induced bystander effects contribute to cellular senescence, as well as tumor promotion and recurrence (146). Bystander effects are defined by a cell's reaction to its neighboring irradiated cell, with consequences of damage to nearby healthy brain regions. Irradiated GBM cells have shown to induce bystander effects including increased cell growth, micronucleus formation, and apoptosis in non-irradiated tumor cells (147–149). These bystander processes can ignite ROS production and mitochondrial dysfunction, leading to persistent or irreparable DNA damage, activation of DNA damage responses, irreversible cell cycle arrest, and culmination of cellular senescence (128).

Radiation-Induced Adaptations Facilitate Aggressiveness of Recurrent GBMs

It has been said that what doesn't kill you makes you stronger, and in certain respects GBM exemplifies such adaptive behavior. Tumors adapt to radiation-induced oxidative stress through several mechanisms, including metabolic shifts (63), elevated antioxidant peptide production, and intra-tumoral hypoxia generation (150, 151).

Hypoxic conditions facilitate tumor radio-resistance and recurrence, as ROS are insufficient for apoptosis induction (152). Radiation-induced stabilization and activation of hypoxia-inducible factor 1 (HIF-1) elicits protective processes through regulating downstream target genes, such as nitric oxide (NO), which can stimulate immunosuppressive and anti-apoptotic responses (62, 67).

Vascular remodeling is a hallmark of IR injury. Radiation affects vascular integrity, causing vasculopathy, vascular depletion, hypoxia, and neo-angiogenesis (153–155). Levels of vascular endothelial growth factor (VEGF) and angiotensin are increased in GBM post-radiation, contributing to angiogenesis and tumor growth (67, 156), while SDF promotes vasculogenesis (114, 156). Radiation induces changes in cell density, tight junctions, and increased BBB permeability to inflammatory cells, and perhaps pharmacologic agents (11, 70, 154, 157).

Radiation-induced alterations in ECM composition have incompletely understood impacts on cognitive function and tumor infiltration and proliferation. Specific proteins

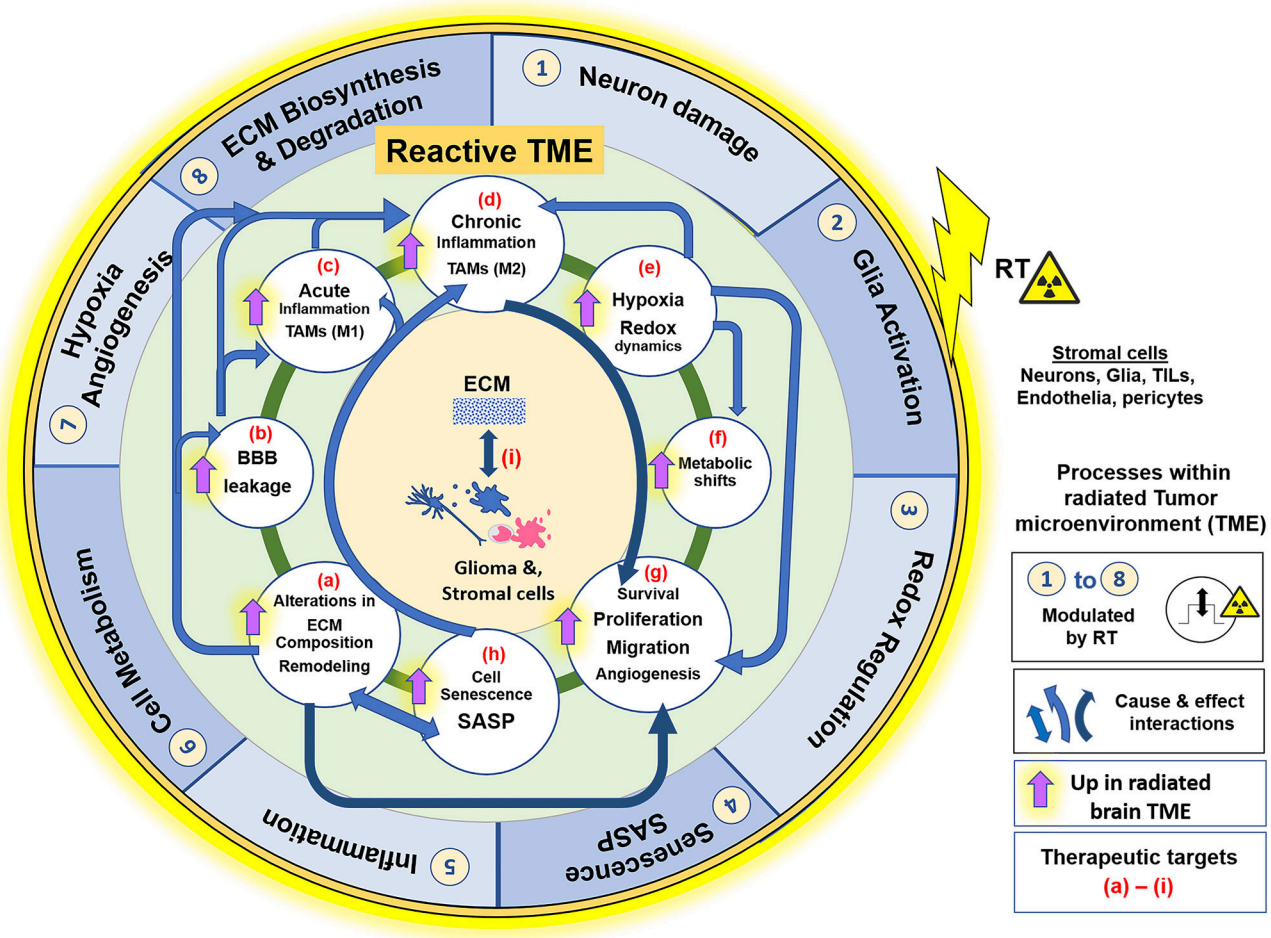

FIGURE 1 | Effects of RT on the glioblastoma (GBM) tumor microenvironment (TME). ECM and its interaction with cellular components such as Glia (microglia and astrocytes), glioma cells, endothelia, pericytes and peripherally derived tumor infiltrating leukocytes (TILs), play a central role in the GBM TME, which contributes to tumor cell survival, proliferation, migration, and invasion. The illustration represents the key pathophysiological processes and their interactions within the radiated TME. Outer blue circle represents biological phenomenon (1–8) that are directly impacted by RT. The inner green circle represents the reactive GBM TME, with its various processes, alterations or adaptive mechanisms that are upregulated in effect of RT, described from **(a–i)**. Blue arrows indicate the "cause and effect" interactions between these processes facilitating GBM pathology and, dark blue arrows signify the primary role of the respective alterations in facilitating cell motility and invasiveness and thus aggressive tumor recurrence. Radiation injury leads to neuronal damage, overactivation of M1 microglia, and elicits acute inflammatory response, with high ROS production in neurons and glia cells. There is alteration in ECM composition, and ECM-cell interactions. MMP/TIMP disbalance degrades Col-IV in basement membranes, which leads to blood brain barrier leakage. Pronounced inflammation causes infiltration of leukocytes (monocyte derived cell populations, macrophages), which along with activated microglia form the TAMs. TME of progressive tumors favor M2 phenotype and establishment of chronic inflammation. Redox dysregulation in effect of RT causes exacerbation of SASP phenotype and tumor cell adaptive processes, like Hypoxia, metabolic shifts, and redox regulation (ROS/NO production), leading to neo-angiogenesis and ECM remodeling. These alterations collectively make the TME permissive to glioma cell migration and invasion, thereby contributing to resistance and an aggressive tumor recurrence. All these biological processes in the reactive TME are potential therapeutic targets for improved glioblastoma care, with having ECM-cell interactions central to the manifestation of each of these phenomenon. RT, Radiation therapy; ECM, extracellular matrix; TAM, Tumor associated macrophages; TILs, Tumor infiltrating leukocytes; MMP, matrix metalloproteases; TIMP, Tissue inhibitor of metalloproteases; Col-IV, collagen-type IV; BBB, blood brain barrier; ROS, reactive oxygen species; NO, Nitric oxide; SASP, Senescence associated secretory phenotype; M1/M2, proinflammatory or immune suppressive phenotypes of TAMs, respectively.

involved in ECM biosynthesis (brevican, vitronectin, tenascin C, hyaluronin, lysyl oxidase) (25–27, 47, 52), degradation (matrix metalloproteinases) (56–59), signal transduction (melanoma differentiation-associated gene 9/Syntenin) (51), and ECM-glioma cell interactions (ICAM-1, DDR-1, integrins) (53–55, 158) are upregulated following radiation. These alterations may facilitate tumor cell infiltration and further exacerbate impacts of radiation on tumor cells themselves. These alterations include induction of a pro-migratory, p53-mediated mesenchymal phenotype (159). Additional p53-independent changes have been reported, including transcriptional regulation (45), integrin expression, MMP expression and activity (56, 57, 59, 160), altered membrane type 1 MMP and tissue inhibitor of MMP-2 expression, and increased BCL-2/BAX

expression (55), resulting in apoptosis resistance and enhanced migration.

Effects of Radiation on Inflammation in the GBM Microenvironment

Radiation-induced vascular permeability leads to infiltration of immune cells into brain parenchyma. RT-induced chronic inflammation promotes high intracellular NO in glioma cells (161), causing stabilization of HIF-1 and inhibition of tissue inhibitor of matrix metalloproteases-1 (TIMP-1) (162, 163). HIF-1-induced expression of stromal-derived factor 1 (SDF-1) promotes recruitment of macrophages following RT (71, 164). Elevated NO also inhibits tissue TIMP-1, contributing to ECM remodeling and tumor cell invasion (60, 165).

Radiation-induced cellular damage induces astrocyte gliosis, characterized by increased GFAP expression (73, 74), mediated by oxidative stress and microglial release of prostaglandins (74). Radiation-induced redox signaling profoundly alters microglial function. Differential expression of iNOS and arginase-1 in M1 vs. M2 profiles provides the basis of redox control in TAM phenotypes (166). Nuclear factor (erythroid-derived 2)-like 2 (NRF2) regulates redox dynamics and favors an M2 phenotype with cytoprotective effects (167, 168). Additionally, glioma cells and stromal cells maintain a pool of antioxidant peptides, such as glutathione and thioredoxin, protecting them against radiation-induced redox (169, 170). Differential ability of glioma cells to modulate redox reactions may be a mechanism by which certain cancer cells are more radioresistant than others. Combinatorial effects of RT on the TME lead to increased aggressiveness of recurrent GBM.

The extent that M1 vs. M2 polarization states relate to radiation-induced changes in microglia remains unclear. The M1 polarization state is most characteristically defined as that exhibited by systemic monocytes upon *in vivo* exposure to lipopolysaccharide, compared to M2 phenotype following IL4 exposure. Though this terminology has been widely extended to microglia, the actual microglia activation states are almost certainly more complex. To date, the transcriptional profiles of radiated mouse microglia have been described 24 h and 1 month after whole brain radiation, yielding phenotypes unique from, but partially overlapping with published M1 and, to a lesser extent, M2 phenotypes. Notably, the degree of ECM changes induced in radiated microglia exceeded both M1 and M2, while closely approximating changes observed in aged microglia (171). How this pertains to radiation-induced changes in the radiated TME is unclear, however, our unpublished observations demonstrate strongest enrichment of radiated microglial genes in the mesenchymal GBM subtype, as well as patients with the worst prognosis. Poorer prognosis of aged patients with GBM is well-documented. Whether the more radiation-like polarization state of aged microglia contributes to such poorer outcomes remains unknown. However, given the recurrent theme of chronic inflammation, in GBM, radiation, aging, and neurodegeneration, efforts to modulate the inflammatory microenvironment of both primary and recurrent GBM are of broad interest to attenuate tumorigenesis and enhance cognitive outcomes following radiation (172, 173). Given the differences in the neuroinflammatory phenotype of rodents and humans, mechanistic studies specifically interrogating human disease will be paramount (174).

THERAPEUTIC IMPLICATIONS AND FUTURE DIRECTIONS

Most mortalities are due to GBM recurrence. Most recurrent tumors arise from the previously radiated location. As such, understanding and combating mechanisms via which RT may augment pro-tumorigenic mechanisms in the GBM microenvironment is necessary to facilitate long-term survival. GBM invasiveness is induced by radiation and facilitates distant failures in the event of prolonged local control. Systemic radiosensitizers in combination with RT are being explored to enhance the effects of radiation with aims of lowering radiation and chemotherapy doses with improved efficacy (175). Whether senolytics may complement other classes of radiation sensitizers remains unknown.

RT-induced ECM alterations for GBM infiltration are potentially important therapeutic targets. Prior studies on inhibition of ECM biosynthetic and degradative processes, and receptor blockade to prevent ECM-cell interactions for cancer prevention, further support this idea (176, 177). Targeting cytoskeletal dynamics is also a proposed therapeutic strategy (178). Targeting microglial-ECM interactions that promote pro-tumorigenic phenotypes in GBM may also offer opportunities for therapeutic intervention. Developing technologies to anatomically direct targeted therapies to a radiated region may provide capabilities akin to limit off-target effects through use of radiation sensitizers, senolytics, or other agents selectively active in radiated tissue (179).

This work has discussed a variety of challenges for recurrent GBM management, highlighting important roles of the TME and associated matrix-cell interactions in instigating pathophysiological processes. Radiation-induced alterations in the microenvironment that can serve as targets for therapeutic intervention are summarized in **Table 1**, whereas the immunostimulatory role of hypofractionated radiation as an immunotherapy component is described by others (180) and us (Rajani et al, article under preparation). Importantly, it should be emphasized that no single therapy will likely be sufficient for tumors as heterogeneous as GBM and combinatorial treatment may be required. Balancing pros and cons of RT in concert with targeted therapies will provide an ongoing focus of therapeutic efforts for glioblastoma. Translational strategies are needed that can yield mechanistic biomarkers of efficacy for optimization of multi-drug approaches.

CONCLUSIONS

Understanding and targeting the GBM microenvironment is no less important than targeting the biology of GBM

cells. GBM infiltration depends on unique features of the CNS microenvironment. Several lines of evidence suggest long-term sequelae of radiation can exacerbate recurrent glioma. Understanding lasting impacts of radiation and other therapies on the TME will be necessary to overcome recurrent disease. Two other take-home points are worth emphasizing:

1) Unlike heterogeneous GBM cells that can out-mutate targeted therapies, the genetic stability and thus inherently greater predictability of tumor stroma should offer a tangible focal point for targeted therapies
2) The many mechanisms by which GBM cells harness the TME to their advantage should encourage multidisciplinary efforts to develop and translate synergistic multi-target therapies optimized to the unique biology of the CNS.

AUTHOR CONTRIBUTIONS

KG proposed the presented concept and idea, wrote and edited the manuscript, and designed the figures. TB proposed the presented concept and idea, reviewed the manuscript and supervised and supported KG. All authors have contributed to the final version of the manuscript.

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REVIEW
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Immunotherapy and Epigenetic Pathway Modulation in Glioblastoma Multiforme

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Glioblastoma Multiforme (GBM) is the most common malignant primary brain tumor. Despite aggressive multimodality treatment it remains one of the most challenging and intractable cancers (1). While current standard of care treatment for GBM is maximal safe surgical resection, systemic chemotherapy with Temozolimide (TMZ), and radiation therapy, the current prognosis of GBM patients remains poor, with a median overall survival of 12–15 months (2, 3). Therefore, other treatments are needed to provide better outcomes for GBM patients. Immunotherapy is one of the most promising new cancer treatment approaches. Immunotherapy drugs have obtained regulatory approval in a variety of cancers including melanoma (4), Hodgkin lymphoma (5), and non-small cell lung cancer (6). The basis of immunotherapy in cancer treatment is linked to stimulating the immune system to recognize cancer cells as foreign, thereby leading to the eventual elimination of the tumor. One form of immunotherapy utilizes vaccines that target tumor antigens (7), while other approaches utilize T-cells in patients to stimulate them to attack tumor cells (8). Despite intensive efforts all approaches have not been overtly successful (9), suggesting that we need to better understand the underlying biology of tumor cells and their environment as they respond to immunotherapy. Recent studies have elucidated epigenetic pathway regulation of GBM tumor expansion (10), suggesting that combined epigenetic pathway inhibition with immunotherapy may be feasible. In this review, we discuss current GBM clinical trials and how immune system interactions with epigenetic pathways and signaling nodes can be delineated to uncover potential combination therapies for this incurable disease.

Keywords: glioblastoma, immunotherapeutic, clinical trial, epigenetic, long non coding RNA

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REVIEW OF CLINICAL TRIALS USING COMBINATION IMMUNOTHERAPY

Multiple immunotherapy clinical trials in GBM have been initiated although few have reached completion. And of those few, the results observed were limited. For example, an upfront (newly diagnosed GBM) phase 2 trial tested the efficacy of a patient specific dendritic cell vaccine termed ICT-107, by observing its ability to significantly change the median survival rate of newly diagnosed GBM patients. In the ICT-107 study, patients in the experimental arm as well as control arm

underwent surgical resection followed by radiation and TMZ treatment for six weeks. Subsequently, white blood cells (WBCs) from GBM patients were extracted from both experimental and control groups and cultured with antigens found in the GBM experimental group and not in the control group. Over the period of several months, the WBCs pulsed with GBM antigen for the experimental arm and WBCs not pulsed with GBM antigen for the control arm were reintroduced as vaccines to patients in the respective groups. The results showed that when compared to patients in the control group, whose extracted WBC were not cultured with GBM antigens, the GBM cultured WBCs increased survival in ICT-107 treated patients by only less than 2 months [**Table 1**, (23)].

Other combination trials using immunotherapy with chemotherapy and/or radiotherapy have been performed in the hopes of attaining statistically significant results, but most have fallen short. Another upfront phase 2 immunotherapy combination trial investigated the effect of immunotherapy with radiation and chemotherapy to determine the efficacy of combination therapy as treatment for GBM with the PEP-3-KLH vaccine for newly diagnosed EGFRvIII-expressing GBM patients. The PEP-3-KLH vaccine is a synthetic peptide derived from a mutated segment of the epidermal growth factor type vIII (EGFRvIII), which is overexpressed in some patients with malignant glioma (24). This mutated segment is then conjugated onto the adjuvant keyhole limpet hemocyanin (KLH), a respiratory protein that is similar to some GBM antigens. The researchers in this study used this vaccine to assess its synergistic ability to elicit an immune response in conjunction with radio- and chemo-therapies when compared to patients who only received radio- and chemotherapies alone. In the first of three arms of this study, the PEP-3-KLH vaccine was administered to the GBM patients after they had completed radiation treatment. In the second and third arms of this study, the PEP-3 vaccine was used after radiation in combination with the oral chemotherapy drug, TMZ (25). The patients in this study who received TMZ, radiotherapy, and vaccine were compared to a matched cohort who were only treated with radiation therapy and TMZ. When compared to the median overall survival (OS) of the matched cohort, which was 15 months (95% CI 11.4 to 19.7 months), the vaccinated patients had an increased median OS of 26 months (95% CI, 21.0 to 47.7 months) (26). While these results are promising, it should be noted that only a subset of GBM patients have the EGFRvIII antigen on their GBM tumor cells, which was present in all participants studied (26). In fact, a study discussing the prognostic significance of EGFRvIII antigen found that only 14 of 73 (19.2%) patients evaluated with primary GBM expressed the EGFRvIII antigen (27). Collectively, these findings suggest that only a select few GBM patients have the potential to benefit from this treatment.

A second upfront combination trial analyzed the efficacy of immunotherapy in conjunction with radiotherapy and chemotherapy. In this trial, patients who had recently undergone tumor resection for their newly diagnosed GBM were first treated with radiotherapy and concurrent chemotherapy, TMZ. After completion of both radiotherapy and chemotherapy, WBCs were collected from these patients and cultured along with dendritic cells with each individual patient's GBM tumor antigens (**Figure 1**). This autologous dendritic cell vaccine was reintroduced to each patient whose GBM antigens were used to culture the vaccine (28). This study showed that only 50% of this carefully selected patient population mounted an immune response resulting in improved survival. In addition, the study noted that there was vast heterogeneity in the immune response (29), meaning that the immune reaction to treatment is different in each individual.

TABLE 1 | A summary of clinical trials utilizing standard of care for GBM in addition to immunotherapy and biomarker immunotherapy in combination with and without targeted therapy.

	Immunotherapy	**Biomarker immunotherapy**
Standard of care, targeted therapy, and immunotherapy: immunotherapy combined with other treatment method(s)	Combination of immunization and radiotherapy for recurrent GBM (InSituVac1) (InSituVac1) (11) Basiliximab in treating patients with newly diagnosed glioblastoma multiforme undergoing targeted immunotherapy and temozolomide-caused lymphopenia (REGULATe) (12) Vaccine therapy in treating patients with newly diagnosed glioblastoma multiforme (ACTIVATe) (13) Phase II feasibility study of dendritic cell vaccination for newly diagnosed glioblasto mamultiforme (14) A study of ICT-107 immunotherapy in glioblastoma multiforme (GBM) (15)	Tremelimumab and durvalumab in combination or alone in treating patients with recurrent malignant glioma (16) Pembrolizumab and vorinostat combined with temozolomide for newly diagnosed glioblastoma (17) Adjuvant dendritic cell immunotherapy plus temozolomide in glioblastoma patients (ADDIT-GLIO) (18)
Immunotherapy and standard of care:	Immunotherapy for patients with brain stem glioma and glioblastoma (19) Tumor lysate pulsed dendritic cell immunotherapy for patients with brain tumors (20) Dendritic cell-based tumor Vaccine adjuvant immunotherapy of human glioblastoma multiforme (WHO grade IV gliomas) (21) A pilot study to evaluate PBR PET in brain tumor patients treated with chemoradiation or immunotherapy (22)	A study of ICT-121 dendritic cell vaccine in recurrent glioblastoma (19)

The latter two upfront GBM trials mentioned above suggest a common theme in the ability to properly treat GBM. Patient outcome is highly variable, which could possibly be due to the heterogeneity of GBM tumors. In 2010, research utilizing data from The Cancer Genome Atlas developed a catalog of genomic abnormalities correlated with GBM tumors and helped to categorize four different GBM tumor subtypes: Classical, Proneural, Mesenchymal, and Neural (30), which has been more recently reclassified into Classical, Proneural, and Mesenchymal as the 3 main the subtypes of GBM (31). The significance of GBM subtype lies within the discovery that each subtype holds distinct genomic abnormalities, tumor microenvironments, and most importantly in terms of patient outcome, treatment response. Furthermore, GBM subtype switching has been observed upon disease recurrence, such as a Proneural to Mesenchymal transition, which been implicated in treatment resistance GBM (32). Thus, GBM subclasses have the potential to influence patient treatment, though the clinical relevance of this proposed classification remains to be determined. This is especially true given recent single cell sequencing of GBM tumors demonstrating that the even within classes of tumors vast heterogeneity exists (33–35). Future single cell sequencing studies will delineate the cell populations that remain after immunotherapy in order to better design combination therapies.

In comparison to upfront GBM trials for combination immunotherapy, recurrent GBM trials have even fewer results. These trials include single therapy dendritic cell vaccines in which patient WBCs are cultured and reintroduced (similar to the ICT-107 trial mentioned above) (19), treatment with immune adjuvants with radiation (11), and combination treatments utilizing checkpoint inhibitors (discussed below) (16). Overall, while there are some other recurrent GBM clinical trials, few have reported results.

PD-L1 IMMUNE SYSTEM BLOCKADE AND CHECKPOINT INHIBITION

The programmed death pathway's role in GBM tissue immunity has been examined and preclinical studies suggest that inhibition of this pathway, termed "checkpoint inhibition," has high therapeutic potential. This pathway involves down regulation of the immune response after it has run its course (36). Specifically, in a healthy individual, it stimulates the inhibition of function in T-cells, and thus prevents T-cells from being overactive in an individual whose immune response needs to be stopped after an infection (37). In the case of tumors, activation of this pathway prevents T-cells, which express the PD-1 receptor, from accessing and acting on tumors. In mice transplanted with human GBM tumors, anti-PD-1 therapy completely eradicated GBM in 44.4% of mice and combination anti-PD-1 therapy with the chemotherapy drug TMZ completely eradicated GBM tissue in all mice. Combined therapy also reduces the frequency of exhausted tumor infiltrating lymphocytes (TIL), or T-cells that have lost their functional ability to develop immune responses (38), without affecting the non-exhausted TIL load that penetrate the BBB and enter GBM tissue. In other words, after combined therapy TIL ($CD8^+$ T-cells) do not highly express the PD-1 receptor and therefore, cannot bind the PD-L1 (ligand) on GBM tissue resulting in the high success rate of this combined therapy (39). However, while these results boast an extremely potent immunotherapy regimen in mice, they have not translated well into efficacy in GBM clinical trials.

One issue that needs to be addressed in immunotherapy trials is the immunosuppressive microenvironment of GBM (40), which can induce angiogenesis leading to tumor growth (41). The PD-L1 ligand can induce and upregulate T-reg cells (42), which are immunosuppressive cells that protect the GBM tumor from the body's immune system (43). T-reg cells are involved in the inhibition of T-cells that recognize and attack self-antigen that are present on both normal tissue and tumor tissue (44). In a normal setting, this prevents a host's immune system from developing autoimmunity, or the condition in which the immune system attacks its own cells. In the case of GBM, induction of T-reg cells prevents the destruction of the tumor tissue due to presence of self-antigen on tumor cells.

The first large scale anti-PD-L1 therapy clinical trial in GBM was conducted using a drug called Nivolumab. Unfortunately, unlike the mouse studies mentioned above, the results were

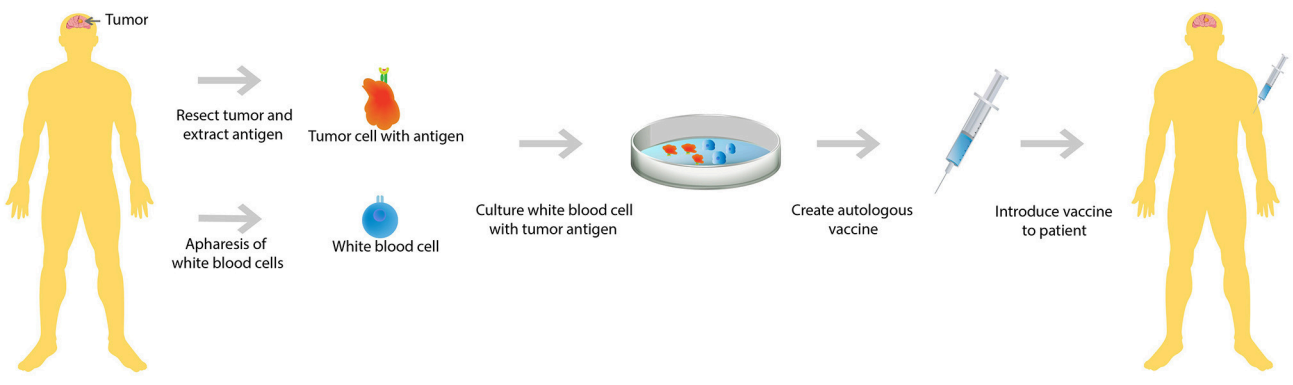

FIGURE 1 | Autologous vaccine therapy. Both GBM tumor antigen and white blood cells are extracted from the patient. Subsequently, the extracted white blood cells are cultured with the GBM tumor antigens. A vaccine is created that is specific to each individual GBM patient's tumor antigen and then the cultured white blood cells are reintroduced to the patient.

not promising. Specifically, nivolumab was determined to be no more effective at increasing overall survival than the anti-VEGF cancer drug, bevacizumab (37). But this lack of success was not a failure because it highlighted a few key points about the immunosuppression of GBM. It appears anti-PD-L1 therapy may not be sufficient to alleviate immunosuppression of GBM patients. In other words, other immunosuppressive factors within the microenvironment of GBM may render T-cells anergic, or unable to produce a functional response, which would result in a failed immune response despite PD-1 pathway inhibition. In addition, this highlights the blood brain barrier (BBB) as a significant limiting factor in GBM treatment. Because nivolumab is too large to cross the BBB, this study supports the assumption that anti-PD-L1 antibody therapy exerts its effect outside of the BBB. The therapeutic effect of anti-PD-1 treatment on T-cells occurs before they cross the BBB and enter the tumor microenvironment. However, if there is an inadequate population of T-cells in the periphery and/or the T-cells have already crossed the BBB and have been rendered anergic by the microenvironment of the GBM tumor, then PD-L1 therapy may not be effective, as was exemplified in this trial (37).

EPIGENETICS

As discussed above, immunotherapy and combined immunotherapy are intensively studied in the treatment of GBM, although they do not seem to be wholly effective in treating GBM nor do they encompass the entire picture of GBM treatment. Recent discoveries have identified epigenetic pathway specific GBM biomarkers, which have enormous potential in terms of treatment because they can be used as targets for therapy. This epigenetic focus encompasses genes and gene regulators that are not dependent on the DNA sequence yet can be inherited and modified by endogenous enzymes. The influence of these epigenetic enzymes facilitates DNA modifications like methylation, acetylation, phosphorylation, and ubiquitination, all of which alter gene expression and change the state of cells within the body. Ultimately, dysfunction in these enzymes can lead to modifications within the cell that can lead to the development of cancer (45).

However, there is hope regarding epigenetic modifications that result in cancer. The good news is that modification of the DNA sequence by epigenetic enzymes is reversible. In fact, it appears as though a key factor in epigenetic regulation are long noncoding RNA transcripts (lncRNAs) that facilitate the molecular processes of epigenetic regulation (46). lncRNAs have been proposed to control activation and modulation of epigenetic enzymes (46, 47), and lncRNAs have been shown to be involved in cancer resistance to immune reaction through antigen release, antigen presentation, immune activation, and immune cell migration and infiltration (45, 48). Additionally, nine lncRNAs as prognostic markers for GBM patient outcome (49). Therefore, inhibition of epigenetic changes by lncRNAs has immense potential as a GBM therapy (50).

Several lncRNAs are differentially expressed in GBM relative to normal brain tissue (51). For instance, the HOX Transcript Antisense Intergenic RNA (HOTAIR) is completely undetectable in normal brain but is overexpressed in GBM tumors. HOTAIR is a lncRNA from the homeobox super family on the HOXC locus on chromosome 12q13.13 (52). It was the first transregulation lncRNA to be found and has been linked to osteoarthritis and cardiovascular disease in addition to multiple cancers (53). HOTAIR interacts with chromatin modeling complexes, which consequently leads to gene regulation and the promotion of tumor cell invasion, metastasis, and maintenance of stemness in cancer cells (54). However, in specific cancers like colorectal cancer (CRC) its exact method of action is unclear. What is known is that knockdown of HOTAIR in CRC cell lines drastically reduces CRC cell proliferation, which has also been observed in mouse GBM models as well (55, 56). Additionally, HOTAIR also plays a role in drug sensitivity in CRC cells. Knockdown of HOTAIR demonstrates that CRC cells display increased sensitivity to Cisplatin, a chemotherapy agent, and HOTAIR was observed to be upregulated in drug resistant CRC cell lines (57).

We and others determined whether HOTAIR levels were significantly correlated with GBM tumors and GBM serum (52, 54, 58). Quantitative real-time-PCR (qRT-PCR) was conducted to detect HOTAIR levels in 15 pairs of GBM tissue and GBM serum samples. The result of the study demonstrated that not only is HOTAIR dysregulated and that the dysregulated lncRNA facilitates GBM proliferation, but that there are also higher levels of HOTAIR in serum exosomes of GBM patients (52). This association between HOTAIR and GBM proliferation and expansion has been demonstrated by others as well in GBM patients *in vivo* (58). Additionally, it was discovered that HOTAIR mediates the ability of GBM cells to migrate and invade through membranes *in vitro* (56). Therefore, there is considerable evidence that not only is HOTAIR related to cancer proliferation, but that it is also an independent negative prognostic marker in GBM (54, 58).

We demonstrated that HOTAIR is part of a proliferative pathway controlled by the bromodomain and extra-terminal domain (BET) epigenetic reader proteins (**Figure 2**). One such protein, Bromodomain Containing 4 (BRD4), was shown to bind to the HOTAIR promoter and in doing so, controlled HOTAIR levels. Specifically, BRD4 binding and activation led to increased levels in HOTAIR (52). As expected then, the use of a Bromodomain and Extraterminal (BET) inhibitor, I-BET151, that reduces BRD4 binding at the HOTAIR promoter was observed to cause a consequential decrease in the expression of HOTAIR in GBM cells (52, 59). Another BET inhibitor, JQ1, was observed to induce G1 cell cycle arrest and apoptosis, which led to reduction of significant GBM genes (c-MYC, hTERT, Bcl-2, Bcl-xL, and P21CiP1/WAF1) (60). JQ1 reduces tumor growth via reduction of PD-L1 expression on tumor cells leading to less T-cell death induced by the PD-L1 pathway (36). This idea of BET inhibitors promoting T-cell immune reactions against cancer cells has been further supported by research that has shown that BET inhibitors promote T-cell infiltration in mouse models. Moreover, it was discovered that epigenetic inhibitors can even rejuvenate the ability of exhausted T-cells to infiltrate tumor cells once again (61). This further exemplifies the ability of

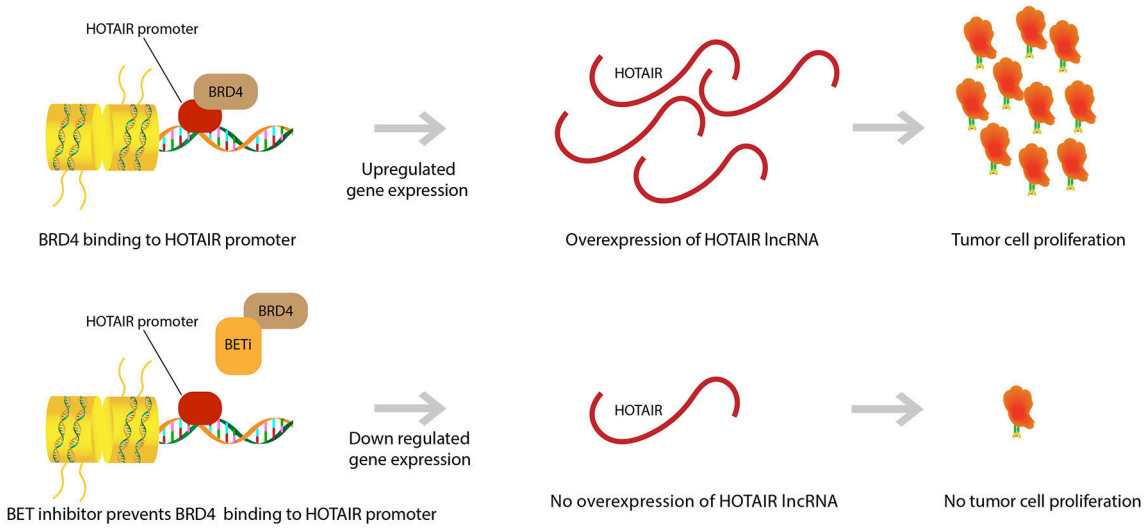

FIGURE 2 | BET inhibitor drugs and HOTAIR level regulation. BRD4 binding to HOTAIR promoter region causes an upregulation in gene expression for the lncRNA HOTAIR. This overexpression in HOTAIR is associated with proliferation and expansion of GBM tumor cells. BET inhibitors prevent binding of BRD4 to the HOTAIR promoter and yield a down regulation in HOTAIR expression. Subsequently, there is prevention of tumor cell proliferation.

epigenetic pathways to control neoplastic activity and highlights the therapeutic potential of epigenetic pathway modulation in GBM.

Another study involving lncRNAs looked at lncRNA LINC00470, which like HOTAIR, is overexpressed in GBM when compared to normal brain tissue. Moreover, it was reported that patients with higher levels of LINC00470 had poorer prognoses in terms of survival time when compared with patients with lower levels of LINC00470 (62). Researchers in this study investigated LINC00470 and its interaction with AKT, a serine/threonine kinase related to cell proliferation, autophagy, and survival. What was discovered was that interaction between LINC00470 and AKT caused an upregulation of AKT activation thus leading to cell proliferation and GBM tumorigenesis (62). Thus, there is strong evidence for the role of lncRNA and cancer proliferation through epigenetic interactions.

Research looking into epigenetic management of cancer has shown that epigenetic inhibitors are safe and may be effective in treating certain neoplasms. For example, Vorinostat, a histone deacetylase (HDAC) inhibitor that works by preventing the action of histone deacetylases (HDACs), has been approved for treatment of Cutaneous T-cell Lymphoma and may benefit patients suffering from prostate cancer, breast cancer, and lung cancer (63). 5-Azacytidine (5-AZA), another epigenetic inhibitor that works through prevention of the enzyme DNA methyltransferase, has been shown to be effective in treating patients with myelodysplastic syndromes (64). Additionally when used in combination with chemotherapy agents, epigenetic inhibitors, such as the DNA methyltransferase inhibitor Decitabine, and chemotherapy drugs, such as DNA synthesis inhibitors Mitoxantrone Hydrochloride, Cytarabine and Etoposide, were effective in increasing overall survival in patients suffering from relapsed or refractory acute myeloid leukemia or high-risk myelodysplastic syndromes (65). Regarding epigenetic and immunotherapy combination trials, researchers are currently assessing the viability and safety of epigenetic inhibitors, like 5-AZA, with the checkpoint inhibitor, like pembrolizumab, in combinations such as 5-AZA with pembrolizumab, entinostat (HDAC inhibitor) with pembrolizumab, and vorinostat with pembrolizumab (66).

CONCLUSIONS AND FUTURE PERSPECTIVES

GBM remains an incurable disease despite increased understanding of the genetic and epigenetic pathways dysregulated in these tumors. While immunotherapy trials have shown minor improvements in overall survival, the actual increase in time for the patient is still only months and treatment is highly limited to certain subtypes of GBM. Importantly, lncRNAs can be detected in patient serum and can be used as biomarkers for efficacy of drugs in patients. Thus, it seems that the pursuit of other treatment options such as combining epigenetic pathway inhibitors along with immunotherapy treatment could bring medicine closer to treating GBM. Though it may be difficult to create a blanket treatment for all types of GBM, it is worth dedicating resources into future research of individualized patient treatment.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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REVIEW
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Macromolecular Drug Carriers for Targeted Glioblastoma Therapy: Preclinical Studies, Challenges, and Future Perspectives

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Glioblastoma, the most common, aggressive brain tumor, ranks among the least curable cancers—owing to its strong tendency for intracranial dissemination, high proliferation potential, and inherent tumor resistance to radiation and chemotherapy. Current glioblastoma treatment strategies are further hampered by a critical challenge: adverse, non-specific treatment effects in normal tissue combined with the inability of drugs to penetrate the blood brain barrier and reach the tumor microenvironment. Thus, the creation of effective therapies for glioblastoma requires development of targeted drug-delivery systems that increase accumulation of the drug in the tumor tissue while minimizing systemic toxicity in healthy tissues. As demonstrated in various preclinical glioblastoma models, macromolecular drug carriers have the potential to improve delivery of small molecule drugs, therapeutic peptides, proteins, and genes to brain tumors. Currently used macromolecular drug delivery systems, such as liposomes and polymers, passively target solid tumors, including glioblastoma, by capitalizing on abnormalities of the tumor vasculature, its lack of lymphatic drainage, and the enhanced permeation and retention (EPR) effect. In addition to passive targeting, active targeting approaches include the incorporation of various ligands on the surface of macromolecules that bind to cell surface receptors expressed on specific cancer cells. Active targeting approaches also utilize stimulus responsive macromolecules which further improve tumor accumulation by triggering changes in the physical properties of the macromolecular carrier. The stimulus can be an intrinsic property of the tumor tissue, such as low pH, or extrinsic, such as local application of ultrasound or heat. This review article explores current preclinical studies and future perspectives of targeted drug delivery to glioblastoma by macromolecular carrier systems, including polymeric micelles, nanoparticles, and biopolymers. We highlight key aspects of the design of diverse macromolecular drug delivery systems through a review of their preclinical applications in various glioblastoma animal models. We also review the principles and advantages of passive and active targeting based on various macromolecular carriers. Additionally, we discuss the potential disadvantages that may prevent clinical application of these carriers in targeting glioblastoma, as well as approaches to overcoming these obstacles.

Keywords: macromolecular, glioblasoma, preclinic study, drug carriers, targeted therapies

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INTRODUCTION

Glioblastoma (GBM) is the most common and the most aggressive primary malignant tumor of the central nervous system. Current therapy regimens are initial surgical resection which is followed by radiation and chemotherapy using the DNA alkylating agent Temozolomide. However, glioblastoma tumors are very aggressive and resistant to multimodal therapies, and the average life expectancy and overall survival is <18 months. Therefore, current clinical therapies are ineffective as they are more palliative in nature than curative. Treatment options are limited since complete surgical resection is impossible and since tumor tissue is heterogeneous and penetrates surrounding healthy brain tissue. As a result, almost all the patients develop recurrent tumors, which are more aggressive and often resistant to anticancer drugs. Furthermore, drug delivery to the brain is hampered by the presence of blood brain barrier (BBB) (**Figure 1**), resulting in poor delivery of drugs to the tumor tissue and dose related systemic toxicity in healthy tissues. Considering limitations and overall ineffectiveness of the current approaches in the treatment of glioblastoma, there is an urgent need for more efficient treatments to achieve improved outcome and increase overall survival in glioblastoma patients.

One of the approaches of tumor specific drug delivery is based on macromolecular drug carriers. Advantages of macromolecular carriers over small molecule drugs include protection of the drugs from degradation, improvement of drug solubility, and blood plasma half-life-time, release of the drugs in the optimal dosage range, and delivery of the anticancer agents specifically to the tumor. Currently used macromolecular drug delivery systems, such as liposomes and polymers, passively target solid tumors by capitalizing on abnormalities of the tumor vasculature, its lack of lymphatic drainage, and the enhanced permeation and retention (EPR) effect. However, to achieve therapeutic efficacy in treating GBM, polymeric carriers must successfully overcome several transport barriers (including BBB), extravasate tumor micro vessel walls, and penetrate the plasma membrane of the tumor cells. In addition to passive targeting, further selectivity of macromolecules can be achieved by active targeting. Active targeting approaches include the application of cancer biomarker proteins that bind to overexpressed cell surface proteins in specific cancer cells. It also includes stimuli-responsive macromolecular carriers which can release anticancer drugs specifically in the tumor tissue or tumor cells in response to internal or external stimuli. Internal stimuli drug release is based on the fact that tumor tissue has a different environment compared to normal tissue; more acidic pH, higher redox potential, and/or overexpressed proteins, and enzymes. In addition, stimuli such as light, ultrasound, a magnetic field, and temperature, can be also applied to the tumor site externally to allow drug to be released and their molecular target in the cancer cells reached.

In the present review, we report the use of macromolecular carriers with different composition, including lipids, proteins, and synthetic nanoparticles and we consider their targeting aspects. We also review selected preclinical brain drug delivery macromolecular carriers and highlight their potential in the clinical treatment of glioblastoma.

ACTIVE TARGETING

Active targeting to tumor sites generally exploits an intrinsic cell characteristic to obtain drug delivery. Utilizing a homing device such as an antibody or ligand, a drug can bind tumor cells through antigens or receptors without affecting any other normal tissues (1).

This classic concept of active targeting has been successful due to its high selectivity and binding affinity to produce a series of antibody-drug conjugates or ADC. Currently, there are several marketed ADC including Brentuximab and Trastuzumab; however, much more promising ADC are under investigation in clinical trials (2). Despite all of the enthusiasm toward this type of approach, ADC strategy is also facing a few problems that must be resolved in order to take a greater step forward. These problems include low efficiency in cellular uptake or in endosomal escape, heterogeneity of tumor cells in the expression of specific receptors, and challenges in manufacturing (3).

Another example of active targeting is stimuli-responsive targeting. As knowledge of tumor biology and technologies advances, a variety of novel, and smart devices have been introduced showing unprecedented efficiency of drug delivery. Environmentally responsive macromolecular drug carriers can release cargo drugs in the targeted tumor tissues as a response of external stimuli such as heat, light, ultrasound, and a magnetic field. This triggered drug release provides advantages over other types of active targeting technologies in that it allows exquisite control over time and location of drug release (4).

Receptor-Mediated Endocytosis

Clathrin-dependent endocytosis is known for a predominant mechanism for the internalization of ADC even though the other pathways including caveolin-mediated, clathrin–caveolin-independent, and cholesterol/macropinocytosis-mediated are also reported in literature (5). To briefly describe this process, once binding to specific receptor, ADC-receptors are invaginated by cells through the formation of clathrin-coated vesicles. With dynamin GTPase, the vesicles are then released from the membrane with some of the mature vesicles fusing with lysosomes to form lysosome-late-endosome hybrids through Ras-related protein 7 (Rab7). While cells perform this whole procedure for acceptance of the ADC-receptors, ADC have a couple of opportunities to release drugs from the antibody. First, ADC can release drugs in the endosomal phase. Acid-labile linkers such as (6-maleimidocaproyl) hydrazine (EMCH) allow ADC to unload drugs in the endosome because of the acidic environment of endosomes. Second, ADC can release drug in the lysosome. The high content of enzymes such as cathepsins and collagenases in lysosomes can digest some dipeptide linkers such as valine-citrulline (vc) or phenylalanine-lysine linkers which are specific for cathepsin B. Third, even without a cleavable linker between antibody and drug, drugs can be released by proteolytic digestion in the lysosome. Some products from the digestion of metabolites

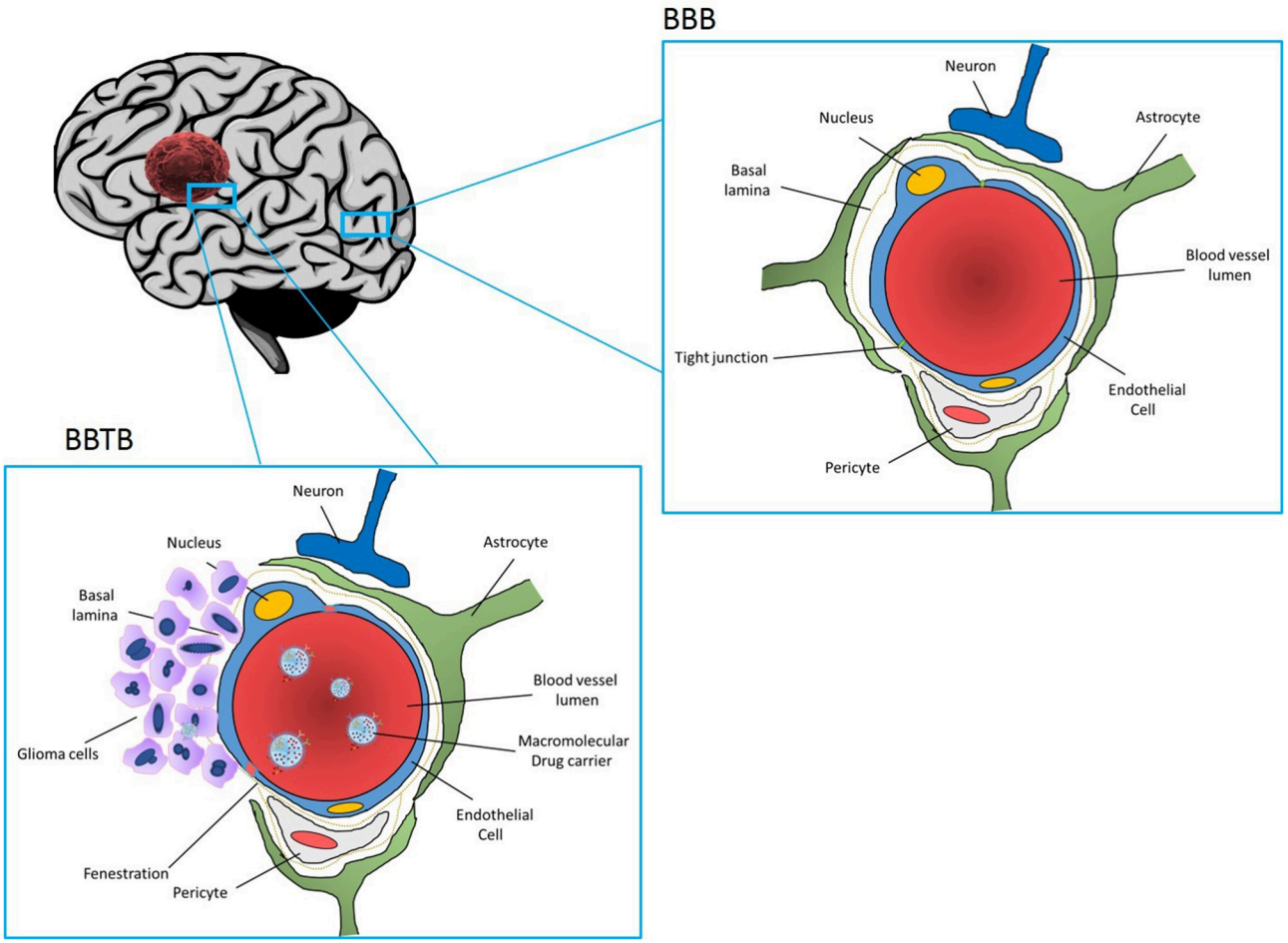

FIGURE 1 | Section of the brain showing normal part of the brain and blood brain barrier (BBB) (upper figure), and part of the brain infiltrated with GBM Blood vessels are enclosed by endothelial cells connected with tight junctions which seal the intercellular space and prevent entrance of drugs from the blood stream to the brain. BBB is further reinforced with a basal lamina. While basal lamina is disrupted by GBM tumor cells (figure the left), endothelial cells are still present forming blood brain tumor barrier (BBTB). Nanoparticles loaded with drugs and coated with receptors specific to the tumor vasculature and glioma cells can interact with the BBTB allowing for transcytosis and delivery of the drugs to GBM tumor cells.

still retain the original activities of the drug and express their activities in the cells or neighboring cells; thebystander effect (6).

Another mechanism involved in internalization of ADC-receptor is autophagy. As a part of the autophagy process, ADC-receptor can be taken up by autophagosomes and digested in autolysosomes releasing drugs afterward (7).

Antibodies

Epidermal Growth Factor Receptor EGFR

The most common genetic aberration associated with malignant glioma is amplification of the epidermal growth factor receptor, with a frequency of about 50% (8).

Targeting the receptor for epidermal growth factor receptor (EGFR) has been rewarding in cancer and many pharmaceuticals are approved alone or in combination with chemotherapy for colorectal cancer, non-small-cell lung cancer, and pancreatic cancer, among others, but not for gliomas (9). It remains unresolved why EGFR targeting has not been successful for glioma as it should be ideally suitable in the context of this disease (9).

Jamali et al. delivered curcumin using Poly (D, L-lactic-co-glycolic acid) nanoparticles (PLGA NPs). Monoclonal antibody targeting epidermal growth factor receptor variant III (EGFRvIII) was incorporated into PLGA NPs showing selective internalization of the NPs by an EGFRvIII overexpressed human glioblastoma cells and increased photodynamic toxicity of curcumin (10).

In another study, etoposide (ETP) was loaded in solid lipid nanoparticles (SLNs) containing a monoclonal antibody for insulin receptors and another monoclonal antibody against EGFR (11). Since insulin receptors are found on human brain microvascular endothelial cells (HBMEC), these dual targeting nanoparticles passed across HBMEC/HA (human astrocytes), an *in vitro* model for blood-brain barrier, and increased cytotoxicity in the treatment of U87MG cells (**Table 1**).

TABLE 1 | Active targeting with antibodies (or ligands) for GBM treatment.

Group	Carrier/Technology	Targeting method	Cargos	References
Nanoparticle	Poly (D, L-lactic-co-glycolic acid), PLGA	Anti-EGFRvIII (A-EGFRvIII-f)	Curcumin	(11)
	PLGA	OX26 type monoclonal antibody for transferrin receptor	Temozolomide	(12)
	Bovine serum albumin-polycarprolactone (BSA-PCL)	Anti-EGFR	Radioiodine	(13)
	(PLGA) and PLGA-polyethylene glycol (PLGA-PEG) polymers	Anti-Fn14 receptor		(14)
	PEGylated-hydrophilic carbon clusters	Epidermal growth factor receptor (EGFR) binding peptide		(15)
	Superparamagnetic iron oxide nanoparticle (SPION) based polymeric nanocomposites	Antibody against nestin, a stem cell marker, and transferrin	Temozolomide	(16)
	PLGA	Human/mouse chimeric anti-GD2 antibody ch14.18/CHO, enabling specific targeting of GD2-positive GBM cells	Letrozole,	(17)
	PEG-PE-based polymeric micelles	The micellar system was decorated with GLUT1 antibody single chain fragment variable (scFv)	Doxorubicin, Durcumin	(18)
	Magnetite particles + PEG	Plant lectin viscumin		(19)
	non-living bacterially-derived minicells	Epidermal growth factor receptor (EGFR) targeting	Doxorubicin	(20)
	Nanorings made of dihydrofolate reductase (DHFR) fusion proteins	A PEGylated EGFR targeting peptide (LARLLT)	Methotrexate	(21)
	Graphene oxide (NGO)	Integrin avß3 monoclonal antibody (mAb)	Pyropheophorbide-a	(22)
	Solid lipid nanoparticles (SLNs)	83–14 Monoclonal antibody and anti-epithelial growth factor receptor	Etoposide (ETP)	(11)
	SLN	Melanotransferrin antibody and tamoxifen	ETP	(23)
	SLN	melanotransferrin antibody	ETP	(24)
	superparamagnetic iron nanoparticles (SPION)	Hsp70-specific antibody (cmHsp70.1)		(25)
	polylysine-DTPA (Diethylenetriamine pentaacetate)	Monoclonal antibody to Connexin 43	Gd(III)	(26)
	iron oxide nanoparticles (IONP)	Anti-EGFRvIII-cetuximab (an EGFR- and EGFRvIII-specific antibody		(27, 28)
	Bovine serum albumin	Monoclonal antibodies against vascular endothelial growth factor (VEGF)	Ferric oxide (Fe_3O_4) as a MRI contrast agent	(29)
	nanoparticles	Fn14 monoclonal antibody		(30)
	Nanogels based on PEG and polymethacrylic Acid block copolymer (PEG-b-PMAA)	Monoclonal antibodies to connexin 43 (Cx43)	Cisplatin	(31)
	Activatable cell-penetrating peptides (ACPP) : cyclic-RGD PLGC(Me)AG-MMAE-ACP	Integrin a(v)ß(3)-binding domain, cyclic-RGD, was covalently linked to the ACPP	Monomethyl-lauristatin E (MMAE)	(32)
	Nanogels (PEG-b-PMAA) diblock copolymer base)	Monoclonal antibodies to connexin 43 and brain-specific anion transporter (BSAT1)	Cisplatin	(33)
Liposome	liposomes	A chlorotoxin peptide fused to human IgG Fc Region without hinge sequence (M-CTX-Fc) targeting CD44	Doxorubicin	(34)
	immunoliposome	Angiopep-2 (An2) and anti-CD133 monoclonal antibody (CD133 mAb)	Temozolomide	(35)
	Lipid nanocapsule	Antibody for CXCR4	Rhenium-188	(36)
	nanometric liposome	LAT1 antibody	WP1066	(37)
	liposomes	iNGR	Doxorubicin	(38)

(Continued)

TABLE 1 | Continued

Group	Carrier/Technology	Targeting method	Cargos	References
	PEGylated liposomes	Anti-VEGF and anti-VEGFR2 monoclonal antibody	Cisplatin	(39)
	liposomes	Anti-CD133 monoclonal antibody	Gemcitabine and Bevacizumab	(40)
	a cationic liposome	Anti-transferrin receptor single-chain antibody fragments	Temozolomide	(41)
	PEGylated liposomes	Anti-EGFR		(42)
ADC	Monoclonal antibody	The single chain variable fragment (scFv) from the D2C7 monoclonal Antibody (mAb) of EGFR	Pseudomonas Exotoxin PE38KDEL	(43)
	Monoclonal antibody	Monoclonal Antibody against uPARAP/Endo180,	Dolastatin derivative, monomethyl auristatin E	(44)
	Monoclonal antibody	Anti-CD40 agonistic monoclonal antibody (FGK45)		(45)
	Monoclonal antibody	Glioblastoma-specific CD68 antibody	Curcumin	(46)

Transferrin Receptor (TfR)

TfR plays a key role in the control of the rate of cellular iron uptake, tuning the amount of iron delivered to the metabolic needs of the cells (47).

The presence of BBB and hard parenchyma of the GBM has been a predominant challenge in chemotherapy in the treatment of GBM. Ever since the finding that iron-loaded transferrin is taken up via receptor-mediated endocytosis at the brain capillaries and transcytosed, many researchers have utilized transferring-transferrin receptor to transfer drugs across the BBB (48).

Kuo et al. also utilized this idea to deliver etoposide for the GBM treatment. They generated solid lipid nanoparticles (SLNs) conjugated with melanotransferrin antibody (MA) and examined its transcytosis efficiency across human brain-microvascular endothelial cells (HBMECs) and the resulting growth inhibition of U87MG cells. The *in vitro* transwell assay strategy triggered melanotransferrin-mediated transcytosis and promoted the growth-inhibitory efficacy in U87MG cells suggesting the MA-ETP-SLNs as a promising delivery system for malignant GBM (24) (**Figure 2**).

The findings that there is a higher reactivity in GBM for anti-TfR and that GBM cells are very sensitive to the effects of anti-TfR mAbs instigated research targeting TfR as a direct way to kill GBM cells rather than a way to bypass BBB (49).

Ramalho et al. developed poly(lactic-co-glycolic acid) nanoparticles functionalized with OX26 type transferrin monoclonal antibody with a purpose to target transferrin receptors on GBM cells (U251 and U87). In this study, the approach facilitated uptake of the nanoparticles by the GBM cells while normal human astrocytes did not internalize the nanoparticles efficiently. However, this encouraging data was not reproduced in comparative cytotoxicity tests with native nanoparticle and TfR-targeting nanoparticle (12).

Antibodies for Cancer Stem Cell

Cancer stem cells (CSCs), a small population of quiescent or slowly dividing cells, significantly contributes to the resistance to therapy, and recurrence of cancer. Targeting CSCs could be a good strategy to improve the outcome of cancer therapy. There have also been extensive research to cure GBM through targeting specific markers of CSCs such as CD44, aldehyde dehydrogenase (ALDH) and CD133 as follows.

Mahmud et al. fused human IgG Fc of CD44 with a chlorotoxin peptide (M-CTX-Fc). The authors verified the superiority of M-CTX-Fc by comparing U251MG-P1 cells (CD44+) with CD44-negative cells (SKBR3) in cellular uptake, *in vitro* cytotoxicities and *in vivo* tumor growth inhibition. Since CD44 positivity represent stemness of a cancer cell line along with other markers such as OCT3/4, SOX2, KLF4, and Nanog, this approach may contribute to the retardation of tumor growth by restricting cancer stem cell population (34).

CD133+/ALDH1+ in glioblastoma stem cells (GSCs) were targeted by Kim et al. to deliver Temozolomide with liposome (35). With additional BBB targeting molecule, angiopep-2 (An2), this dual-targeting immunoliposome encapsulating TMZ (Dual-LP-TM) increased *in vitro* cytotoxicity and apoptosis in U87MG GSCs. This approach suggests a potential use of Dual-LP-TMZ as a therapeutic modality for GBM demonstrating significant *in vivo* tumor reduction in intracranial U87MG-TL GSC xenografts (**Table 1**).

pH-Responsive Drug Carriers

One of the most widely used intrinsic stimulus for controlled drug release is pH difference between normal tissues and tumor tissue, as well as between cellular compartments. Since tumor metabolism is very active and requires considerable energy for tumor growth, there is increased production of hydrogen ions (H^+) and lactate resulting in an acidic tumor environment (pH 6.5)(50, 51). Since normal tissue has a pH 7.4, this difference can then be exploited for triggering drug release

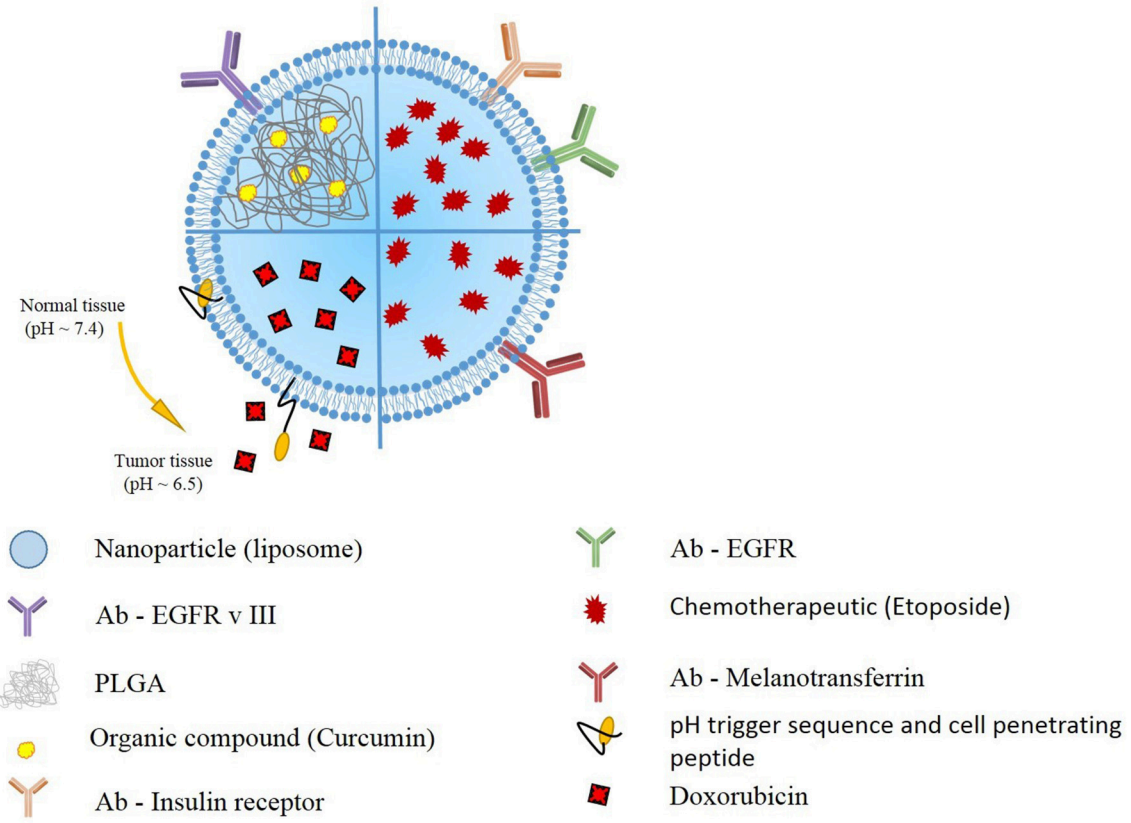

FIGURE 2 | Schematic presentation of selected liposomal nanoparticles. Liposomal nanoparticles are versatile, and can be loaded with wide variety of anti-oncogenic compounds, such as curcumin, etoposide, and doxorubicin). To further enhance the targeting, the outer layer includes antibodies targeting GBM cells, or pH responsive and cell penetrating peptides.

in the more acidic tumor tissue. Furthermore, the difference in pH between cellular compartments at the cellular level, between endosomes (pH 5.5) or lysosomes (pH 5.0) can be also used to trigger drug release in the cytoplasm. Drug release is usually accomplished by incorporation of an acid sensitive spacer between carrier and drug, which enables drug release at slightly acidic tumor environment or endosomes, and lysosomes of cancer cells.

While these reports use only a pH-triggered drug release mechanism to locally release drug at the tumor site, to further increase specificity Miller at al. (52) constructed a pH-responsive micelle conjugated with a novel moiety against overexpressed cell surface platelet derived growth factor receptor (PDGFR). These micelles are loaded with Temozolomide (TMZ), targeted to PDGFR on glioblastoma cells, resulting in pH-dependent release of TMZ preferably in tumor tissue, thereby reducing systemic toxicity. *In vitro* studies have shown that these micelles exhibit specific uptake and increased cell killing in glioblastoma cells, and *in vivo* studies demonstrated increased accumulation of micelles in brain tumor tissues. Although these results are promising, addition of *in vivo* tumor reduction efficacy and survival experiments would greatly improve the potential of this approach in clinics (**Table 2**).

An interesting approach to target glioblastoma, reported by Zhao et al. (55) used tumor-specific pH-responsive peptide $H_7K(R_2)_2$ as a targeting ligand. This peptide contained the pH trigger sequence polyhistidine H_7 and cell penetrating peptide arginine rich sequence $(R_2)_2$ and exhibited activity at an acidic pH environment due to the ionization of the histidine thus switching from hydrophobic to hydrophilic conditions. This peptide was used to modify pH-sensitive liposomes loaded with doxorubicin (DOX-PSL-$H_7K(R_2)_2$). The pH-triggered doxorubicin release from the pH-sensitive liposomes and targeting effect under acidic conditions was demonstrated in *in vitro* experiments. Furthermore, *in vivo* experiments in C6 tumor-bearing mice and U87-MG orthotopic tumor-bearing nude mice confirmed the anti-tumor activity of pH-responsive peptide modified liposomes loaded with doxorubicin. Results showed that the DOX-PSL-$H_7K(R_2)_2$ (37 days) significantly improved the survival rate of mice compared with control animals (23 days) or doxorubicin treated animals (24 days).

Since doxorubicin is a highly effective anticancer therapeutic for the treatment of many malignancies, there is a great interest in using it in the treatment of glioblastoma. Marrero et al. (53) examined the hydrazine-conjugated doxorubicin

TABLE 2 | Stimuli responsive targeting macromolecules for GBM treatment.

Carrier	Composition	Targeting mechanism(s)	Drug delivered	Animal model	Outcome	References
Polymer based carriers	Albumin	(6-maleimidocaproyl) hydrazone conjugate of doxorubicin	Doxorubicin	U87-luciferase expressing orthotopic xenografts	Aldoxorubicin, U87-luc tumors were 10-fold smaller when compared to control animals, and median survival of Aldoxorubicin treated mice was 62 days, compared to 26 days median survival of control or doxorubicin treated animals	(53)
	Elastin-like polypeptide	Thermo-responsive	c-Myc inhibitory peptide	Rat C6 Glioma orthotopic model	Thermal targeting of the Bac-ELP1-H1 polypeptide to the tumors resulted in significant delayed onset of neurological deficits, 80% tumor volume reduction, and doubled survival.	(54)
	1,2-Distearoyl-sn-glycero-3-phosphoethanolamine (methoxy(polyethylene glycol)-2000) (DSPE-PEG)	pH-sensitive liposomes modified with tumor-specific pH-responsive peptide $H_7K(R_2)_2$	Doxorubicin	Rat C6 glioma, U87MG human glioblastoma	*In vitro* experiments show pH-triggered DOX release from the pH-sensitive liposomes under acidic conditions The anti-tumor activity has been confirmed in C6 tumor-bearing mice and U87-MG orthotopic tumor-bearing nude mice	(55)
Liposomes	Superparamagnetic iron oxide 1,2-distearoyl-sn-glycero-3-phosphocholine; 1,2-Distearoyl-snglycero-3-phospho-rac-glycerol sodium salt	Magnetic responsive	Doxorubicin	Rat C6 glioma orthotopic model		(56)
	Chitosan-PEG copolymer coated iron oxide nanoparticles , cross-linked and functionalized with BG	Tumor targeting peptide chlorotoxin, redox responsive	MGMT inhibitor O^6-benzylguanine (BG)	Primary GBM6 xenograft tumor model which expresses high levels of MGMT	Treatment with nanoparticles and TMZ showed a 3-fold increase in median overall survival in comparison to TMZ treated and untreated animals.	(57)
Nanoparticles	Poly(ethylene Glycol)-b-poly(trimethylene carbonate-co-dithiolane trimeth-ylene carbonate)-b-polyethylenimine (PEG-P(TMC-DTC)-PEI,	Tumor targeting peptide angiopep-2 (ANG), redox responsive	Protein toxin saporin	U-87 MG-Luc cells orthotopic xenografts	Treatment with polymersomes resulted in 2-fold increase in median overall survival in comparison untreated animals.	(58)
	Human serum albumin (HSA) NPs stabilized with intramolecular disulfide bonds	Tumor targeting peptide substance P (SP) redox responsive	Paclitaxel (PTX)	U-87 MG-Luc cells orthotopic xenografts	The *in vitro* PTX release from NPs occurred in a redox-responsive manner. Treatment *in vivo* showed pro-apoptotic effect and resulted prolonged survival period of treated animals	(59)
	distearoyl phosphoethanolamine-PEG-2000-amine and N-palmitoyl homocysteine	Peptide targeting PDGF receptor, pH-responsive	Temozolomide (TMZ)	U-87 MG-Luc cells orthotopic xenografts	*In vitro* studies have shown that micelles have specific uptake and increased cell killing in glioblastoma cells, and *in vivo* studies reported selective accumulation of micelles in orthotopic glioblastoma model.	(52)
	iron oxide nanoparticles ferumoxytol	MMP-14 activatable peptide, enzyme-responsive	Azademethylcolchicine	pcGBM39 or pcGBM2- orthotopic xenografts	*In vivo* studies demonstrated significant apoptosis of cancer cells and prolonged survival of pcGBM39-bearing mice and complete tumor remission of pcGBM2-bearing mice.	(60)
	poly(ethylene glycol)-poly(ϵ-caprolactone) block copolymer (PEG-PCL)	protamine (LMWP) MMP2/MMP9 activatable peptide, enzyme-responsive	Paclitaxel (PTX)	C6 glioma cells in Orthotopic xenografts in mice	Specific accumulation of PEG-PCL, increased median survival of 48 days when compared to control group (21 days)	(61)

derivative, Aldoxorubicin, which binds selectively to Cysteine34 of blood circulating serum albumin, and releases doxorubicin selectively at the tumor site in response to low pH tumor environment. Aldoxorubicin-treated mice exhibited high levels of doxorubicin within the tumor tissue, accompanied by apoptosis of glioblastoma cells and a 3-fold decrease in tumor cell proliferation. Effectiveness of Aldoxorubicin treatment was confirmed in *in vivo* experiments, which demonstrated that when mice were treated with Aldoxorubicin, U87-luc tumors were 10-fold smaller when compared to control animals, or 8-fold smaller when compared with tumors in animals treated with doxorubicin. Importantly, median survival of Aldoxorubicin treated mice was 62 days, compared to 26 days median survival of control and doxorubicin-treated mice. These encouraging results provide a strong rationale to further investigate this approach for the treatment of glioblastoma.

Redox-Responsive Drug Carriers

Redox-responsive drug delivery carriers exploit the difference in redox potential between the tumor and intracellular environment and normal tissue and blood plasma. Tumor tissues have four times more glutathione (GSH) than normal tissue (62). Furthermore, intracellular concentration of GSH is 3–4 magnitudes higher as compared to the extracellular environment (63). Redox-responsive drug carriers are based on macromolecules containing disulfide bonds which encapsulate drugs. After these redox-responsive carriers are exposed to GSH, disulfide bonds are reduced to sulfhydryl groups resulting in release of encapsulated drugs.

Macromolecular carriers based on different materials, such as proteins, lipids, and polysaccharides have been used as redox-responsive drug delivery systems to target glioblastoma. For example, Stephen et al. (57) developed superparamagnetic iron oxide nanoparticles coated with cross-linked, redox-responsive chitosan PEG copolymers loaded with O^6-benzylguanine (BG). The aim of the study was to selectively deliver BG to the glioblastoma in mice, inhibit the DNA repair protein O^6-methylguanine-DNA methyltransferase (MGMT), and overcome Temozolomide resistance. To further improve tumor targeting, particles were also modified with the tumor-targeting peptide chlorotoxin (CTX). *In vitro* studies confirmed that BG was released from the particles in the reducing environment, and glioblastoma cells were more responsive to TMZ. *In vivo* studies, have shown that treatment with such constructed nanoparticles and TMZ showed a 3-fold increase in median overall survival in comparison to TMZ treated and untreated animals.

In another report, Jiang et al. (58) synthesized redox-responsive virus-mimicking polymersomes (PS) which can efficiently deliver saporin (SAP), a highly potent natural protein toxin, to orthotopic human glioblastoma engrafted in nude mice. To enhance delivery of the drug, polymeromes were modified with angiopep-2 (ANG), a peptide that binds with high affinity to low-density lipoprotein receptor-related protein-1 (LRP-1) which is often overexpressed in glioblastoma cells and brain capillary endothelial cells (64, 65). *In vivo* anti-glioblastoma efficacy experiments have shown that ANG-PS-SAP-treated mice had approximately 7-fold lower tumor bioluminescence intensity than control mice, indicating efficient tumor reduction by ANG-PS-SAP. This was confirmed with 2-fold improvement in median survival time from 22 days in control group compared to 43 days in animal treated with ANG-PS- SAP.

A different approach using human serum albumin (HSA) nanoparticles stabilized with intramolecular disulfide bonds and modified by substance P (SP) tumor-targeting peptide to deliver paclitaxel (PTX) to U87 orthotopic xenografts (59). Animals treated with SP-HAS-PTX nanoparticles exhibited antitumoral effect and prolonged survival time of treated mice when compared to control group.

Enzyme-Responsive Drug Carriers

Enzymes play important roles in all metabolic and biological processes and dysregulation of enzyme activity and expression is exhibited in many diseases including glioblastoma. Therefore, exploiting overexpression of enzymes and their selective catalytic activity as a trigger to release the drug at the tumor site is a very promising approach.

Mohanty et al. (60) applied this concept to deliver the azademethylcolchicine potent active vascular-disrupting agent. They designed an enzyme-responsive carrier consisting of three main elements: (1) theranostic cross-linked iron oxide nanoparticle backbone, (2) matrix metalloproteinase 14 MMP-14 cleavable linker, and (3) drug azademethylcolchicine. The iron core of nanoparticles enabled *in vivo* tracking of the carrier with MRI imaging, which demonstrated significant accumulation of drugs in the glioblastoma tumors in mice.

Treatment with nanoparticles in combination with Temozolomide achieved tumor remission and increased survival pcGBM2-bearing mice by more than 2-fold compared with treatment with temozolomide alone. Thus, this synergistic combination therapeutic strategy may have significant potential for clinical translation to improve long-term outcomes of glioblastoma patients.

Besides MMP-14, some glioblastomas have upregulated MMP-9 and MMP-2. To exploit increased expression of these proteases, Gu et al. (61) constructed nanoparticles composed of poly(ethylene glycol)-poly(ϵ-caprolactone) block copolymer (PEG-PCL) as the matrix conjugated with activatable cell penetrating peptide protamine (ALWMP, E_{10} -PLGLAG-VSRRRRRRGGRRRR). Positive charges on the LWMP necessary for transduction were at first masked by a polyanionic peptide (E10) via a MMP-2/9-cleavable peptide linker sequence PLGLAG. Once the nanoparticles were exposed to proteolytic activity of the MMPs, transduction activity of cell penetrating peptides was restored. As a result, these particles loaded with paclitaxel (PTX) exhibited elevated MMP-dependent intracellular accumulation in C6 cells, and improved cytotoxicity. *In vivo* imaging demonstrated specific accumulation of the particles in intracranial C6 glioma model in nude mice. Specific accumulation of PEG-PCL nanoparticles in glioblastoma was reflected in increased median survival of 48 days when compared to control group (21 days) or taxol (24) alone. These results are promising, and encourage further *in vivo* experiments in different animal models which would open new modalities for the

treatment of glioblastoma based on enzyme-responsive targeted drug release.

Magnetic and Ultrasound

The integrity of the brain is compromised not only by the highly invasive nature of glioblastoma multiforme tumors, but also further exacerbation occurs with standard surgical resection of the tumor. Surgical resection is followed by radiotherapy and chemotherapy, and efficacy of the therapy is monitored by imaging techniques. There are ongoing efforts in clinics to develop approaches to monitor specificity of the therapy and image the tumor at the same time. These tools are called "theranostics," and they integrate imaging and therapeutic modality in the singe macromolecule. Wide application and

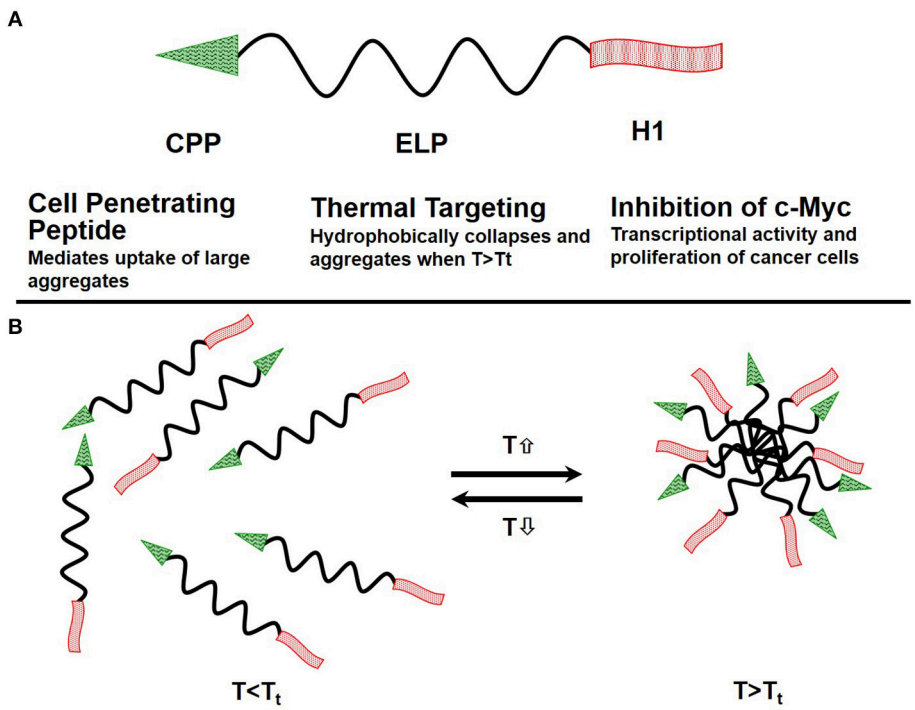

FIGURE 3 | Schematics of the ELP-based drug delivery vector**. (A)** The delivery system consists of the cell penetrating peptide (CPP) Bac, which promotes cellular uptake of the polypeptide, the thermally responsive elastin-like polypeptide, and a c-Myc transcriptional activity inhibitory peptide (H1), which inhibits cancer cell proliferation. **(B)** ELP remains a soluble monomer when the solution temperature is at or below body temperature. When solution temperature is raised above body temperature $T > T_t$, it hydrophobically collapses and forms aggregates.

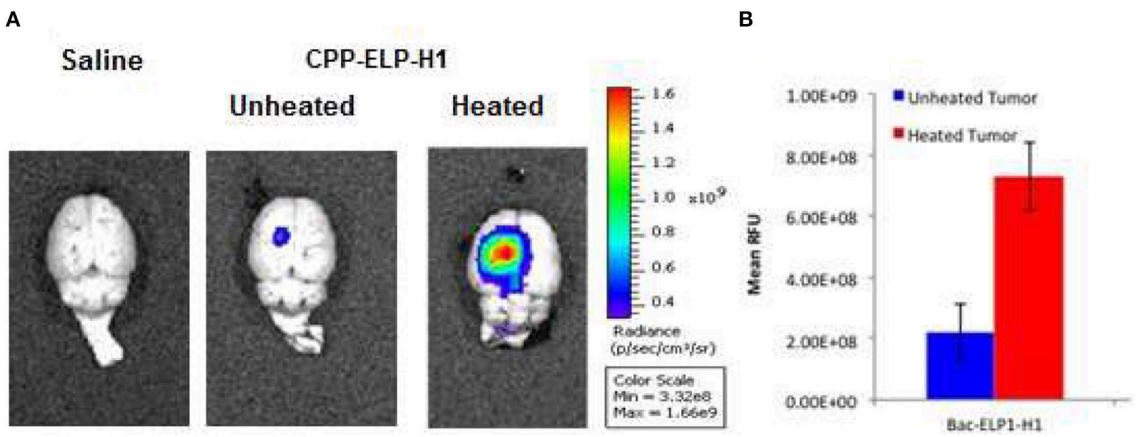

FIGURE 4 | Enhancement of CPP-ELP1-H1 Tumor Uptake by Thermal Targeting. Following IV administration of Alexa750-labeled CPP-ELP1-H1 with or without hyperthermia, construct levels in tumors and organs were determined by *ex vivo* whole organ fluorescence imaging. **(A)** Representative brain images from each treatment group. **(B)** Quantitation of tumor fluorescence from each group. Bars, s.e.m. *, Fluorescence levels differ statistically (p, 0.01, one way ANOVA with *post-hoc* Bonferroni; $n = 4$ rats/group).

versatility of theranostic complexes have led to design and production of various different theranostic compounds.

To use the maximum potential of such theranostic compounds, several groups have included ultrasound in their methodology. Effective drug delivery to the brain tumor is primarily hampered by blood-brain barrier and to overcome BBB, it has been reported that focused ultrasound (FUS) can be used for temporarily opening of the BBB (66, 67). One such approach was used by Fan et al. (56), 2016 for local drug delivery in a rat glioma model. The group has fabricated Superparamagnetic iron oxide (SPIO) conjugated with doxorubicin and embedded in lipid microbubbles (MBs), namely SD-MBs. SD-MBs compounds were used for augmented drug delivery to the brain tumor. The animals underwent FUS sonication after bolus injection of SD-MBs, with the purpose of opening BBB and easier tumor perfusion. The FUS sonication was followed by magnetic resonance imaging (MRI) for SD-MBs visualization, simultaneously with magnetic targeting (MT) for increased drug delivery to the tumor site.

Although theranostic tools are very promising and versatile, future studies should be further focused on efficiency of tumor reduction and survival in glioblastoma animal models as well as treatment safety. These more extensive preclinical studies would justify applying this approach in future clinical treatments.

Similar strategy has been used in another study with thermo-responsive liposomes (68). Liposomes were modified with gadolinium and rhodamine and could therefore be used for both ultrasound-mediated drug delivery as well as MRI and optical imaging. The group synthesized t liposomes with different transition temperature (Tt), the temperature at which liposomes undergoes gel-to-liquid crystalline phase transition. One thermoresponsive liposome, The New Thermosensitive liposome (NLP), was designed with a Gadolinium–DOTA lipid bilayer and a Tt of 42°C. The second thermosensitive liposome The Conventional liposome (CLP), was designed with Gd-DTPA-BSA lipid and a Tt of 60°C (68, 69). At determined Tt the transmembrane permeability of liposomal complex was increased.

Using light microscopy to show that the designed liposomes accumulated in the flank of a murine glioma model, they further modified the liposome surface with biotin and rhodamine, which tightly binds to Gli36 glioma cells expressing biotin acceptor peptide (BAP). Significantly higher accumulation of liposomes was observed in BAP-expressing tumors, indicating efficient tumor targeting and imaging capabilities using MRI.

Since the designed liposome are thermo-responsive they have a potential to be targeted to the tumor tissue and release the drug when external mild heat is applied. To further demonstrate drug delivery potential, additional experiments including drug encapsulation and determination of stability of liposomes in plasma and efficacy in orthotopic glioma model are necessary to advance this technology to its full potential.

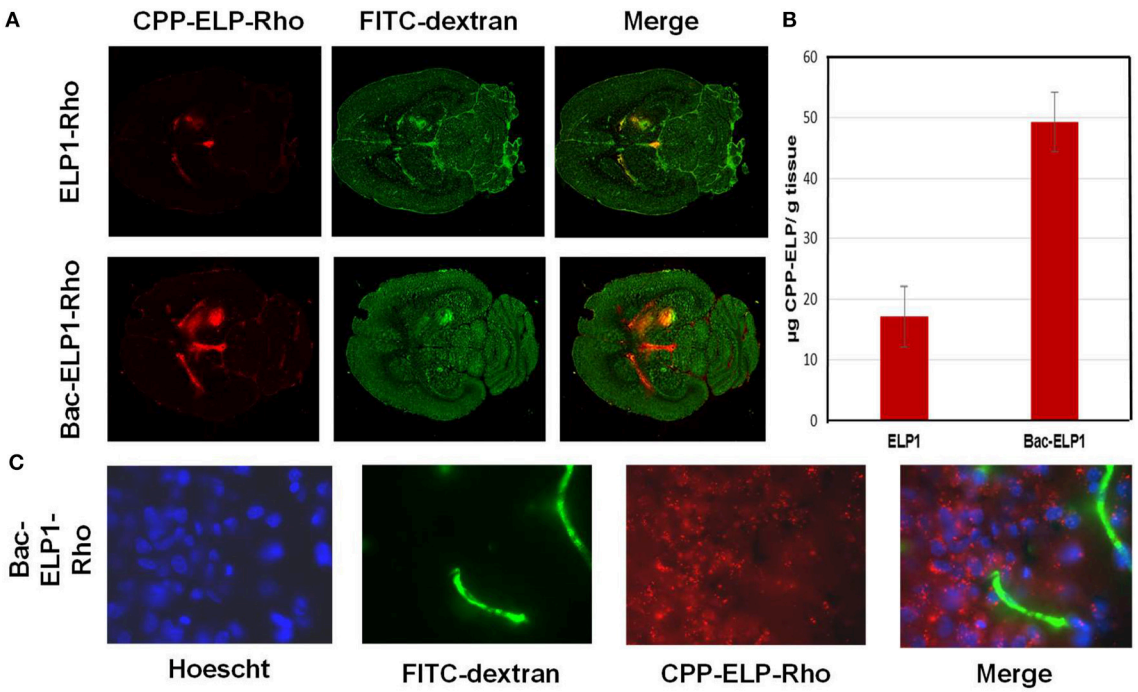

FIGURE 5 | Tumor and Cellular Uptake of CPP-ELP. **(A)** Distribution of rhodamine-labeled polypeptides in tumor and normal brain relative to perfused vasculature. Rhodamine fluorescence was used to follow the localization of the polypeptide within the tumor (left panel); the perfused vasculature was marked by infusion of high molecular weight dextran 1 min prior to euthanasia (middle panel). **(B)** Tumor levels 4 h after IV administration of rhodamine-labeled ELP or CPP-ELPs. Bars, s.e.*, Tumor levels are significantly enhanced (p, 0.01, one way ANOVA with *post-hoc* Bonferroni, $n = 6$ rats/group). **(C)** Microscopic images of tumor sections were collected after staining cell nuclei with Hoechst 33342 using a 60 X magnification objective.

Temperature-Responsive Drug Carriers

Thermo-responsive drug delivery carriers are one of the most investigated stimuli-responsive strategies for targeted, stimuli-responsive drug delivery. Temperature-responsive drug carriers undergo phase transition and rapid change in their physical property at certain temperatures; lower critical solution temperature (LCST). Below LCST, drug carriers are soluble but upon heating they become insoluble, which may increase drug carrier accumulation or trigger drug release in the heated tumor area. Moreover, LCST may be modulated by incorporation of hydrophilic or hydrophobic monomers to achieve LCST temperature corresponding to mild hyperthermia (37–42° C). This temperature range is desirable, since it is higher than normal temperature, but lower than temperatures which may damage healthy tissue. Furthermore, mild hyperthermia can be effectively localized and contained within the tumor site without spilling into adjacent normal tissue. As tumors have a defective vascular architecture and impaired lymphatic drainage, the application of mild heat results in the preferential retention and increased concentration of drugs. Additionally, hyperthermia is a mature clinical modality currently used in clinics, rendering the methods, and techniques necessary to employ targeting of thermally sensitive polypeptides available in the clinical setting. Further hyperthermia increases blood flow, resulting in an increased permeability of the tumor, as compared to normal vasculature and hyperthermia increases tumor vasculature pore size, enhancing extravasation of macromolecules (70, 71) and cellular uptake (72, 73).

Elastin-Like Polypeptides

One class of thermo-responsive drug carriers, which was developed in our lab, is based on the thermally responsive biopolymer elastin-like polypeptide (ELP). An ELP, soluble at physiological temperatures, undergoes a phase transition and aggregates in response to an externally applied mild hyperthermia (40–41°C). Our ELP's coding sequence was modified by adding a cell penetrating peptide (CPP) Bac, to enhance polypeptide delivery across the blood brain barrier (BBB) and to facilitate cell entry. Also added was a peptide, derived from helix 1 (H1) of the helix-loop-helix region of c-Myc (H1-S6A, F8A), to inhibit c-Myc transcriptional activity and cell proliferation. Schematic of the ELP based drug delivery vector was presented in **Figure 3**.

ELPs are genetically engineered biopolymers that, in addition to all the benefits of macromolecular drug delivery systems, provide a number of additional advantages: (1) ELPs are thermally responsive biopolymers which undergo a sharp (2–3°C range) phase transition, leading to desolvation and aggregation of the biopolymer when the temperature is raised above their Tt (74, 75), rendering them suitable for thermal targeting; (2) ELPs are genetically encoded, providing control over the ELP sequence and molecular weight (MW) to an extent impossible with synthetic polymer analogs which allows ELP molecular weights to be precisely specified, resulting in monodisperse polymers, a feat difficult to achieve with synthetic polymers; (3) ELP composition can be encoded at the gene level, allowing an ELP sequence to be modified by adding cell penetrating peptides, and therapeutic peptides. These targeting peptides can then be used to define tissue distribution, tumor penetration, and sub-cellular uptake/localization. Together, these properties make ELPs a promising class of biopolymers for targeted drug delivery.

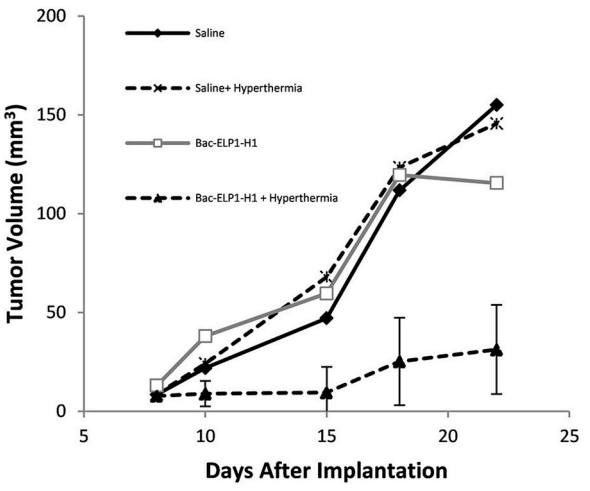

FIGURE 6 | Inhibition of Glioma Progression by the Thermally Targeted c-Myc Inhibitory Polypeptide. Sprague Dawley rats bearing intracerebral C6 tumors were treated with 4 daily IV injections of the Thermally Targeted c-Myc Inhibitory Polypeptide, with MRI monitoring of tumor volume. Average tumor volume for each treatment group. $n = 6$-8 animals per group; *, Tumor volume was significantly reduced compared to control tumors (one way ANOVA, *post hoc* Bonferroni).

Thermally targeting increases delivery of CPP-ELP1-H1 to intracerebral gliomas

We tested the ability of the CPP-ELP1-H1 polypeptide to be thermally targeted to tumors. Rats bearing intracerebral tumors were injected IV with Alexa750-labeled BacELP1-H1. Tumors were heated using the described thermal cycling protocol (54), and tumor deposition was determined by *ex vivo* imaging of rat brains 4 h after injection using an IVIS Spectrum animal imager. Polypeptide accumulation in tumors occurred at a high level relative to adjacent normal brain (**Figure 4A**). Moreover, tumor polypeptide levels noticeably increased when Bac-ELP1-H1 treatment was combined with tumor hyperthermia. Quantitation of tumor fluorescence intensity revealed that thermal targeting increased Bac-ELP1-H1-Alexa750 tumor accumulation by 3.3-fold (**Figure 4B**, $p = 0.0004$, Student's *t*-test).

CPP-ELP1-H1 can penetrate the BBB and enter GBM cells

A major barrier to GBM treatment is posed by the BBB, which any proposed therapeutic must penetrate. To assess the ability of CPP peptides to do so, and to determine their capacity to mediate ELP drug carrier delivery into C6 brain tumors, rats bearing intracranial C6 tumors were IV injected with Rhodamine-labeled CPP-ELP1 or an ELP1 control. At 4 h after injection, a 500 kDa FITC-dextran was injected to mark perfused vasculature, the animal sacrificed, and the

brain removed, frozen, and sectioned (**Figure 5A**). Slides were scanned with a ScanArray Express slide scanner (Perkin Elmer), with fluorescence intensity determined using Image J software. Tumor intensity, expressed relative to plasma concentration at time zero (RFU/C_0), was averaged for all animals. ELP passive accumulation in C6 tumors was higher than in normal brain tissue; however, adding a CPP greatly enhanced tumor fluorescence relative to unmodified ELP. Comparing CPP-ELP-Rhodamine fluorescence with FITC-dextran fluorescence (**Figure 5B**) showed: (1) slightly greater enrichment of perfused vessels in tumors than in normal brain, and (2) significantly greater polypeptide accumulation in the tumor than in normal neural tissue. Microscopic examination of tumor sections showed the presence of CPP-delivered ELP in the blood vessels, extravascular space, and within tumor cells (**Figure 5C**). These data indicate the ELP polypeptide's passive accumulation in brain tumors in this rat model, as well as the enhancement conferred for total tumor levels and deposition throughout the tumor, relative to a non-CPP containing control, by using the CPP.

Reduction of intracranial C6 tumor proliferation by Bac-ELP-H1

After demonstrating that CPP-ELP-H1 can enter C6 tumors in brain, the construct's effects on tumor progression and animal survival were evaluated.

Rats bearing intracerebral C6 tumors were treated daily for 4 days beginning on day 9 after implantation. The CPP-ELP1-H1 polypeptide, or control polypeptides lacking the H1 peptide (Bac-ELP1) or utilizing the non-thermally responsive version of ELP (Bac-ELP2-H1), was injected IV. In the hyperthermia groups, hyperthermia was applied to the tumor site using a thermal cycling protocol immediately after each injection. Tumor progression was monitored using multi-slice 3D T1 trans-axial

Imaging with a gadolinium-based contrast on days 10, 15, 18, and 22. As shown in **Figure 5**, the C6 tumors progressed rapidly in all treatment groups except those in the Bac-ELP1-H1+ hyperthermia group; in this group, tumor volumes were 80% smaller, with a mean volume of 31 mm^3 ($p = 0.004$, one-way ANOVA, **Figure 6**).

Control polypeptides without H1 peptide (CPP-ELP1) had no effect on tumor reduction, while the non-thermally responsive ELP (CPP-ELP2-H1) resulted in a 30% tumor reduction (data not shown).

These results are significant, since they demonstrate that it is feasible to increase brain tumor uptake of thermally responsive ELP drug carriers with focused hyperthermia, but also thermal targeting of the Bac-ELP1-H1 polypeptide to the tumors resulted in significant delayed onset of neurological deficits, 80% tumor volume reduction, and at least doubled survival.

While these results demonstrate that use of ELP to thermally target the H1 peptide, similar approach may be used to apply ELP technology for delivery of other therapeutic peptides for glioma. Future studies should expand this testing into other GBM models, including mouse orthotopic xenografts of human glioblastoma cells.

CONCLUSION AND FUTURE PERSPECTIVES

The current treatment of glioblastoma is particularly challenging not only because of the delivery of therapeutics to the brain, but also because of the tumor heterogeneity, aggressiveness, and recurrence. Although prognosis for the glioblastoma patients remains poor, recent developments in drug delivery approaches provide hope for the successful treatment of glioblastoma. This article reviewed recent progress and potential of macromolecular drug carriers. Macromolecular carriers increase efficacy, stability and plasma half-life of anticancer drugs, and reduce toxicity to healthy tissues. Tumor targeting of macromolecular carriers mostly rely on the passive tumor targeting via the enhanced permeability and retention effect. However, in addition to passive targeting, numerous macromolecular carriers have been developed to deliver and/or release drugs in response to internal or external stimuli, including pH, enzymes, redox potential, magnetic field, ultrasound, and temperature. These stimuli-responsive macromolecules provide active targeting for anticancer drugs and further improve delivery of the drugs specifically to the tumor tissue. However, despite the progress which has been achieved in development of macromolecular carriers, some challenges for their successful clinical application remain.

Beside heterogeneity of tumors across the patients and tumor types, such as difference in pH and expression of specific enzymes, both of which may influence drug delivery in response to internal stimuli, there is also the issue of non-specific biodistribution of macromolecular carriers in other organs, such as liver and kidneys. Furthermore, complex design of some of the carriers and difficulties in scaling up their production may present further limitations in clinical applications. Due to these reasons there are only a limited number of macromolecular carriers presently used in clinics. Substantial progress may be possible if the research efforts are also focused not only on developing efficient macromolecular carriers, but also on development and selection of clinically-relevant animal models and assays which can more precisely predict their potential toxic effects.

AUTHOR CONTRIBUTIONS

DR wrote the initial drafts of the manuscript. SD and JR wrote subsequent drafts of the manuscripts, and produced figures. All authors reviewed and approved the final submitted manuscript.

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Functional Blockade of Small GTPase RAN Inhibits Glioblastoma Cell Viability

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Glioblastoma, the most common malignant tumor in the brain, lacks effective treatments and is currently incurable. To identify novel drug targets for this deadly cancer, the publicly available results of RNA interference screens from the Project Achilles database were analyzed. Ten candidate genes were identified as survival genes in 15 glioblastoma cell lines. RAN, member RAS oncogene family (RAN) was expressed in glioblastoma at the highest level among all candidates based upon cDNA microarray data. However, Kaplan-Meier survival analysis did not show any correlation between RAN mRNA levels and patient survival. Because RAN is a small GTPase that regulates nuclear transport controlled by karyopherin subunit beta 1 (KPNB1), RAN was further analyzed together with KPNB1. Indeed, GBM patients with high levels of RAN also had more KPNB1 and levels of KPNB1 alone did not relate to patient prognosis. Through a Cox multivariate analysis, GBM patients with high levels of RAN and KPNB1 showed significantly shorter life expectancy when temozolomide and promoter methylation of O^6-methylguanine DNA methyltransferase were used as covariates. These results indicate that RAN and KPNB1 together are associated with drug resistance and GBM poor prognosis. Furthermore, the functional blockade of RAN and KPNB1 by importazole remarkably suppressed cell viability and activated apoptosis in GBM cells expressing high levels of RAN, while having a limited effect on astrocytes and GBM cells with undetectable RAN. Together, our results demonstrate that RAN activity is important for GBM survival and the functional blockade of RAN/KPNB1 is an appealing therapeutic approach.

Keywords: RAN, glioblastoma, importazole, cell survival, glioblastoma prognosis, KPNB1, glioblastoma treatment

INTRODUCTION

Glioblastoma (GBM) is an aggressive tumor generally found in the cerebral hemispheres of the brain. Spanning 16% of the cases of all primary tumors in the brain and ∼50% of all malignant brain tumors, GBM is the most common malignant type in the central nervous system (1, 2). The average length of survival for GBM patients is ∼15 months, and only about 5.5% of patients will

live longer than 5 years after diagnosis and aggressive treatments, such as chemotherapy, radiation therapy, and surgical resection (1, 3–5). However, surgery is not sufficient for a clean and complete resection of the tumor mass due to the infiltration of tumor cells into the normal brain parenchyma. The remaining tumor cells are often refractory to chemo drugs and radiation, thereby contributing to the high incidence of tumor recurrence that is robustly associated with a poor prognosis of GBM (6–9). Therefore, more effective treatments are needed.

To identify novel therapeutic targets for GBM, we and other research groups used RNA interference (RNAi) screening, a technique that allows a simultaneous analysis of genes in a genome for their functions in a particular setting. For example, we performed a genome-wide RNAi screen using a diphtheria toxin negative selection approach (10) and uncovered a molecular pathway that controls the transcription of activating transcription factor 5, a key survival factor for GBM (11). Identification of this molecular survival pathway has led to a phase I clinical trial, in which a combination of radiation and sorafenib, an inhibitor of RAF kinase that suppresses the expression of activating transcription factor 5, was used to treat GBM patients (12). In another study, we carried out a kinome RNAi drop-out screen, through which 20 kinases were identified as survival kinase genes (7). Among these candidates, casein kinase 1 epsilon (13) and phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit beta (14, 15) were further verified as essential survival factors and appealing drug targets for GBM. Studies from other groups have also revealed possible therapeutic targets (e.g., PFKFB4, PLK1, SGK1, NLK, etc.) for GBM using RNAi screens (16–19). Hence, RNAi screening is a proven, useful tool for identifying novel drug targets for GBM.

Recently, the Broad Institute initiated a program termed Project Achilles (20–23). This project aims to complete genome-wide RNAi or CRISPR-Cas9 screens in more than 1,000 different cancer cell lines in order to unveil survival genes in cancer cells and to provide a comprehensive cancer dependency map, allowing for the elimination of tedious and repetitive work of RNAi screens in different laboratories so researchers can further analyze the RNAi screen results to uncover cancer survival genes and develop effective cancer treatments. The principle of these so-called "drop-out" screens is based on the hypothesis that short hairpin RNAs (shRNAs) or guide RNAs (gRNAs) of genes that are essential for cancer cell survival induce cell death; hence, cells with these shRNAs or gRNAs will be depleted over time. By comparing the sequencing reads of shRNAs or gRNAs in cells at the initial and end time point, shRNAs or gRNAs that are lost or under-represented (due to the depletion of cells) will be identified. Results of RNAi screens in more than 500 cancer cell lines, including 15 GBM cell lines, have recently been made available to the public, offering us an opportunity to search for more survival genes in GBM.

In this report, we analyzed RNAi screen results in 15 GBM cell lines and identified 10 candidate genes that are important for the survival of GBM cells. Further comprehensive analyses revealed one gene, RAN (RAN, member RAS oncogene family), as the top candidate because this gene was highly expressed in GBM and its activity was robustly associated with drug resistance and poor prognosis in GBM. RAN is a small GTPase protein that provides energy for nucleocytoplasmic transport and mitotic spindle assembly by hydrolyzing guanosine triphosphate into guanosine diphosphate (24–28). Through this released energy, RAN regulates the activities of the importin protein complexes that mediate nuclear import and export (27–29). Hence, this protein has been implicated in the genesis and disease progression of numerous different types of cancer (30–38). However, the role of RAN in GBM has not yet been extensively explored, despite being shown in two studies as a regulator of apoptosis through blocking Bcl-2-associated X protein and activating survival pathways in GBM cells (39, 40). Directly and selectively targeting RAN is difficult and has not been very successful so far (40, 41). It has been recently shown that importazole, a small molecule inhibitor of RAN and KPNB1, blocks the interactions between RAN and KPNB1 based upon results from fluorescence resonance energy transfer, nuclear localization of fluorescent proteins, and co-immunoprecipitation. The disruption of RAN/KPNB1 complexes represses RAN/KPNB1-mediated nuclear transport (42). We therefore chose importazole to test whether a blockade of RAN activity would inhibit GBM cell viability. While importazole has been tested in different types of cancer, this drug (43–46) has not yet been applied to GBM. We found that blocking the activity of this candidate gene activated cell death and induced a potent inhibition of cell growth in GBM cell lines as well as primary GBM cells, presenting a possibility as an effective drug target for GBM.

METHODS AND MATERIALS

Materials

GBM cell lines, primary GBM cells, and normal human astrocytes were cultured as described previously (7, 14, 47). In brief, GBM cell lines A172, LN-18, SF-268, SF-295, T98MG, U251, and U87MG were maintained in Dulbecco's Modified Eagle Medium (DMEM, Life Technologies) supplemented with 10% EquaFETAL® bovine serum (Atlas Biologicals, Inc.) and 100 μg/ml streptomycin and 100 IU/ml penicillin (Gibco). Primary cells VTC-001, VTC-002, VTC-004, VTC-037, VTC-056, VTC-058, VTC-084, and VTC-103 were cultured in DMEM supplemented with 15% fetal bovine serum (Peak Serum, Inc.) and penicillin/streptomycin. Normal human astrocytes were cultured in MCDB-131 Medium (Sigma) containing 3% fetal bovine serum (Peak Serum, Inc.), 10 X G-5 Supplement (Gibco), and penicillin/streptomycin. Cell lines have been authenticated by the ATCC authentication service utilizing Short Tandem Repeat (STR) profiling. Primary GBM cells were kept at low passages (no more than 10). Antibodies of RAN and GAPDH were purchased from Santa Cruz Biotechnology, Inc. Importazole was purchased from Cayman Chemicals, Inc. Stock solution of importazole was prepared at 50 mM using dimethyl sulfoxide (DMSO). Working solution was further diluted using cell culture media.

Analysis of RNAi Screen Results From the Project Achilles

RNAi screen results (Achilles_v2.4.6.rnai.gct) were retrieved from the Project Achilles database at the following website: https://portals.broadinstitute.org/achilles. The screen contains more than 50,000 short hairpin RNAs (shRNAs) that target the human genome and the results were presented as fold changes of shRNA loss (log2). The lower the fold change of a particular shRNA, the stronger the depletion of the shRNA in GBM cells. This shRNA depletion is, as hypothesized, due to the loss of cells over time. Results of these shRNAs in 15 GBM cell lines (A172, DBTRG05MG, DKMG, GB1, LN229, LN340, LN382, LN428, LN443, LN464, SF172, SNU1105, U343, U87MG, and YKG1) were first sorted by two or more shRNAs targeting one single gene. More than 4,000 genes were targeted by two or more shRNAs. Next, the fold changes of shRNA loss were averaged. Candidate shRNAs with an average of fold change <-4.0 and a fold change <-3.0 in all 15 GBM cell lines were selected.

Gene Expression Analyses Using Online Databases

cDNA microarray data were retrieved from BioGPS (http://biogps.org/#goto=welcome), Oncomine (https://www.oncomine.org/resource/login.html), Glioblastoma Bio Discovery Portal (https://gbm-biodp.nci.nih.gov), and The Cancer Genome Atlas (TCGA) database (http://www.cbioportal.org/index.do). Data from BioGPS were reanalyzed. The arbitrary units of mRNAs of candidate genes in GBM cell lines were divided by those in astrocytes, yielding fold changes (GBM/Astrocytes). Regarding data from the Oncomine database, fold changes of candidate gene mRNAs in GBM tissues normalized with those in normal brain tissues from three different studies (Shai Brain, Murat Brain, and Brendel Brain 2) were recorded and summarized in **Table 1**. *P*-values that determine the statistical significance of fold changes were included as well. mRNA levels of candidate genes in different subtypes of GBM were retrieved from Glioblastoma Bio Discovery Portal. Patient numbers of classical, mesenchymal, and proneural GBM subtypes were 199, 166, and 163, respectively. Levels of candidate gene mRNAs in GBM subtypes were then averaged. RAN levels and MGMT promoter methylation status in GBM patients were retrieved from the TCGA database and were re-analyzed using JMP software.

Kaplan-Meier Survival Analysis

Kaplan-Meier survival analyses of GBM patients from the TCGA database have been reported in GlioVis (http://gliovis.bioinfo.cnio.es), Glioblastoma Bio Discovery Portal, and The Human Protein Atlas (http://www.proteinatlas.org). The survival results were retrieved from these databases and presented together with the Log-rank *P*-values.

Cox Univariate and Multivariate Analysis

Gene expression data and clinical information of GBM patients were retrieved from the TCGA database (http://www.cbioportal.org/index.do). The correlation between mRNA levels and GBM patient survival was determined by Cox univariate or multivariate analysis using the JMP software as previously described (7). Hazard ratios (HR, chance of death) with *P*-values determining HR probabilities larger than Chi-squares were shown. The lower and upper 95% confidence intervals were plotted as well.

MTS Cell Viability Assay

The MTS cell viability assay was described previously (14, 47, 48). In brief, GBM cell lines, primary GBM cells, and astrocytes were dissociated as single cells and then plated at 500, 1,000, or 4,000 cells per well, respectively, in 100 μl of culture media in a 96-well plate. Next day, cells were treated with importazole at 12.5 μM or at various concentrations (3.125, 6.25, 12.5, 25, or 50 μM, respectively) for 3 or 6 days. A 0.1% DMSO solution was used as the control. At the end point, stock MTS reagent (Promega) was diluted in culture media at 1:10 and added to each well. Two hours later, the absorbance at 490 nm (detecting the color change of MTS in live cells) was measured using a microplate reader (Molecular Devices). Percentages of cell viability were obtained by dividing the MTS readings in importazole-treated cells with those in DMSO-treated cells. *P*-values were determined using the two-way ANOVA.

Caspase 3/7 Activity Assay

Apoptosis was determined using the caspase 3/7 activity assay as described previously (14, 47, 48). GBM cells and astrocytes were dissociated to single cells and plated at 500 or 4, 000 cells per well in 100 μl of culture media in a 96-well plate. Next day, cells were treated with either a 0.1% DMSO solution or 12.5 μM of importazole. After 3 days, caspase 3/7 activity assay reagent (Promega) was diluted in culture media at 1:1 and added to each well. After 1 h incubation, the luminescence of caspase 3/7 activity reagent was recorded using a microplate reader. Fold changes of caspase 3/7 activity were obtained by dividing luminescence readings in cells treated with importazole with those in cells treated with DMSO. *P*-values were determined using the student *t*-test.

Immunoblotting

Protein levels were determined using immunoblotting as described in detail previously (14, 47, 49, 50). Briefly, 25–50 μg of total protein was loaded onto an SDS-PAGE gel and then transferred onto a PVDF membrane. Antibodies were diluted as follows: anti-RAN antibody (1:500; Santa Cruz Biotechnology, Inc.), and anti-GAPDH antibody (1:200; Santa Cruz Biotechnology, Inc.).

Statistical Analyses

Significance of difference in means among different treatment groups was determined using either student *t*-test or two-way ANOVA. The software Prism 7 was used.

TABLE 1 | Levels of candidate genes in GBM tissues compared to normal brain.

Gene symbol	Shai brain		Murat brain		Brendel brain 2	
	Fold change (GBM/Normal brain)	*P*	Fold change (GBM/Normal brain)	*P*	Fold change (GBM/Normal brain)	*P*
NHP2L1	N/A	N/A	1.198	0.006	−2.08	1
PSMB2	2.068	<0.001	1.541	0.007	1.448	0.001
PSMD1	1.226	0.006	−1.328	0.98	−1.001	0.501
RAN	3.375	<0.001	1.265	<0.001	1.512	0.004
RPL23A	1.714	0.006	1.193	<0.001	N/A	N/A
RPS13	1.37	0.003	1.358	0.014	1.278	0.012
RPS15A	1.617	<0.001	1.985	<0.001	1.916	<0.001
RPS7	1.928	<0.001	1.4	<0.001	1.161	0.077
UBB	−1.002	0.505	−1.252	0.992	−1.642	0.994
KPNB1	1.37	<0.001	1.171	<0.001	1.098	0.009
KPNA2	2.063	<0.001	1.67	<0.001	1.608	0.003

Data were retrieved from the Oncomine database. mRNA fold changes (GBM/normal brain) and P-values that determine the significance of fold changes are shown. Positive numbers indicate more mRNAs in GBM and negative numbers indicate less mRNA in GBM. Candidates showing significantly high levels in GBM in all three studies are highlighted in red.

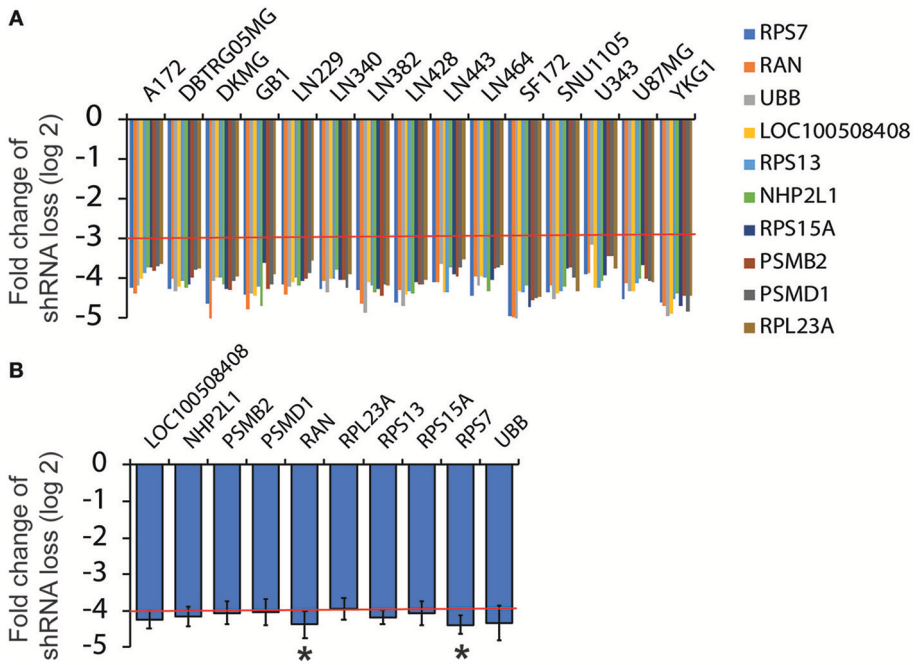

FIGURE 1 | Analysis of RNAi screen results from Project Achilles. RNAi screen results were retrieved from Project Achilles. Candidate genes were selected based on the following criteria: (1) Genes are targeted by two or more different shRNAs; (2) The fold changes of shRNA loss (log2) are lower than −3; (3) The average fold changes of shRNA loss (log2) are lower than −4. Ten candidates were selected. The fold changes of shRNA loss (log2) in each GBM cell lines are shown in **(A)** and the average fold changes of shRNA loss are shown in **(B)**. Red lines indicate cut-off numbers *$P < 0.05$.

RESULTS

Analysis of Loss-of Function Screens in GBM Cell Lines

As described earlier, Broad Institute has published drop-out RNAi screens in more than 500 cancer cell lines including 15 GBM cell lines. To identify survival genes from RNAi screens in 15 GBM cell lines, we followed the following criteria: (1) Candidate genes should be targeted by two different shRNAs; (2) The fold change of shRNA loss (log2) should be <-3.0 in every GBM cell line tested (**Figure 1A**, red line); and (3) The average fold change of shRNA loss (log2) across the 15 cell lines should be below −4.0 (**Figure 1B**, red line). From more than 50,000 shRNAs, we identified 10 survival genes in GBM.

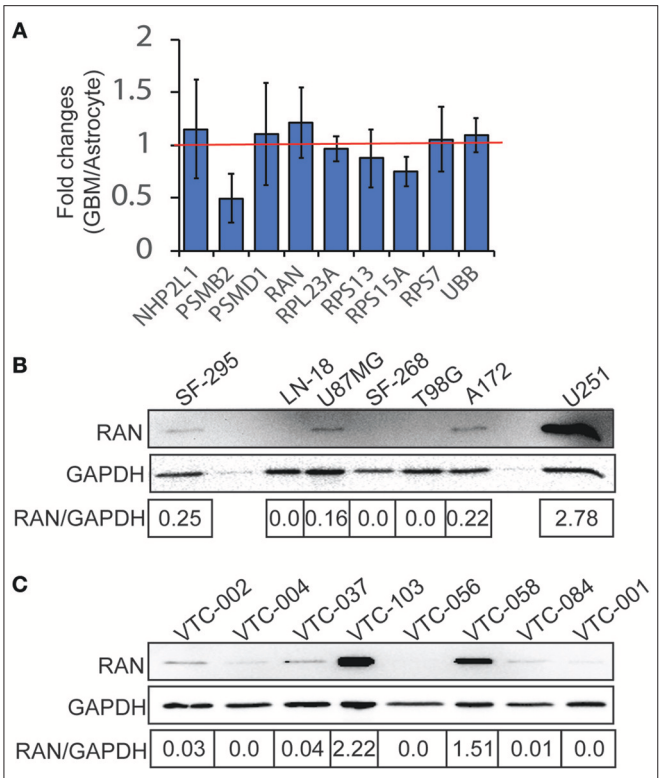

FIGURE 2 | Expression of candidate genes in GBM. **(A)** cDNA microarray data were retrieved from the BioGPS database. The intensities of probes detecting candidate mRNAs in GBM cell lines were divided by those in astrocytes, yielding fold changes (GBM/Astrocyte). Fold changes of NHP2L1, PSMD1, RAN, RPS7, and UBB were above 1 (red line), suggesting that levels of these candidate mRNAs were high in GBM cell lines. Among these candidates, RAN levels in GBM are the highest. Error bars represent standard deviations from six GBM cell lines. **(B)** RAN protein levels in GBM cell lines. RAN and GAPDH proteins were detected in 7 GBM cell lines as indicated using immunoblotting. Band intensities were quantified using Image J. Fold changes (RAN/GAPDH) were obtained by dividing intensities of RAN with those of GAPDH. **(C)** RAN protein levels in primary GBM cells.

Expression of Survival Genes in GBM

Because these genes are important for cell survival, it is likely that they are highly enriched in GBM. To test this possibility, we retrieved cDNA microarray data for GBM cell lines and astrocytes from the online database BioGPS (51–53). By comparing mRNA levels of candidate genes in GBM cell lines and in astrocytes, we found that levels of PSMB2, RPL23A, RPS13, and RPS15A in GBM were lower than those in astrocytes, whereas LOC100508408 was not detected in both GBM and astrocytes. In contrast, mRNA levels of NHP2L1, PSMD1, RAN, RPS7, and UBB in GBM cells were higher than those in astrocytes (**Figure 2A**, fold change >1.0 as indicated by the red line). Levels of RAN (RAN, member RAS oncogene family) in GBM were the highest among these candidates. We next inquired another online database, Oncomine (54, 55), where tissue microarray results were collected. In three different studies (Shai Brain, Murat Brain, and Brendel Brain 2), fold changes (GBM/normal brain) of RAN, PSMB2, RPS13, and RPS15A were >1 with *P*-values lower than 0.05 (**Table 1**). In contrast, levels of other candidate genes were not significantly high in GBM. To corroborate the above results, we measured protein levels of RAN in multiple GBM cell lines or primary GBM cells derived from patient specimens (14, 47) using immunoblotting (**Figures 2B,C**). RAN was detected in SF-295, U87MG, A172, U251, VTC-103, and VTC-058 cells (RAN/GAPDH >0.15; designated as RAN-positive cells), whereas LN-18, SF-268, T98G, VTC-002, VTC-004, VTC-037, VTC-056, VTC-084, and VTC-001 cells did not express RAN or expressed RAN at a very low level (RAN/GAPDH <0.15; designated as RAN-negative cells). Our results validate the detectability of RAN protein levels in GBM and reveal variations in RAN protein levels amongst GBM cell lines. We have also shown that primary GBM cells proliferate at different rates [see Supplementary Data in (14) for details]. For example, VTC-002 and VTC-103 grew much faster than VTC-056. Intriguingly and consistent with our results shown in **Figure 2C**, RAN was detectable in VTC-103 and VTC-002, but not in VTC-056. These results suggest that GBM cells expressing RAN have a high-index of proliferation, indicative of a detectable activity of RAN.

RAN and GBM Prognosis

The role of RAN in GBM has not yet been extensively explored. To address this, we tested the hypothesis that RAN, as a survival gene, correlates with GBM patient survival. By querying the TCGA GBM data using The Human Protein Atlas and the Glioblastoma Bio Discovery Portal, we found that RAN levels did not correlate with patient survival (**Figure 3A**, $P = 0.909$). We further looked into the correlation between RAN and the survival of GBM subtypes and found no statistically significant trend between RAN mRNA levels and the prognosis of classical, mesenchymal, or proneural GBM subtypes (**Figures 3B–D**, $P = 0.427$, 0.505, or 0.688, respectively).

These results indicate that mRNA levels of RAN are not associated with GBM prognosis. However, given that nuclear transport is more active in cancer cells due to the high proliferation index (31, 33, 56), the activity of RAN and its functional partners may be more important for GBM survival. RAN regulates nuclear transportation through interacting with importin α, encoded by karyopherin subunit alpha 2 (KPNA2), and importin β1, encoded by karyopherin subunit beta 1 (KPNB1) (57–62). We therefore examined the levels of RAN together with KPNA2 and KPNB1 in GBM cell lines and tissues. Similar to RAN (**Figure 4A**), mRNA levels of KPNA2 (**Figure 4B**) and KPNB1 (**Figure 4C**) were elevated in LN-18, SF-268, SF-295, and U87MG cells. Linear regression analysis revealed that there was a strong trend between levels of RAN and KPNA2 (**Figure 4D**, $R^2 = 0.4798$) and levels of RAN and KPNB1 (**Figure 4E**, $R^2 = 0.7591$). Congruently, KPNA2 and KPNB1 were also enriched in GBM tissues compared to normal brain tissues (**Table 1**). In addition, results from the TCGA database showed that levels of RAN, KPNA2, or KPNB1 did not vary among different GBM subtypes (**Figure S1**).

Next, we determined the relationship between KPNA2 or KPNB1 and GBM prognosis. Based on the TCGA data

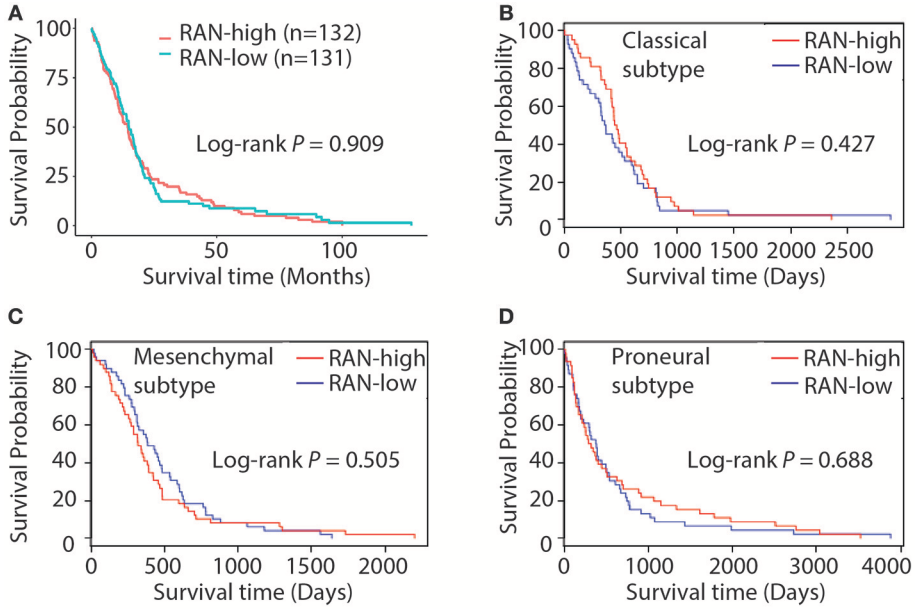

FIGURE 3 | Kaplan-Meier analysis of RAN expression and GBM patient survival. **(A)** Survival curve of GBM patients with different levels of RAN. Results were retrieved from the GlioVis database. Survival curves of classical **(B)**, mesenchymal **(C)**, and proneural **(D)** GBM patients with different levels of RAN were retrieved from the Glioblastoma Bio Discovery Portal. Log-rank *P*-values are shown.

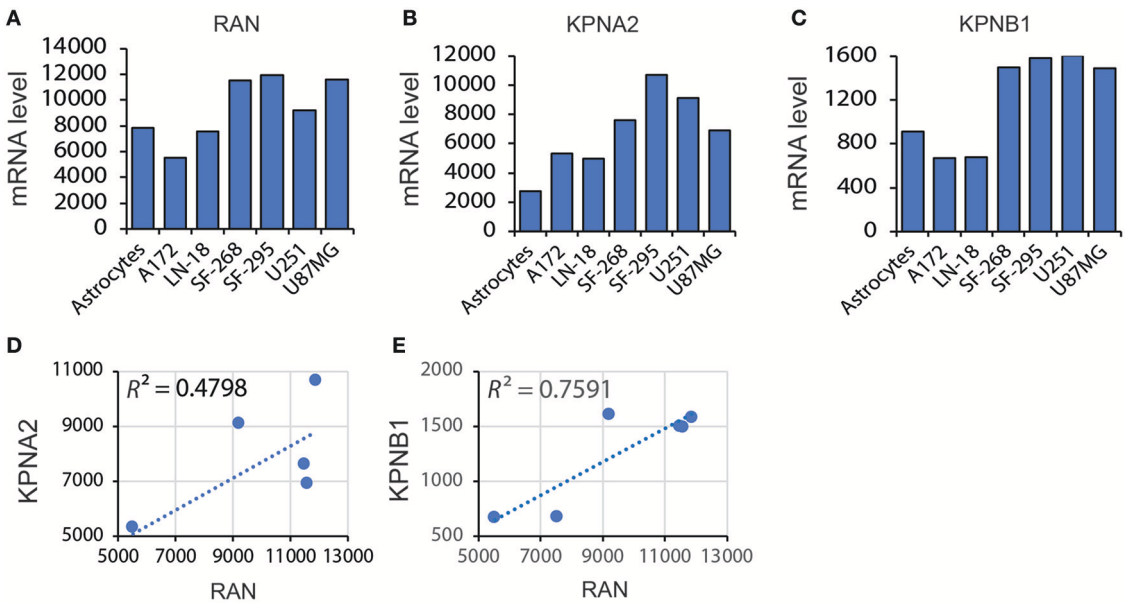

FIGURE 4 | Correlation between levels of RAN and KPNA2 or KPNB1 in GBM. **(A)** Levels of RAN mRNA in astrocytes and GBM cell lines. Data were retrieved from the BioGPS database. The intensities of probes that detect RAN mRNA are shown. **(B)** KPNA2 mRNA levels in astrocytes and GBM cell lines. **(C)** KPNB1 mRNA levels in astrocytes and GBM cell lines. **(D)** Correlation between mRNA levels of RAN and KPNA2 in GBM cell lines. **(E)** Correlation between mRNA levels of RAN and KPNB1. A linear regression model was used. *R* square (R^2) is the coefficient of determination.

analyzed using The Human Protein Atlas, we found that levels of KPNA2 (**Figure 5A**) or KPNB1 (**Figure 5B**) alone were not significantly correlated with patient survival (P = 0.134 and 0.106, respectively), consistent with the results for RAN (**Figure 3A**). Because levels of RAN and KPNB1 were more closely correlated with each other (**Figure 4E**), we next interrogated the relationship between levels of RAN and KPNB1 and GBM patient survival. The Cox univariate analysis showed

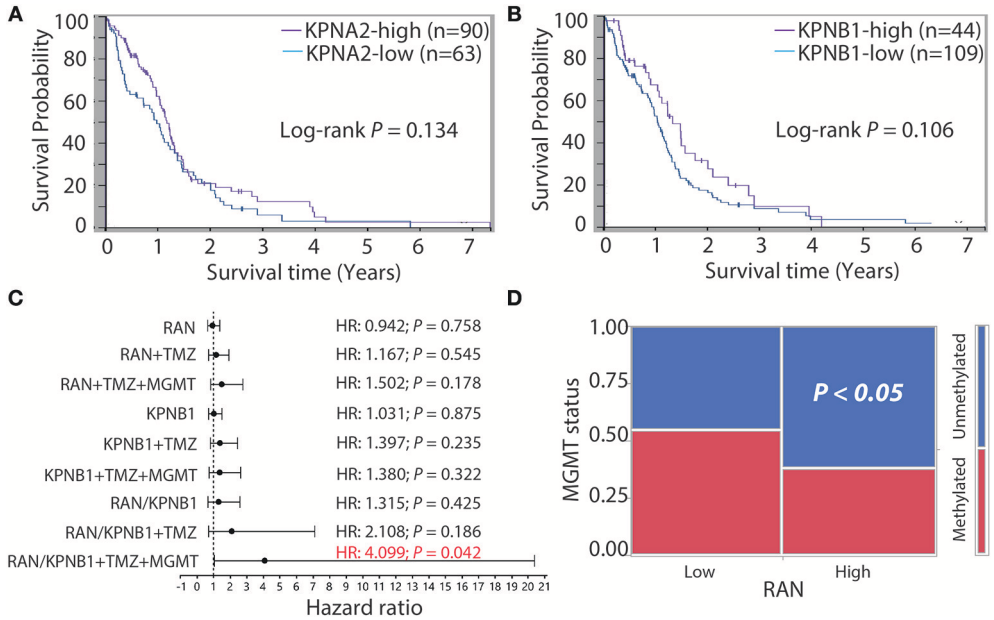

FIGURE 5 | GBM patients with more RAN and KPNB1 exhibit MGMT-dependent TMZ resistance and have shorter life expectancies. Survival curves of GBM patients with different levels of KPNA2 **(A)** or patients with different levels of KPNB1 **(B)** were retrieved from the Human Protein Atlas. Log-rank *P*-values are shown. **(C)** Cox univariate and multivariate analyses of GBM patients with different levels of RAN and/or KPNB1. Data were retrieved from the TCGA database and re-analyzed using JMP software. Hazard ratios (HRs) that determine chances of death are shown. *P*-values indicate the statistical significance of HRs. TMZ treatment (TMZ) and promoter methylation status of MGMT (MGMT) were used as covariates. **(D)** MGMT promoter methylation in GBM patients expressing different levels of RAN. $P < 0.05$ indicates that GBM patients with high levels of RAN often have an unmethylated MGMT promoter.

that GBM patients with high levels of RAN or high levels of KPNB1 had a hazard ratio (HR, risk of death) of 0.942 or 1.031, respectively (**Figure 5C** and **Table S1**, panel RAN and KPNB1). In contrast, the HR of GBM patients with high levels of both RAN and KPNB1 increased to 1.315 with a *P*-value of 0.425 (**Figure 5C** and **Table S1**, panel RAN/KPNB1). To further understand whether this increase suggests a possible link between levels of RAN/KPNB1 and GBM prognosis, we introduced drug resistance into this study as a covariate. Temozolomide (TMZ) is a front-line chemo drug for GBM; however, patients often develop TMZ resistance due primarily to the consequences of promoter unmethylation of O-6-methylguanine DNA methyltransferase (MGMT), an enzyme that repairs TMZ-induced DNA damage (63–65). The poor prognosis of GBM patients is, therefore, closely associated with MGMT promoter methylation. Indeed, GBM patients with high levels of RAN often had an unmethylated MGMT promoter (**Figure 5D**). We therefore used TMZ treatment (TMZ) and/or MGMT promoter methylation as covariates in a Cox multivariate analysis model. When TMZ was used as a covariate, HRs of GBM patients with high levels of RAN, KPNB1, or RAN/KPNB1 were 1.167, 1.397, or 2.108 with a *P*-value of 0.545, 0.235, or 0.186, respectively (**Figure 5C** and **Table S1**, panel RAN+TMZ, KPNB1+TMZ, and RAN/KPNB1+TMZ). By adding MGMT promoter methylation (MGMT) as an additional covariate, HRs of GBM patients with high levels of RAN or high levels of KPNB1 were 1.502 or 1.380 with a *P*-value of 0.178 or 0.322 (**Figure 5C** and **Table S1**, panel RAN+TMZ+MGMT and KPNB1+TMZ+MGMT). In contrast, the HR of GBM patients with high levels of RAN and KPNB1 was elevated to 4.099 with a *P*-value of 0.042 (**Figure 5C** and **Table S1**, highlighted in red). These results indicate an inverse correlation, associated with MGMT-dependent TMZ resistance, between high levels of RAN and KPNB1 and poor prognosis of GBM patients. Our results together demonstrate that the activity, rather than the expression levels, of RAN is strongly linked to GBM patient survival.

Functional Blockade of RAN Using Importazole

The results shown above suggest that targeting RAN is a potentially appealing approach to impeding GBM disease progression. Our results also indicate that RAN activity in nuclear transport is important for GBM patient survival. We therefore chose importazole to test whether a blockade of RAN activity would inhibit GBM cell viability. We first treated astrocytes and GBM cell lines with 12.5 μM of importazole and monitored cell viability using the MTS cell viability assay. As shown in **Figure 6A**, importazole decreased the viability of the RAN-positive GBM cell lines A172, U87MG, U251, and SF-295 by >3-fold with a $P < 0.0001$, whereas the RAN-positive GBM cell lines LN-18, SF268, and T98G were much less sensitive to importazole (<3-fold) with a $P < 0.001$. Hence, the statistical analysis shows that the significance of importazole-induced growth inhibition is much stronger in RAN-positive cells ($P < 0.0001$) than in RAN-negative cells ($P < 0.001$).

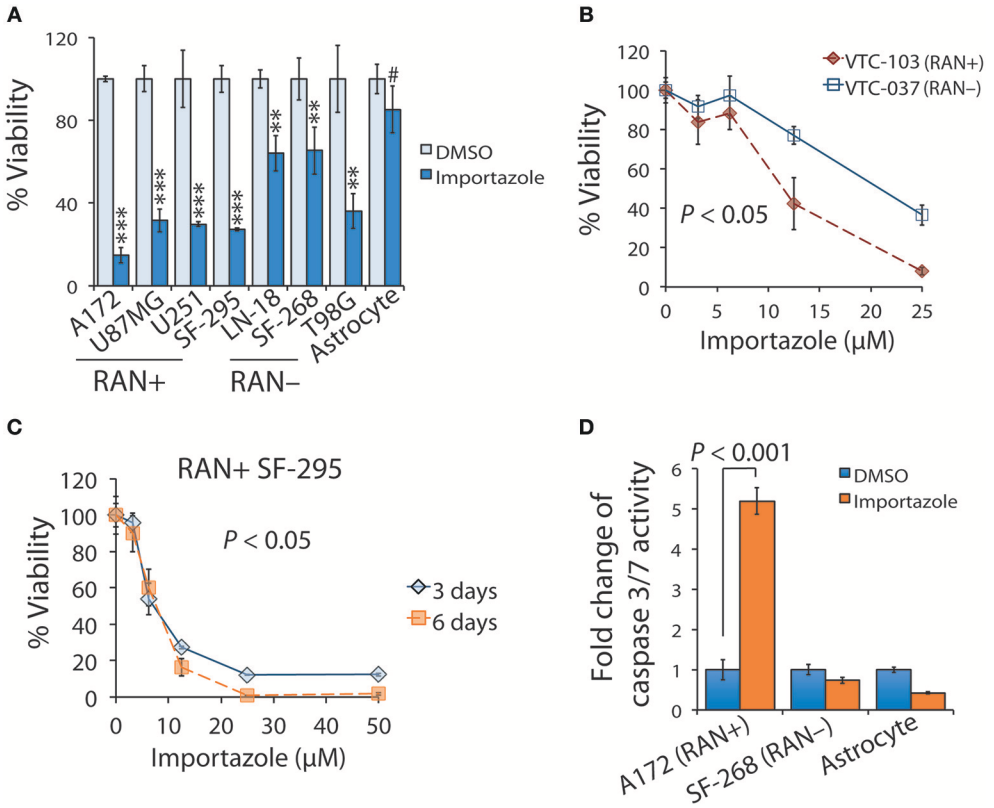

FIGURE 6 | Functional blockade of RAN by importazole induces growth inhibition and activates apoptosis in RAN-expressing GBM cells. **(A)** Viability of GBM cells expressing different levels of RAN and astrocytes when treated with importazole. Cells were incubated with DMSO (light blue bars) or 12.5 μM importazole (dark blue bars) for 3 days. Cell viability was determined using the MTS viability assay. Percentages of viability were obtained by dividing the MTS absorbances of importazole-treated cells with those of DMSO-treated cells. RAN+: RAN-positive; RAN–: RAN-negative. Statistical significance between DMSO and importazole in each cell line was determined using a student *t* test. #$P > 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. **(B)** Viability of primary GBM cells when treated with importazole. Primary GBM cell lines VTC-103 (RAN+; red line) and VTC-037 (RAN–; blue line) were incubated with importazole at different concentrations ranging from 0 to 25 μM. *P* value that determines the statistical significance between responses of VTC-103 and VTC-037 to importazole at different doses was obtained using a two-way ANOVA analysis. **(C)** Viability of RAN+ SF-295 cells when treated with importazole at different time points. RAN+ SF-295 cells were treated with importazole at different doses ranging from 0 to 50 μM for 3 or 6 days. *P*-value that determines the statistical significance between different time points was obtained using a two-way ANOVA analysis. **(D)** Importazole-induced apoptosis in astrocytes and GBM cells expressing different levels of RAN. Cells were incubated with DMSO or 12.5 μM importazole for 3 days. Apoptosis was assessed using the caspase 3/7 activity assay. Fold changes of caspase 3/7 activity were obtained by dividing luminescence intensities of importazole-treated cells with those of DMSO-treated cells. *P*-value was obtained using the student *t*-test. Standard deviations (error bars) were derived from three independent experiments.

More importantly, importazole only decreased the viability of astrocytes by 15% with no statistical significance ($P > 0.05$). These results suggest that targeting RAN activity is an appealing approach with potentially low side effects. To corroborate these results, we treated RAN-positive or RAN-negative primary GBM cells with importazole at different doses. While both RAN-positive VTC-103 and RAN-negative VTC-037 cells showed a dose-dependent response, VTC-103 cells were more robustly sensitive to importazole than VTC-037 cells (**Figure 6B**; red line vs. blue line; $P < 0.05$), particularly when cells were treated with importazole at 12.5 or 25 μM. These results were consistent with those obtained from cell lines.

To determine whether importazole response is also time-dependent, we treated RAN-positive SF-295 with importazole at different doses and treatment lengths. We found that the cell viability of a 6-day treatment of importazole was lower than the cell viability of a 3-day treatment, particularly at high doses (**Figure 6C**). Two-way ANOVA analysis revealed a statistically significant difference between two time points ($P < 0.05$). Hence, the cytotoxic effect of importazole is also time-dependent. Finally, we tested whether the inhibition of cell viability by importazole is due primarily to cell death such as apoptosis. By using the caspase 3/7 activity assay, we found that importazole activated apoptosis, as manifested by the remarkable increase of caspase 3/7 activity in RAN-positive A172 cells, while failing to activate apoptosis in RAN-negative SF-268 cells and astrocytes (**Figure 6D**). Our results suggest that importazole induces apoptosis in RAN-expressing cells, thereby suppressing cell viability. Taken together, our results demonstrate that a functional blockade of RAN by importazole activates apoptosis

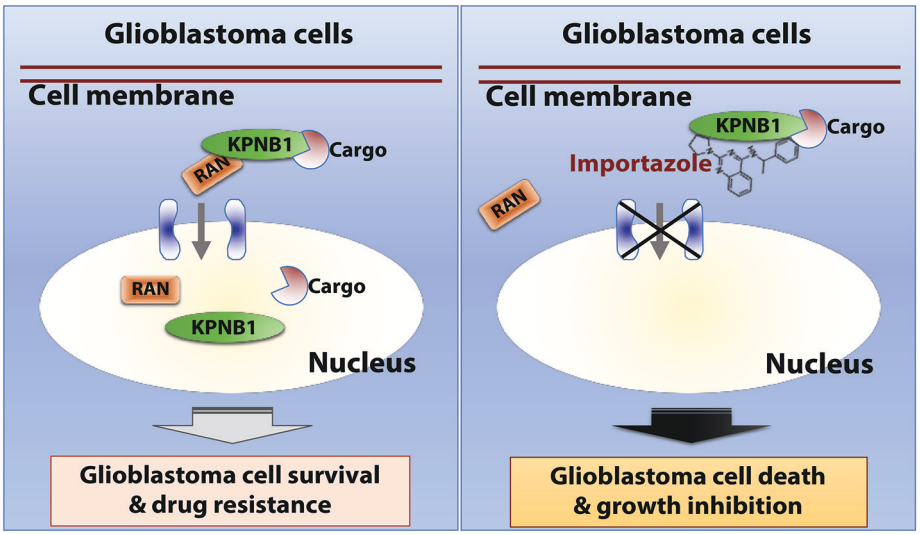

FIGURE 7 | The model of action of RAN in glioblastoma. RAN and its partner KPNB1 regulate nuclear import to promote glioblastoma cell survival and to induce drug resistance in patients **(Left)**. Importazole blocks interactions between RAN and KPNB1, thereby inhibiting nuclear import. The consequences of this blockade are induction of cell death and growth inhibition in glioblastoma **(Right)**.

in RAN-expressing GBM cells and suppresses GBM cell growth via a time/dose-dependent manner.

DISCUSSION

In this report, we re-analyzed RNAi screen results from Project Achilles and identified RAN as an important survival factor for GBM. Our further investigation of GBM patient data revealed a robust correlation between levels of RAN/KPNB1 and GBM poor prognosis associated with MGMT-dependent TMZ resistance. Moreover, the application of importazole, an inhibitor of RAN/KPNB1 activity, substantially induced cell death and growth inhibition in RAN-expressing GBM cells. Based upon our results together with results from other research groups (31, 33, 56–62), we proposed a model illustrating the mode of action of RAN in glioblastoma (**Figure 7**). RAN and its partner KPNB1 regulate nuclear import of their cargos to promote glioblastoma cell survival and to induce drug resistance in patients (**Figure 7**, left panel). Importazole blocks interactions between RAN and KPNB1, thereby inhibiting nuclear import. The consequences of this blockade are the induction of cell death and inhibition of growth in glioblastoma (**Figure 7**, right panel).

RAN GTPase and proteins involved in nuclear transport have been implicated in cancer progression, drug resistance, and cancer therapeutic development (30, 33, 38, 66). Deng et al., found that RAN was highly expressed in pancreatic cancers with high risk of metastasis (67). Furthermore, depletion of RAN substantially inhibited the migration of metastatic pancreatic cancer cells and the capability of these cells to metastasize to the liver. Congruently, ectopic expression of RAN activates PI3K/AKT signaling and promotes the invasive potential of non-small cell lung cancer cells (36). In a different study, Yuen et al. inactivated RAN in breast cancer cells and significantly increased the sensitivity of these cells to gefitinib (68). The role of RAN and nuclear transport mediated by RAN has not yet been widely explored in glioblastoma. In particular, whether RAN mediates TMZ resistance is not clear. Guvenc et al. examined the expression of RAN and survivin in primary GBM specimens and found that GBM patients with high levels of RAN and survivin were resistant to TMZ (40). They further developed a small chemical compound LLP-3 that disrupted the interaction between RAN and survivin. Incubation of TMZ-resistant GBM cells with LLP-3 diminished TMZ resistance.

These results are consistent with our findings presented above. Our results that demonstrate a strong link between high levels of RAN/KPNB1 and MGMT-dependent TMZ resistance (**Figure 5C**) are of particular interest. As we described earlier, TMZ is a front-line GBM treatment, but patients often become relapsed despite the reception of TMZ treatment due to the presence of MGMT proteins that repair TMZ-induced DNA damage (63, 69–78). Given that ∼45% of GBM patients express MGMT (63), it is therefore critical to overcome MGMT-dependent TMZ resistance. Recent development of MGMT inhibitors has shown modest effect on restoring TMZ sensitivity in MGMT positive GBM patients (79–82). Our findings demonstrate that blocking the activity of RAN/KPNB1 is perhaps an effective approach to enhancing the responsiveness of GBM patients to TMZ, thereby providing a better and more promising therapeutic option for TMZ-resistant GBM patients.

Importazole has also been used in treating other cancers before. Multiple myeloma cell lines RPMI 8226 and NCI-H929 exhibited a strong response to importazole with a 50% inhibitory concentration (IC50) of 4.43 and 4.78 μM, respectively (45).

As a comparison, importazole also displayed IC50s at similar range in GBM cell lines and primary tumor cells (**Table S2**). Given that most cancers, including GBM, demonstrate a hyper-dependency on nuclear transport (31, 44), a selective inhibition of RAN/KPNB1 activity by importazole may represent an innovative and effective treatment for GBM.

While our study unveils the crucial role of RAN in GBM cell survival, important questions remain to be addressed to establish that targeting RAN is an effective treatment option for GBM, particularly those with TMZ resistance. Future studies will reveal whether RAN is a biomarker that predicts MGMT-dependent TMZ resistance in GBM, elucidate how RAN contributes to TMZ resistance, and determine whether importazole or functional blockade of RAN circumvents TMZ resistance and inhibits GBM progression.

AUTHOR CONTRIBUTIONS

RTV, SL, and ZS conceived the project and wrote the manuscript. KLS and KJP performed all experiments.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2018.00662/full#supplementary-material

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PARP Inhibitors for Sensitization of Alkylation Chemotherapy in Glioblastoma: Impact of Blood-Brain Barrier and Molecular Heterogeneity

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Prognosis of patients with glioblastoma (GBM) remains dismal despite maximal surgical resection followed by aggressive chemo-radiation therapy. Almost every GBM, regardless of genotype, relapses as aggressive recurrent disease. Sensitization of GBM cells to chemo-radiation is expected to extend survival of patients with GBM by enhancing treatment efficacy. The PARP family of enzymes has a pleiotropic role in DNA repair and metabolism and has emerged as an attractive target for sensitization of cancer cells to genotoxic therapies. However, despite promising results from a number of preclinical studies, progress of clinical trials involving PARP inhibitors (PARPI) has been slower in GBM as compared to other malignancies. Preclinical *in vivo* studies have uncovered limitations of PARPI-mediated targeting of base excision repair, considered to be the likely mechanism of sensitization for temozolomide (TMZ)-resistant GBM. Nevertheless, PARPI remain a promising sensitizing approach for at least a subset of GBM tumors that are inherently sensitive to TMZ. Our PDX preclinical trial has helped delineate *MGMT* promoter hyper-methylation as a biomarker of the PARPI veliparib-mediated sensitization. In clinical trials, *MGMT* promoter hyper-methylation now is being studied as a potential predictive biomarker not only for response to TMZ therapy alone, but also PARPI-mediated sensitization of TMZ therapy. Besides the combination approach being investigated, IDH1/2 mutant gliomas associated with 2-hydroxygluterate (2HG)-mediated homologous recombination (HR) defect may potentially benefit from PARPI monotherapy. In this article, we discuss existing results and provide additional data in support of potential alternative mechanisms of sensitization that would help identify potential biomarkers for PARPI-based therapeutic approaches to GBM.

Keywords: PARP (poly(ADP-ribose) polymerase, chemo-radiation sensitivity, DNA Damage, replication stress, DNA repair activity

BACKGROUND

Glioblastoma (GBM) is a fatal disease with less than 2% of patients surviving 5 years after initial diagnosis and treatment (1, 2). GBM therapy, which includes aggressive surgical resection, high dose external beam radiation therapy (RT) and temozolomide (TMZ) chemotherapy, is associated with a median time to progression of approximately 6 months and a median overall survival of 15 months (3). Sensitizing strategies to enhance efficacy of radiation and chemotherapy may prolong patient survival. TMZ, used as standard of care for newly diagnosed GBM, is a mono-alkylating agent that induces cytotoxic lesions including N_7-methylguanine (N7MeG), N_3-methyladenine (N3MeA) and O_6-methylguanine (O6MeG) (4, 5). N7MeG and N3MeA are repaired by base-excision repair (BER) and contribute minimally to overall cytotoxicity of TMZ, while O6MeG is repaired by O_6-methylguanine-DNA-methyl transferase (MGMT), found suppressed by promoter methylation in ∼40% of GBM tumors. Lack of MGMT expression results in persistent O6MeG lesions that trigger replicative stress and cytotoxicity via futile cycles of mismatch repair (MMR) (5, 6). The poly (ADP ribose) polymerase (PARP) family of enzymes coordinates the DNA damage response. Binding of PARP1 to nicked DNA provides the necessary scaffold that recruits BER components (7, 8). Therefore, PARP inhibitors (PARPI) were thought to potentiate TMZ by disrupting BER (9). Indeed, PARPI potentiate TMZ efficacy in numerous pre-clinical models (4, 9), providing a rationale for clinical development of PARPI to potentiate TMZ therapy in GBM. In addition to the established role of PARP in BER, destabilization of stalled replication forks by allosteric trapping of PARP also contributes toward mechanisms of TMZ sensitization by PARPI (10).

However, like other novel drugs for GBM, several promising PARPI agents have limited distribution across the blood-brain barrier (BBB) or demonstrate heterogeneous *in vivo* response (11). For example, talazoparib and rucaparib are potent PARPI that are substrates for the efflux transporters P-glycoprotein (PgP) and/or breast cancer resistance protein (BCRP) that are active in brain endothelial cells (12, 13). In keeping with poor brain penetration, these drugs have limited distribution and no appreciable TMZ sensitization in orthotopically implanted GBM patient-derived xenografts (PDXs). In contrast, the PARPI veliparib is brain penetrant and an effective TMZ-sensitizer in a subset of GBM PDX models (4, 14, 15). Based on previously published data and additional experimental results, the focus of this article is to explore potential biomarkers critical to a PARPI-based sensitization approach to GBM therapy.

Discordance Between *in vitro* Versus *in vivo* Preclinical Data

Numerous preclinical studies have investigated the combination of PARPI with RT, TMZ or RT/TMZ and other chemotherapy agents in glioma models (14, 16, 17). Models including established glioma cell lines (16, 18–20), zebrafish embryos (21), genetically engineered mouse models (GEMM) (22) and PDXs (14) have been used. While each of these models has helped to characterize PARPI combinations, discordance between *in vitro* vs. *in vivo* data needs to be considered when developing therapies based on preclinical studies. Specifically, the *in vitro* sensitizing effects of the PARPI veliparib were pronounced in TMZ-resistant models, while these models did not benefit from the combination *in vivo*. In contrast, *in vivo* sensitization by veliparib was pronounced in TMZ-sensitive models, although the *in vitro* sensitization was limited (4). This discordance is due to *in vivo* drug achievability, which was lower than concentrations required for DNA damage induction in resistant tumors (4). These results highlight the importance of using clinically relevant concentrations of both TMZ and PARPI for *in vitro* assays and raise the possibility that molecular mechanisms defined by using supratherapeutic drug concentrations may not be applicable to *in vivo* sensitization.

PDX models are translationally relevant because they preserve the genetic characteristics of the tumor, and orthotopically implanted PDXs represent tumor microenvironment and vascular structures found in human GBM (23–25). Furthermore, pharmacokinetic profiles of PARPI in murine models mimic drug exposures reported in human clinical trials (12, 18). GEMMs are ideal to study gliomagenesis; however, GEMMs cannot recapitulate genetic heterogeneity or epigenetic features, such as *MGMT* promoter methylation found in human GBM. Use of large panels of PDXs for drug evaluation may accurately model tumor heterogeneity and the variability in response. As reported previously, veliparib-mediated *in vivo* sensitization is associated with inherent TMZ sensitivity (4, 14). This concept was further tested in a preclinical PDX trial using orthotopic therapy models of 28 different GBM PDX lines with or without *MGMT* promoter methylation, a marker of TMZ sensitivity (15). In this study, profound survival extension with TMZ/veliparib over TMZ alone was observed in ∼45% of PDX models with *MGMT* hyper-methylation, while *MGMT* unmethylated models had no meaningful survival benefit (15). This result helped delineate *MGMT* promoter methylation as a predictive biomarker for veliparib-mediated sensitization (15).

Mechanism of PARPI-Mediated Sensitization:

Understanding mechanisms of sensitization is important to delineate biomarkers and new therapeutic targets. Synthetic lethality of PARPI with HR is the hallmark of single-agent PARPI therapy in breast and ovarian cancers (26, 27). PARPI also potentiate efficacy of genotoxic agents, including DNA alkylating agents and RT (28). Mechanistically, enzymatic activation of PARP consumes NAD+ and generates poly-ADP-ribose (PAR) moieties to modify interacting proteins and itself via a phenomenon known as PARylation (29). PARP auto-PARylation at DNA lesions initiates recruitment of repair proteins, while also keeping PARP-DNA interactions unstable allowing repair machinery access to the lesion (7, 30). PARPI blocks auto-PARylation and prevents dissociation of PARP-DNA interactions, thereby trapping PARP at the damage site, leading to

replicative stress and replication-associated double strand DNA breaks (10, 31).

Prior studies suggest that PARPI-mediated *in vivo* sensitization of TMZ depends on replicative stress caused by persistent O6MeG (4, 14, 15). The significance of PARP trapping to O6MeG-mediated replicative stress is unclear as PARP is known to engage at N7MeG and N3MeA lesions. PARPI-mediated BER inhibition and PARP trapping contribute more robustly at supratherapeutic drug concentrations used *in vitro*; whether PARPI concentrations achievable *in vivo* induce detectable PARP trapping remains to be seen (30, 32, 33). Furthermore, PARPI with high trapping capacity are not well tolerated in combination with TMZ (30), and dose-reduced regimens tested have not shown greater sensitization than veliparib, a weak trapping agent (12–15). In a head-to-head comparison of PARPI agents, the trapping capacity was found to be inversely correlated with *in vivo* efficacy (30), suggesting that the trapping ability of PARPI may not be fully exploited for TMZ sensitization. However, this can be important to PARPI monotherapy or combinations where higher doses of trapping agents can be safely administered. Robust *in vitro* radio-sensitizing effects of PARPI talazoparib used at clinically relevant concentrations have been reported (34). However, evaluation of radio-sensitizing effects of talazoparib in *in vivo* orthotopic GBM models will be important as talazoparib concentrations in intracranial tumors may not reach clinically relevant concentrations based on plasma level (12).

Veliparib-mediated *in vivo* sensitization is limited to a subset of tumors that are inherently sensitive to TMZ (4, 14), suggesting that N7MeG or N3MeA lesions may have little effect on *in vivo* sensitization (4, 12). Consistent with this idea, here we demonstrate that depletion of XRCC1 or MPG, the essential proteins in the BER pathway, had no further increase in sensitization at clinically relevant veliparib concentrations in U251TMZ cells (**Figures 1A,B**). However, knockdown (KD) of *BRCA1* or *RAD51* in U251TMZ cells increased sensitivity to veliparib or TMZ alone, but also led to robust TMZ sensitization (**Figures 1A,B**). Surprisingly, *BRCA2* KD had no increase in sensitivity toward veliparib or TMZ; additionally, veliparib-mediated sensitization was modest in *BRCA2* KD cells compared to that in *BRCA1* or *RAD51* KD cells (**Figures 1A,B**). The differential response among *BRCA2* vs. *BRCA1* or *RAD51* KD cells was intriguing as HR efficiency was equally suppressed in *BRCA1*, *BRCA2* or *RAD51* KD cells (**Figure 1C**). BRCA1, BRCA2 and RAD51 have also been reported to regulate replication fork stability, a function considered unrelated to HR (35, 36). Thus, compromised fork protection by BRCA1 or RAD51 depletion can be a new mechanism of PARPI-mediated TMZ sensitization.

Available RNA-Seq data from the PDX lines, used in the previously reported preclinical trial (15), showed that among analyzed HR and BER pathway genes, the expression of *BRCA1* was trending lower in all five TMZ/veliparib responsive lines as compared to 10 non-responsive GBM lines that were analyzed ($p = 0.10$, **Figure 1D**). Interestingly, the *BRCA1* expression, found upregulated in GBM, appears to be a prognostic factor in a Rembrandt GBM patient data set (**Supplementary Figure 1**). Our limited RNA Seq results suggest that low *BRCA1* expression could be useful to identify tumors likely to respond to PARPI-mediated sensitization. The mechanism of BRCA1 downregulation in responder PDXs is not yet clear, although the promoter hyper-methylation, microRNA or the epigenetic modifier RBBP4, have been previously reported to influence *BRCA1* expression (37–40). Similarly, analysis of available whole exome-seq data for PDX lines used in our preclinical trial showed a significantly higher average mutation burden in responder lines than non-responder lines (**Figure 1E**), suggesting that the GBM tumors with genomic instability are likely to respond to PARPI-mediated sensitization. This idea that *BRCA1* downregulation in responder PDXs correlates with increased mutation frequency will need further validation.

TMZ induces replicative stress via futile attempts of MMR at O6MeG:T mismatches, while PARPI may further enhance the stress by compromising stability of stalled replication forks (41, 42). Association of BRCA1 levels with TMZ/veliparib response in the PDX trial indicates that BRCA1 synthetic lethality with PARPI can be important to fork protection (43) in the context of TMZ/veliparib treatment. Understanding the relationship between PARP and other proteins involved in fork protection may reveal key determinants of PARPI-mediated sensitization. **Figure 1F** shows an overview of potential mechanisms of PARPI-mediated sensitization.

Efflux Liability and Delivery Across BBB: A key Determinant of *in vivo* Sensitizing Effects

Drug exclusion from the brain by the BBB undermines the efficacy of many CNS-directed pharmaceutical agents including PARPI (11, 44). The BBB is a complex neurovascular unit comprised of specialized brain capillary endothelium expressing ATP-binding cassette transporters. The distribution of contrast enhancement agents on magnetic resonance imaging (MRI) is commonly used to assess BBB integrity in gliomas. However, infiltrating GBM cells invade brain tissues beyond margins of contrast enhancement (45, 46). The invasive front of GBM tumors is not accessible to cyto-reductive surgery or chemotherapies that do not adequately penetrate the brain. BBB breakdown in GBM is regional and heterogeneous (44), and therefore drug distribution can be significantly lower at infiltrating edges as compared to the necrotic tumor core (44). Thus, delivery to infiltrating glioma cells is limited for many chemotherapy drugs in GBM (13, 47–49). Considerable effort has been made to understand the brain pharmacokinetics of PARPI, and several PARPI, especially the trapping agents, talazoparib and rucaparib, have efflux liabilities at the BBB and therefore lack sensitizing activity in orthotopic tumor models despite their excellent activity in heterotopic tumor models (13, 30). These findings are consistent with the notion that the delivery of targeted drugs into normal brain or orthotopically implanted tumors can model their efficacy in GBM (11, 49).

We have previously reported that the talazoparib concentration in a normal mouse brain (0.5 ng/g, or 1.3 nmol/L) after drug administration was lower than required for effective PARP inhibition *in vitro*. Comparing the pharmacokinetics of

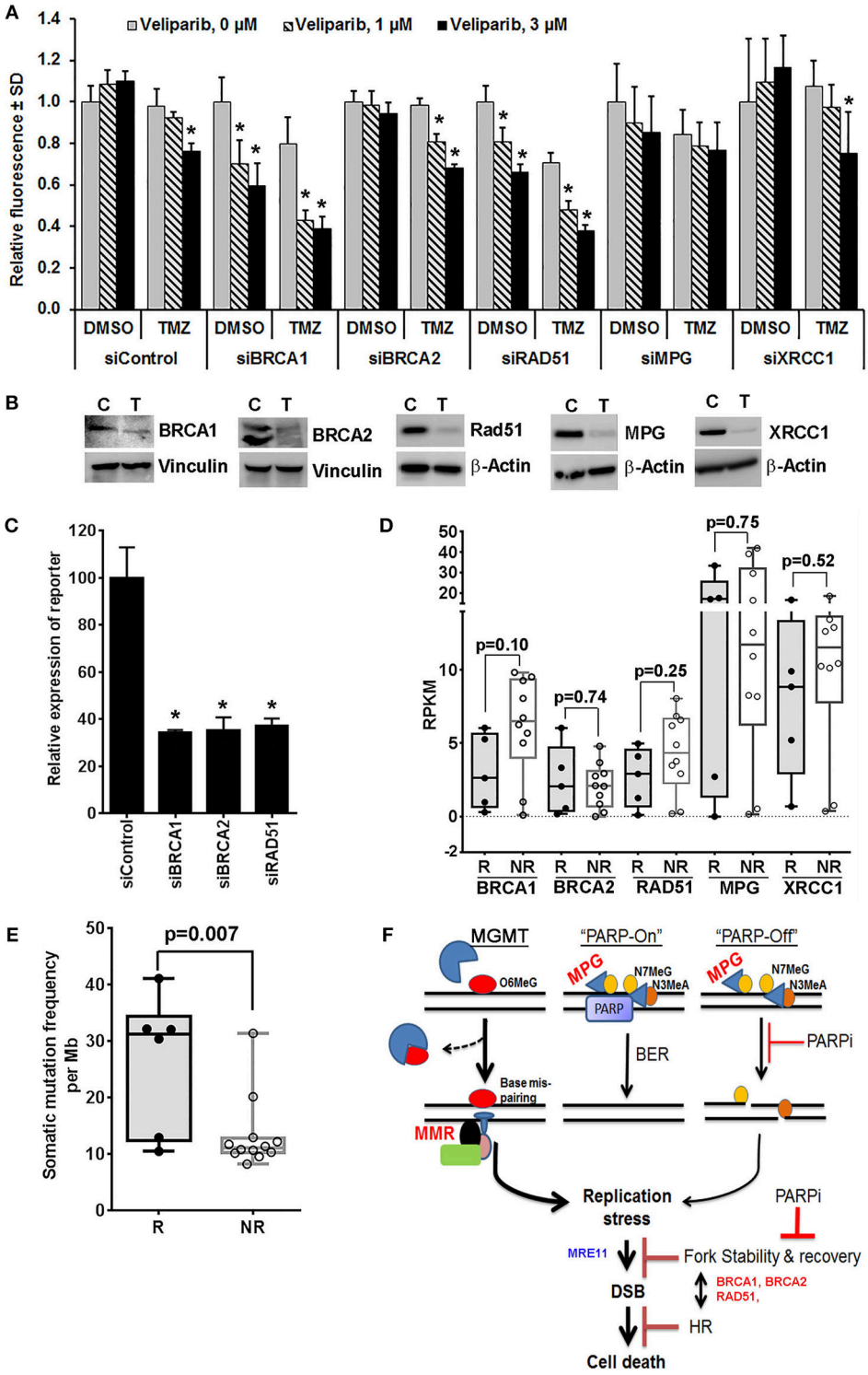

FIGURE 1 | Mechanistic insights into veliparib-mediated sensitization of TMZ therapy in GBM cells (see Supplementary Material for Materials and Methods). **(A)** Effect of homologous recombination (HR) vs. base excision repair (BER) pathway disruption on veliparib-mediated sensitization in U251TMZ cells. Cells transfected with specified siRNA were seeded in 96 well plates (500 cells per well), treated with the vehicle or 30 μM TMZ ± 1 or 3 μM veliparib for 5 days and cell growth measured by CyQuant assay. Bar graphs demonstrate change in average fluorescence intensity relative to control, error bars represent standard deviation calculated from 3 replicates in a representative experiment, and *$p < 0.05$ compared to corresponding control. **(B)** Western blot analysis to determine level of knockdown for cells used in **(A)**, lanes marked with T represent cells transfected with targeted siRNA and C represent cells transfected with control siRNA. **(C)** Bar graphs showing effects of BRCA1, BRCA2 or RAD51 knockdown on HR efficiency. U251TMZ-DRGFP cells were transfected with specific siRNA along with plasmid pCBAScel encoding I-SceI

(Continued)

FIGURE 1 | restriction enzyme and incubated for 72 h followed by quantification of GFP expressing population by FACS analysis. *$p < 0.05$ as compared to control. **(D)** Box plots showing expression levels for specified genes, RKPM values were extrapolated from RNA-Seq data of PDX lines differentially sensitized by veliparib in preclinical PDX trial. Data shown are for 5 of 6 responders (R) lines, which had significantly improved survival vs. 10 of 16 non-responder (NR) lines, which had no significant survival improvement with veliparib/TMZ therapy over TMZ alone in preclinical PDX trial reported previously. two tailed *p*-values reported were calculated by unpaired *t*-test. **(E)** Box plots showing mutation burden based on whole exome seq data available for 21 MGMT methylated PDX lines used in PDX pre-clinical trial and plotted grouped as TMZ/veliparib Responsive (R) vs. Non-responsive (NR) models. SNVs and INDELs across 346 genes involved in DNA damage recognition or repair were analyzed for mutation burden, two tailed *p*-values reported were calculated by unpaired *t*-test. **(F)** Hypothetical model of potential mechanism of the sensitizing effect of PARP inhibition on TMZ therapy *in vivo*. O6MeG, O_6-methylguanine; N7MeG, N_7-methylguanine; N3MeA, N_3-methyladenine; MPG, methyl purine glycosylase; PARP, poly-ADP-ribose polymerase; PARPi, poly-ADP-ribose polymerase inhibitor; MMR, mismatch repair; DSB, double strand DNA breaks; MRE11, Meiotic Recombination 11 Homolog; BRCA1 and BRCA2, BReast CAncer genes 1 and 2; and HR, homologous recombination.

talazoparib to other PARPI in healthy rodents, the brain-to-plasma concentration ratio for talazoparib (0.02) was lower than that of rucaparib (0.11), which also lacks efficacy in orthotopic glioma models (13). Olaparib is another PARP trapping agent known to have efflux liability and restricted delivery across the BBB (50, 51). Although a phase I clinical trial in patients with recurrent GBM has shown that olaparib can reach the core and the margins of GBM tumors (50), this data has to be interpreted cautiously because GBM cells invade tissues beyond the margins defined by the MRI. Veliparib, on the other hand, has a much higher brain-to-plasma concentration ratio (0.47) than either talazoparib or rucaparib despite the efflux liability of veliparib to MDR1 and BCRP (15, 52). Furthermore, unlike talazoparib and rucaparib, veliparib sensitized orthotopic GBM models despite being significantly less potent in terms of PARP trapping (15). A comparison of the properties of drugs from the same class provides insight on the relative significance of variables such as drug potency, BBB penetrability, and efflux liability for efficacy in orthotopic glioma models. These considerations emphasize the importance of brain pharmacokinetics, drug tolerability, and efficacy evaluation in animal models for the successful design of novel therapies for GBM.

Clinical Trials of PARPI in GBM

PARPI have shown significant promise as a specific RT and/or TMZ-sensitizing strategy. Ever since the rucaparib/TMZ combination was found safe to administer in patients with solid tumors (53), several studies have been launched to assess the safety and efficacy of various PARPI in patients with GBM (**Table 1**). The majority of early clinical trials involved patients with recurrent GBM. However, recently launched trials have involved not only newly diagnosed patients, but have also stratified patients by *MGMT* promoter methylation status to enrich the patient population likely to benefit from the therapy (NCT02152982, PARADIGM-2, and NCT03150862). Phase I or phase I/II studies in patients with recurrent GBM are helpful in determining MTD and toxicity. For example, phase I trial NCT00770471 showed that combining veliparib with RT/TMZ is not adequately tolerated (54), and based on this data, later studies planned to evaluate veliparib in combination with RT alone and/or velipaib combined with adjuvant TMZ (NCT03581292, NCT02152982), thus avoiding toxicities reported with the triple combination. Although triple combination of veliparib has been excluded from further development, other PARPI agents in combination with RT/TMZ continue to be tested (NCT03212742, NCT03150862 and PARADIGM-2).

Another important phase I trial has been OPARATIC (NCT01390571), demonstrating that olaparib reaches tumor core and the margins in patients with recurrent GBM, and that the olaparib combined with low dose extended TMZ is well tolerated (50). This data has generated enthusiasm for the olaparib combinations in GBM. A second phase I trial (PARADIGM-2) stratifies newly diagnosed GBM based on *MGMT* hypermethylation to receive olaparib/TMZ/radiation (*MGMT* methylated) or olaparib/radiation (*MGMT* unmethylated) (55). Besides these clinical trials evaluating olaparib combinations, phase II studies NCT03233204 and NCT03212274 aim to investigate single-agent activity of olaparib in pediatric patients with mutated or altered DNA damage repair genes (NCT03233204) or in patients with IDH1/2-mutant tumors (NCT03212274). A phase-II study plans to compare the antitumor activity of olaparib combined with cediranib, an inhibitor of VEGF receptor, vs. bevacizumab monotherapy in patients with recurrent GBM (NCT02974621). An ongoing phase I-II study is investigating PARPI talazoparib combined with TMZ (NCT02116777). Children with refractory or recurrent solid tumors on this trial will receive talazoparib orally either once or twice daily on days 1–6 and TMZ on days 2–6, with therapy repeating every 28 days for up to 24 cycles until disease progression or unacceptable toxicity occurs. Due to limited distribution into the CNS in preclinical mouse models for several of these PARPI agents, concerns remain about the effectiveness of these therapies in gliomas that all have at least a partially intact BBB (56).

Delineation of Predictive Biomarkers to PARPI-Mediated Sensitization

HR deficiency (also known as *BRCAness)* and *PARP* expression are predictive biomarkers for PARPI efficacy (57–59). However, unlike breast and ovarian cancers, *BRCAness* is uncommon in GBM. Although homozygous PTEN deletion, mutant STAG2, or IDH-mutations found in GBM have been reported to disrupt the HR pathway, these studies were performed in established cell lines (60–62). In a PDX preclinical trial, PTEN alterations had no correlation with the TMZ-sensitizing effects of veliparib (15). Similarly, veliparib had neither single agent activity nor any significant sensitization in two different IDH1-mutant GBM PDX models (data not shown). These results suggest that HR deficiency, a

TABLE 1 | Clinical trials of various PARP inhibitors in patients with low grade gliomas and GBM.

Clinical trial identifier	Sponsoring Agency	Description	Biomarker(s) as eligibility criteria
PHASE I STUDIES			
NCT01390571 (OPARATIC)	Cancer Research UK	Olaparib and Temozolomide in Treating Patients with Relapsed Glioblastoma. https://clinicaltrials.gov/ct2/show/NCT01390571	None
NCT01294735	Merck Sharp & Dohme Corp.	Study of the Safety and Efficacy of MK-4827 Given with Temozolomide in Participants with Advanced Cancer (MK-4827-014 AM1). https://clinicaltrials.gov/ct2/show/NCT01294735	None
NCT00770471 (NABTT0801)	Sidney Kimmel Comprehensive Cancer Center, Johns Hopkins	ABT-888, Radiation Therapy, and Temozolomide in Treating Patients with Newly Diagnosed Glioblastoma Multiforme. https://clinicaltrials.gov/ct2/show/NCT00770471	None
PARADIGM-2	University of Glasgow	OlaPArib and RADIotherapy or olaparib and radiotherapy plus temozolomide in newly-diagnosed Glioblastoma stratified by MGMT status: 2 parallel phase I studies http://www.crukctuglasgow.org/eng.php?pid=paradigm_2	MGMT hyper-methylation to establish olaparib MTD in combination with radiotherapy and temozolomide. MGMT unmethylated - to establish olaparib MTD in combination with radiotherapy.
PHASE I/II STUDIES			
NCT03212742	Center Francois Baclesse, France	Study of Concomitant Radiotherapy with Olaparib and Temozolomide in Unresectable High-Grade Gliomas Patients (OLA-TMZ-RTE-01). https://clinicaltrials.gov/ct2/show/NCT03212742	None
NCT01026493 (RTOG0929)	Radiation Therapy Oncology Group	A Randomized Phase I/II Study of ABT-888 in Combination with Temozolomide in Recurrent (Temozolomide Resistant) Glioblastoma. https://clinicaltrials.gov/ct2/show/NCT01026493	None
NCT01514201	NCI	Veliparib, Radiation Therapy, and Temozolomide in Treating Younger Patients with Newly Diagnosed Diffuse Pontine Gliomas. https://clinicaltrials.gov/ct2/show/NCT01514201	None
NCT03150862	BeiGene USA, Inc.	Study to Assess the Safety, Tolerability and Efficacy of BGB-290 in Combination with Radiation Therapy (RT) and/or Temozolomide (TMZ) in Subjects with First-line or Recurrent /Refractory Glioblastoma**.** https://clinicaltrials.gov/ct2/show/NCT03150862	MGMT promoter methylation status (unmethylated vs. methylated)
NCT02116777	Talazoparib	Talazoparib and Temozolomide in Treating Younger Patients with Refractory or Recurrent Malignancies. https://clinicaltrials.gov/ct2/show/NCT02116777	None
PHASE II STUDIES			
NCT03212274	NCI	Study of the PARP inhibitor olaparib in IDH1 and IDH2 Mutant Advanced solid tumors. https://clinicaltrials.gov/ct2/show/NCT03212274	IDH1/IDH2 mutations
NCT02974621	NCI	Cediranib Maleate and Olaparib Compared to Bevacizumab in Treating Patients with Recurrent Glioblastoma**.** https://clinicaltrials.gov/ct2/show/NCT02974621	None
NCT03233204	NCI	Olaparib in Treating Patients with Relapsed or Refractory Advanced Solid Tumors, Non-Hodgkin Lymphoma, or Histiocytic Disorders with Defects in DNA Damage Repair Genes (A Pediatric MATCH Treatment Trial). https://clinicaltrials.gov/ct2/show/NCT03233204	Molecular Analysis for Therapy Choice (MATCH) to APEC1621H based on the presence of an actionable mutations
NCT03581292	NCI	Veliparib, Radiation Therapy, and Temozolomide in Treating Participants with Newly Diagnosed Malignant Glioma without H3 K27M or BRAFV600E Mutations. https://clinicaltrials.gov/ct2/show/NCT03581292	wild-type for H3K27M, BRAFV600E, and IDH1/2
PHASE II/III STUDIES			
NCT02152982 (A071102)	NCI	Temozolomide with or without Veliparib in Treating Patients with Newly Diagnosed Glioblastoma Multiforme. https://clinicaltrials.gov/ct2/show/NCT02152982	MGMT promoter hypermethylation

conventional marker of PARPI sensitivity, may not be a robust biomarker for veliparib-mediated *in vivo* sensitization in GBM.

As reported previously, PARPI-mediated *in vivo* sensitization is associated with inherent TMZ sensitivity (4, 14), whereas *MGMT* hypermethylation is a marker of TMZ sensitivity (5, 63). We assessed the utility of *MGMT* methylation status as a biomarker of veliparib-mediated sensitization in a PDX preclinical trial involving 28 GBM PDX models (15). In this preclinical trial, PDX lines with unmethylated *MGMT* had no survival benefit with TMZ/veliparib over TMZ alone, while profound survival extension with TMZ/veliparib was observed in ∼45% of PDX lines with *MGMT* promoter hyper-methylation (15). Based on this result, the A071102 clinical trial uses *MGMT* promoter methylation as selection criterion for a randomized clinical trial of adjuvant TMZ combined with veliparib or placebo (NCT02152982). *MGMT* promoter methylation status has been integrated in clinical trial designs for at least two other studies testing TMZ/PARPI in GBM (PARADIGM-2, NCT03150862). However, as only a fraction of patients with *MGMT* hyper-methylation expected to benefit from TMZ/PARPI therapy, refinement of predictive biomarkers is necessary to guide optimal use of PARPI in GBM.

Lack of Schlafen Family Member 11 (SLFN11) is known to confer resistance to DNA damaging agents (64, 65). Mechanistically, SLFN11 interacts with replication protein A (RPA), destabilizes RPA-ssDNA complexes and inhibits HR (66). Like *MGMT*, *SLFN11* expression is epigenetically suppressed through promoter hypermethylation in nearly 50% of solid tumors (67). In a recent study, *SLFN11* expression correlated with *in vivo* tumor response to talazoparib in patient-derived xenograft (PDX) models of small cell lung cancer (SCLC) (68). Interestingly, in this study, response to TMZ/Talazoparib had no clear association with SLFN11. However, a phase II clinical trial testing TMZ plus veliparib (or placebo) in patients with SCLC showed that SLFN11-positive tumors, as defined by immunohistochemistry ($n = 12$) had improved progression-free and overall survival relative to patients with SLFN11-negative tumors (69). Based on this promising data in SCLC, SLFN11 is a potential biomarker to be examined in GBM.

IDH1 mutations are oncogenic mutations found in 74% of low-grade gliomas and 9% of GBM (70). Mechanistically, 2-HG produced by the neomorphic mutant-IDH1 enzyme inhibits α-ketoglutarate (αKG)-dependent ALKBH2-3 enzymes and prevents repair of endogenous DNA damage, rendering vulnerability to alkylation therapies (71). A recent study by Salkowski et al. suggests that 2-HG can disrupt HR activity and sensitize cells to PARPI (62). This finding was further confirmed in GBM cell lines modified to express mutant IDH1 constructs (72). However, exogenously expressed mutant IDH1 may not recapitulate all the genetic and phenotypic changes that occur in IDH1-mutant gliomas. NAD+ deficiency is one of the striking features of IDH1-mutant glioma cells, which are highly vulnerable to NAD+ depletion via TMZ treatment or NAMPT inhibition (73). Since NAD+ is consumed by PARP activation during genotoxic therapy, PARPI can be counterproductive. This hypothesis was proven by Tateishi et al. whereby TMZ/olaparib had lesser cytotoxicity than TMZ alone in glioma cells *in vitro* (74, 75). Comprehensive analysis of metabolic vulnerability is necessary to understand conflicting results of PARPI sensitivity in IDH1 mutant gliomas.

CONCLUSIONS AND FUTURE DIRECTIONS

Increased DNA repair compromises therapeutic efficacy of anti-cancer genotoxic therapies (76). Based on the pleotropic role of PARP in DNA repair, there is immense interest in clinical development of PARPI as cancer monotherapy (for HR defective tumors) and as a chemo-radiation sensitizer (76). Preclinical studies using orthotopic GBM models suggest that the efficacy of PARPI in GBM may be limited due to restricted delivery across the BBB and heterogeneous tumor response (4, 12, 13, 15). Pronounced TMZ sensitization by the brain penetrant PARPI veliparib was observed in a subset of tumors inherently sensitive to TMZ, while TMZ-resistant tumors lacked *in vivo* sensitization, suggesting that potentiation of replication stress rather than BER inhibition or PARP trapping is a key mechanism involved in *in vivo* sensitization (4, 14, 15). Based on these findings, *MGMT* promoter methylation was delineated as a predictive biomarker and is being increasingly used in PARPI clinical trials in GBM. However, as only a fraction of *MGMT* methylated tumors responded in preclinical trial, discovery of precise biomarkers is necessary.

One particular area of interest is to dissect the role of PARP in replication stress resolution. Whereas TMZ induces replicative stress via repetitive MMR at O6MeG:T sites, PARPI may potentially compromise stability of stalled replication forks (5, 7, 77). However, compromised fork protection is a complex biological process, where PARPI may act more robustly in context of vulnerabilities such as loss of BRCA1 or other factors involved in fork protection. Identification of critical regulators of fork protection in context the of TMZ/PARPI combinations will help identify new biomarkers. Endogenous replicative stress in cells with compromised fork protection may result in genomic instability and higher mutation burden. Analysis of mutation burden in the context of TMZ/PARPI therapy can be another crucial marker of PARPI-mediated sensitization. Ongoing PARPI trials are poised to generate data and biospecimens that will allow correlative analysis of putative biomarkers identified through preclinical studies in GBM models.

AUTHOR CONTRIBUTIONS

SG and JS conception and design. SG, ES, AM, ST, and PD acquisition of data. SG, JS, ST, and GK analysis and interpretation of data. SG, JS, SK, and GK writing, review, and/or revision of the manuscript. SG and JS study supervision.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2018.00670/full#supplementary-material

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REVIEW
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Treatment Strategies in Diffuse Midline Gliomas With the H3K27M Mutation: The Role of Convection-Enhanced Delivery in Overcoming Anatomic Challenges

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Diffuse midline gliomas harboring the H3 K27M mutation—including the previously named diffuse intrinsic pontine glioma (DIPG)—are lethal high-grade pediatric brain tumors that are inoperable and without cure. Despite numerous clinical trials, the prognosis remains poor, with a median survival of ∼1 year from diagnosis. Systemic administration of chemotherapeutic agents is often hindered by the blood brain barrier (BBB), and even drugs that successfully cross the barrier may suffer from unpredictable distributions. The challenge in treating this deadly disease relies on effective delivery of a therapeutic agent to the bulk tumor as well as infiltrating cells. Therefore, methods that can enhance drug delivery to the brain are of great interest. Convection-enhanced delivery (CED) is a strategy that bypasses the BBB entirely and enhances drug distribution by applying hydraulic pressure to deliver agents directly and evenly into a target region. This technique reliably distributes infusate homogenously through the interstitial space of the target region and achieves high local drug concentrations in the brain. Moreover, recent studies have also shown that continuous delivery of drug over an extended period of time is safe, feasible, and more efficacious than standard single session CED. Therefore, CED represents a promising technique for treating midline tumors with the H3K27M mutation.

Keywords: diffuse intrinsic pontine glioma (DIPG), convection-enhanced delivery (CED), H3K27M mutation, blood brain barrier (BBB), alternative delivery method

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INTRODUCTION

Clinical Presentation of Diffuse Intrinsic Pontine Glioma (DIPG)

Primary pediatric brain tumors are rare entities, with an incidence of ∼2,200 cases annually (1–3). Diffuse intrinsic pontine glioma (DIPG), which makes up ∼20% of these primary pediatric primary brain tumors, carries among the direst prognosis (4, 5). A diffusely infiltrative lesion situated in the brainstem of children, these tumors often present with a constellation of symptoms including headache, nausea, cranial nerve dysfunction, cerebellar signs, and long tract sings, with some patients demonstrating hydrocephalus (6, 7). These tumors occur primarily at a median age of seven (8). DIPGs are one of the few central nervous system (CNS) neoplasms for which diagnosis

can be made with radiographic imaging alone, as the diffuse, non-enhancing T2 signal change in the brainstem, encompassing over half of the pons, is so highly characteristic and biopsy of the lesion carries risk of neurologic deficit (**Figure 1**) (6). However, recent reports have shown that biopsies of these tumors are safe, and molecular analysis from this tissue has greatly increased our understanding of the unique tumor biology (9, 10). Prognosis of these tumors remains uniformly poor, with a median survival of around 1 year from the time of diagnosis despite extensive efforts to improve this (4, 11, 12). Patients eventually develop worsening neurologic deficits, brainstem dysfunction, and hydrocephalus, before ultimately succumbing to their disease.

Current Therapies

The anatomic location of the tumor severely limits any opportunity for meaningful surgical resection, and treatment usually consists of standard fractionated radiation to a dose of 54-59 Gy (over 30 fractions) (6). Multiple regimens involving monotherapy and combination chemotherapy have been trialed, with uniformly poor results (1, 4, 13). More recently, advances have been made in the field of chimeric-antigen receptor (CAR) T cells as a targeted therapy targeting anti-GD2 for DIPG; however, these studies remain in early stages (14). With these limited treatment options, there remains a critical need to develop novel therapeutics and effective delivery mechanisms for DIPG.

H3K27M Mutation

Several key mutations appear to define these tumors. The substitution of a lysine for methionine at position 27 in histone H3 (specifically in either histone 3.3 or 3.1 genes) resulting in a H3K27M mutation, is the most commonly found mutation, and is associated with a worse prognosis than wild-type tumors (15–17). In the largest study of classically defined DIPG tumors that have been biopsied in the molecular era (91 patients), researchers in France found all but one tumor had either a somatic mutation in H3K27M and/or loss of H3K27 trimethylation, highlighting the importance of histone H3 in the pathology of this disease (10). This has implications for chromatin remodeling on a wide scale, with epigenetic silencing and activation of various elements of the genome broadly impacted, as the lysine 27 residue is a critical site for epigenetic regulation (18, 19). This mutation is sufficiently characteristic of these tumors that the World Health Organization recently redefined these tumors as "diffuse midline glioma, H3K27M mutant" in the latest criteria (6, 20). For simplicity and historical reasons we will continue to use the term DIPG tumors throughout this review. Crucially, this single histone mutation and subsequent epigenetic changes presents a potentially druggable target for the treatment of DIPG, and its prevalent expression in DIPG implies an essential role in tumorigenesis and growth, further raising its appeal as a therapeutic target (10, 16). Drugs that modify the histone epigenome have recently been identified as promising targeted therapies including the Histone Deacetylase (HDAC) inhibitor panobinostat and the bromodomain inhibitor (JQ1) have shown some early evidence as promising targeted therapies (21–23). However, the anatomic location of these tumors presents a challenge for effective delivery of medications, requiring novel drug delivery strategies.

Abbreviations: DIPG, Diffuse intrinsic pontine glioma; CED, Convection-enhanced delivery; BBB, blood brain barrier.

THE BLOOD-BRAIN BARRIER (BBB)

Drug delivery to lesions in the brain presents a number of specific challenges; chief among them is getting drugs past the blood brain barrier (BBB). The BBB, a unique tissue-specific modification of the capillary endothelium and basal lamina, serves to exclude nearly all macro molecules and most small molecules from extravagating into the brain parenchyma (24). Highly polar or charged particles are excluded, as are molecules as small as 100 Da (24). This poses obvious challenges in systemic administration of drug, requiring that molecules or mechanisms of drug delivery be specifically engineered in order to bypass the BBB. Even in the case of tumors with significant contrast enhancement (such as glioblastoma), indicative of BBB disruption, effective delivery of drug through the systemic circulation remains a challenge (25). The BBB in cases of DIPG is frequently preserved, as evidenced by the general lack of enhancement in these tumors (26). Further, the BBB is variable throughout the CNS, with some areas (such as the circumventricular organs) that have a reduced or absent barrier (27). In contrast, there is some evidence that the brainstem may be home to an even more robust BBB, further restricting the range of drugs that may be effectively delivered to the region. Using dynamic contrast-enhanced MRI, Subashi et al. demonstrated reduced BBB permeability in brainstem gliomas relative to identical tumors implanted supratentorially in a mouse model (28). There is also some evidence, particularly in mice, that the brainstem has a lower density of capillaries than cortical regions or basal ganglia, which would also imply increased difficulty in delivering effective therapeutic payloads through the circulation (29, 30).

ALTERNATIVE DELIVERY METHODS

Given the challenges presented by the BBB, significant effort has been put into finding means to bypass or disrupt the BBB in a controlled fashion for enhanced delivery of therapeutics. Direct intracranial delivery provides an attractive means to circumvent the BBB, as surgical resection remains the mainstay of treatment of many brain tumors and presents an opportunity for direct inoculation of therapeutic agents into the brain parenchyma. Carmustine wafers have been one such technology, though their efficacy and degree of tissue penetration are somewhat limited (31–34). Such an approach is of limited use in tumors with limited surgical accessibility, including DIPG. Intra-arterial (IA) infusion of therapeutics is an area of active research, as such a route of administration circumvents fist-pass metabolism by the liver, broadening the scope of pharmacologic tools available to cross the BBB. IA therapy also allows for selective infusion of medication into end-arteries in the brain, allowing for

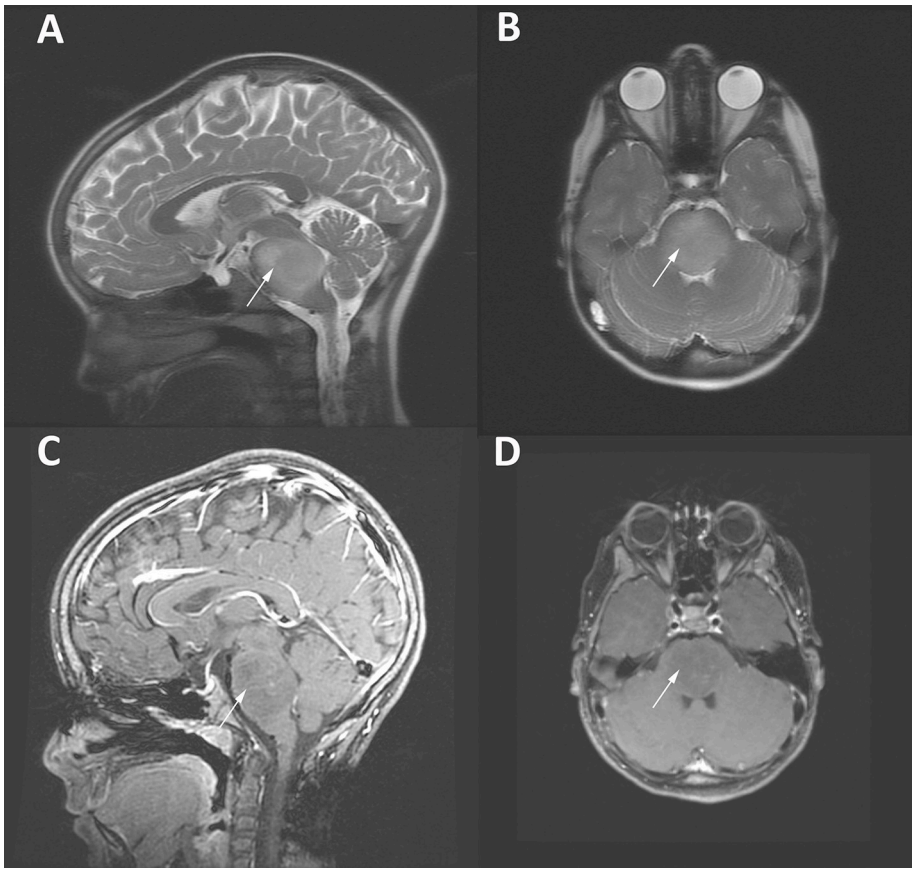

FIGURE 1 | MRI imaging of an 8 year-old girl with a DIPG tumor (white arrows). T2-weighted sagittal **(A)** and axial **(B)** images demonstrate the enlargement of the brainstem and highlight the diffuse infiltrative characteristic of DIPG tumors. **(C,D)** Gadolinium-enhanced T1-weighted MRI images of the same patient demonstrating scant patchy enhancement.

administration of higher dosages of chemotherapy than can be administered systemically (35, 36). Certain transport proteins important for BBB function, including P-gp, are also expressed at lower levels on the arteriolar side of the circulation (29). Co-administration of mannitol with IA chemotherapy has been investigated as a means of crossing the BBB (36, 37). Often studied in conjunction with an IA delivery mechanism, focused ultrasound has shown some promise in circumventing the BBB (38–40). However, this technique has proven inconsistent (41). Infusion of microbubbles coupled with focused ultrasound can allow for focused disruption of the BBB, allowing medications to temporarily cross (42). Alli et al. recently demonstrated the feasibility of this technique in disrupting the BBB to allow for increased local delivery of doxorubicin (43). Some drugs may also be loaded into these microbubbles, creating a packaging system the protect drugs until they reach their destination, providing a mechanism to control their release in a specified location (44). Intranasal delivery has also been advanced as means of improving drug delivery to the brain, though such a route precludes targeting toward specific brain regions (45).

CONVECTION-ENHANCED DELIVERY

Convection-enhanced delivery (CED) is a therapeutic strategy that addresses some of the key pitfalls in the treatment of brain tumors. It allows for targeted treatment of a specific region via a cannula that can be placed in difficult to access areas, and allows for direct intraparenchymal infusion of drug, bypassing the BBB. Fundamentally, CED is the process of continuously infusion drug at a steady rate over a prolonged period of time, allowing a constant pressure head to drive infusate penetration into surrounding tissue via bulk flow and avoid reflux into the infusing cannula, treating a spherical or elliptical region of tissue (46). In this way a small point of access can be used to treat a relatively large volume of tissue, an appealing characteristic for treating tumors in privileged locations such as DIPG. Further, infusion via CED proceeds in a highly predictable fashion, with a sharp drop-off in drug dosage beyond the predicted volume of the infusate, makes it ideal for treating a specific region while avoiding treatment of uninvolved surrounding structures (46, 47). Infusion in this manner proceeds best along white matter tracts, which would likely be of benefit in treating DIPG (48, 49).

Catheter Placement

In using CED to treat DIPG, effective placement of the infusing catheter is a critical step, given the need for the catheter to be fixed in a stable position over a prolonged period, the challenge in placing the catheter into the brainstem without creating a neurologic deficit, and positioning the catheter in such a way as to allow treatment of the entire tumor with infusate. Long-term catheter placement of CED in the brainstem of rodents and primates has been successfully carried out by a number of groups (**Figure 2**), reviewed extensively by Goodwin et al (49, 50). Large human trials involving CED for supratentorial high grade glial tumors have demonstrated an ability to place catheters for treatment in humans safely, though the most notable example, the PRECISE trial, did not monitor distribution of drug over the course of therapy (51). Further, in the PRECISE trial, catheter placement was scored based on depth from brain surface, distance from pial surfaces, and distance from resection cavity/ependymal surface, and only 51% of catheters had adequate placement (52). However, these criteria to determine adequate placement have not been prospectively validated (53). More recently, CED catheters have now been placed into the brainstem in humans and, recent studies have shown this technique to be safe (54–57). Much of the foundational work in this area has been conducted in Bristol, UK. Baura and colleagues used robotic assistance to place a catheter for CED carboplatin treatment in a large pontine tumor in a 5 year-old patient and were able to achieve infusate to 95% of the tumor (57). This group has also worked to develop bone-anchored ports and multiple-catheter systems (up to four catheters), allowing for chronic intermittent CED to a highly-tailored area (58, 59). Improved stereotactic placement of catheters and increasing use of stereotactic biopsy in obtaining tissue for diagnosis and study in DIPG placement has also increased facility and demonstrated the safety of these techniques, which require similar expertise and carry similar attendant risks as CED treatment to the brainstem (60, 61). To validate the real-world application of CED to the pons, Souweidane and colleagues report their results of the first Phase I trial in DIPG tumor patients. CED of the radionuclide [^{124}I]-8H9 for treatment of DIPG in 28 patients was well-tolerated without any dose-limiting toxicities observed in the study, with one patient experiencing transient hemiparesis (trial NCT01502917) (54).

Variables in CED

CED is a robust and tunable platform allowing for infusion of a range of agents of varying sizes over a range of tissue volumes. Such malleability requires optimization for a given therapeutic agent in order to achieve optimal delivery, however.

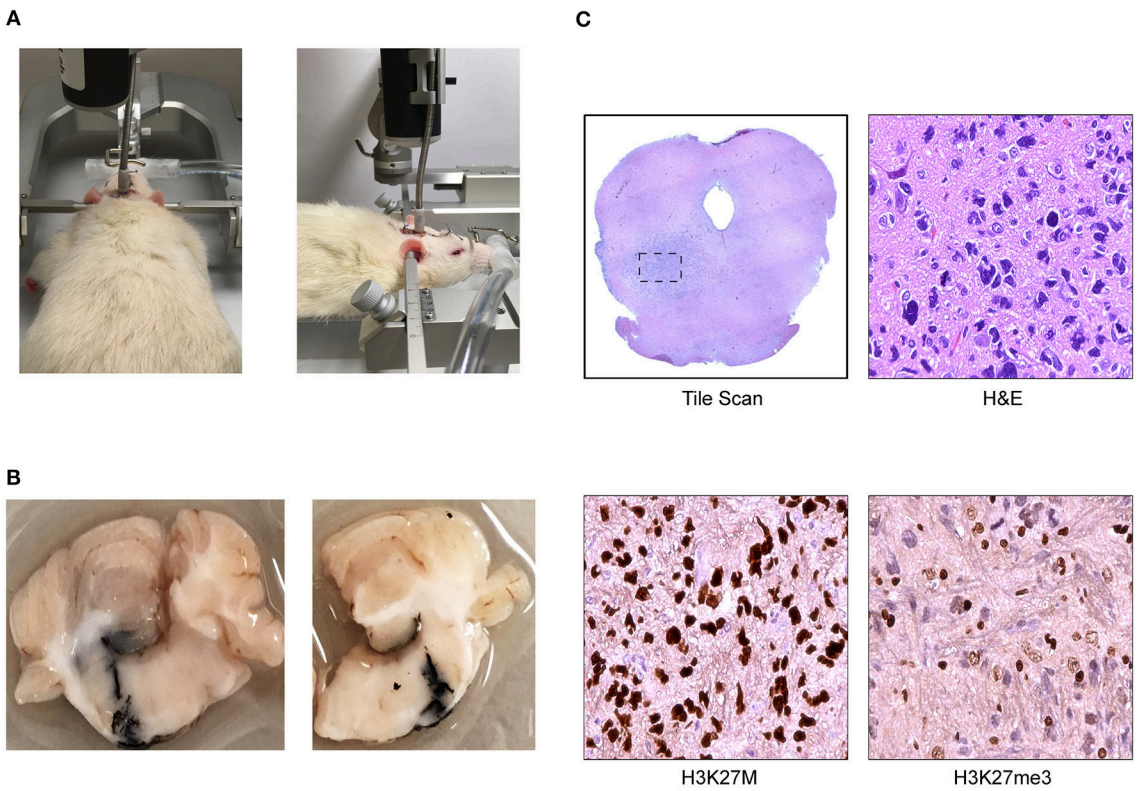

FIGURE 2 | Cannula-guided convection enhanced delivery in the rat pons (Daniels Laboratory—Mayo Clinic). **(A)** Infusion pump is attached to the cannula installed on rat brain where the infusate was delivered at a constant rate over time. **(B)** Photograph of ink solution injected at 8 mm of depth with a Hamilton syringe through the cannula validating V_d. **(C)** Coronal section of athymic nude rat brainstem with DIPG patient derived xenograft showing representative images of low magnification scan of H&E and high magnification scan of H3K27M and H3K27me3 immunohistochemical (IHC) staining.

The volume of tissue treated depends on the volume and rate of infusion, with most CED studies utilizing rates between 0.5 and 6 μL/min (56). These characteristics are essential for ensuring an adequate volume of distribution (V_d) and avoiding reflux into the infusing catheter. The tissue being treated dictates these parameters to a large degree—tumors frequently have high interstitial pressures that need to be overcome in order to achieve adequate infusate delivery, and this resistance can drive infusate away from the desired region (53). Tumors also lack normal vasculature, further exacerbating this outward-directed pressure gradient that can drive the dispersion of drug delivered to the tumor bed (53, 62). The size and shape of the infusing catheter is critical for optimizing both the degree of tissue penetration and avoiding backflow. While many studies have been conducted with traditional end port cannulas, improved infusion profiles have been observed with porous tipped catheters and those with a step-off design, reviewed in detail by Lewis et al. (63). Lewis et al. have also recently described a recessed step catheter that allows for "controlled reflux" of infusate, and may allow for even more tailored delivery of therapeutics moving forward (64). The properties of the infusate itself and its therapeutic payload are also critical variables, and will be discussed in detail below (64).

Advantages for Drug Delivery

In light of the myriad variables in developing an effective CED platform, developing a CED platform for use in real-world situations is an ongoing challenge. However, there are key advantages to this technique that make its use appealing. Most notably in treating patients, ensuring the safety and reliability of these systems is critical. While select cases have made use of CED to the brainstem, the unique properties of every infused therapeutic makes the volume of distribution hard to predict (53). However, in cases where CED infusion in the pediatric brainstem resulted in neurologic changes, cessation of the infusion halted these effects (53, 65). As discussed above, reliable catheter placement remains an area of ongoing growth, but the ability to administer prolonged indwelling infusions via CED has been well-established. Treating a large volume of tissue with a relatively small amount of infusate is advantageous, particularly in treating DIPG, where the volume of therapeutic that can be infused may be limited due to tumor location. Particularly for larger molecules, CED can result in a V_d many times what would be predicted by diffusion alone (47, 53). CED allows for a homogenous distribution of infusate as well, ensuring the targeted area receives therapeutic levels of the administered drug (63).

PROPERTIES OF INFUSATE

The CED cannula itself, the volume of infusate (V_i), and the rate of infusion are not the only critical factors in effective CED administration—the drug and infusate itself must be optimized for ideal distribution. Most critically, the drug infused but be optimized for CED. Size is a critical factor; as smaller molecules will distribute more readily through tissue (47). Mechanisms of clearance include active transport by various ATP-binding cassette (ABC) transport proteins, or CSF spaces that rapidly clear infusate (53, 66, 67). Interstitial pressures in tumors may also be higher than in the surrounding tissue, generating an outward pressure gradient leading to increased clearance of infusate (25, 68). Nano-scale particles (<100 nm) seem to be the ideal size for achieving a large V_d /V_i ratio (67). Hydrophobic molecules also struggle with achieving large V_d when administered via CED, as do those that are positively charged (67). Surface modification of drugs, such as coating relatively hydrophobic molecules with albumin, can improve V_d as well (67, 69). Development of liposomal or nanoparticle formulations of drugs in order to improve CED pharmacokinetic profiles is an active area of development, and some current clinical trials are underway utilizing such formulations (70). Such formulations allow for controlled release of therapeutic over time, prevent premature degradation of drug, and allow for hydrophobic medications to traverse the extracellular space (70). Coupling drugs that are inherently nonspecific for tumor cells, such as toxins, chemotherapeutics, or radionuclide, to tumor-specific antibodies is another promising strategy, adding a degree of tumor specificity (71). Lastly, the viscosity of the infusate itself can be adjusted for improved CED. In some cases, increasing the viscosity of the carrier fluid can improve the V_d of drug, and can readily be achieved by the addition of sucrose or polyethylene glycol (PEG) (67, 72, 73). This is likely due to more efficiently convective forces in higher viscosity fluids, as low viscosity fluids may be more likely taken up by surrounding cells or reflux into the catheter (46, 73).

VISUALIZATION OF CED

The ability to accurately track the distribution of drug administered via CED is an essential challenge in advancing the methodology to clinical applications. Some therapeutics, particularly radionuclides, maybe tracked by positron-emission tomography/CT (PET/CT) in order to evaluate the volume of tissue treated by the therapeutic being administered (71). However, most small molecule or nanocarrier-packaged therapeutics administered by CED lack such an intrinsic ability to be tracked on imaging. Older studies made use of infusion-associated T2 signal changes on MRI to evaluated the area of tissue treated (57). As reviewed in detail recently by Lonser, many current studies co-administer a gadolinium agent such as Gd-DTPA in the infusate with the therapeutic, allowing for visualization of the area treated by CED via MRI (74). Similarly, iodine-based contrast agents such as iopamidol and iopanoic acid can be used for CT-based imaging of CED (74). However, as has been discussed, substances of differing sizes, charge, and hydrophobicity can have very different V_d when administered with a given V_i, and so the use of co-administered gadolinium may not accurately reflect the distribution of the therapeutic agent. Efforts have been made to administer surrogate agents of similar size to the therapeutic agents being administered—Szerlip and colleauges co-infused viral particles and the iron-based contrast agent

ferumoxtran-10, both ∼24 nm in size, for imaging via MRI (75). However, such an approach still makes use of a surrogate marker for visualization.

CURRENT ANIMAL MODELS FOR CED

A number of well-established models have been developed for studying CED. Rodent models in mice and rats (**Figure 2**) have been utilized for some time, as have models in larger organisms including pigs and primates (67, 76–79). A schematic model of CED in the mouse pons is diagrammed in **Figure 3**. Studying the dynamics of CED in these larger systems is critical in order to study distribution volumes at a scale relevant to human therapy. This is not only a function of size, but as discussed previously, CED bulk flow dynamics behave differently in different brain regions, particularly gray vs. white matter (46). Mice and rat brains have particularly limited amounts of white matter, limiting the generalizability of CED data derived from these models (67). A number of brainstem-specific models of CED have also been developed (49). Occhiogrosso et al. demonstrated that long term (24 h infusion) CED to the rodent brainstem was feasible (77). Sewing and colleagues demonstrated the ability to deliver carmustine via CED in the mouse brainstem (78). Zhou and colleagues have demonstrated the ability to infuse therapeutic agents, including kinase inhibitors, to the mouse brainstem with a favorable toxicity profile (80). Developing effective animal models of DIPG has also been an area of active development. Tumor models to study CED in animal models have also been developed, with much work done in the rat glioma models, including the F98 and 9L glioma lines (81, 82). However, more recent efforts have focused on developing brainstem-specific models to better study DIPG. Inoculating tumors in an anatomic position in these models is a challenge given the size and fragility of the brainstem, particularly in a small animal model, however several groups have successfully done so (49, 78). More recently, a genetically engineered mouse model of brainstem glioma has been developed driven by the H3K27M mutation, overexpression of platelet-derived growth factor (PDGF), and loss of p53 (83). Such a model, with *in situ* formation of tumors in the brainstem, may provide a critical tool for evaluating CED of therapeutics in a physiologically-relevant setting.

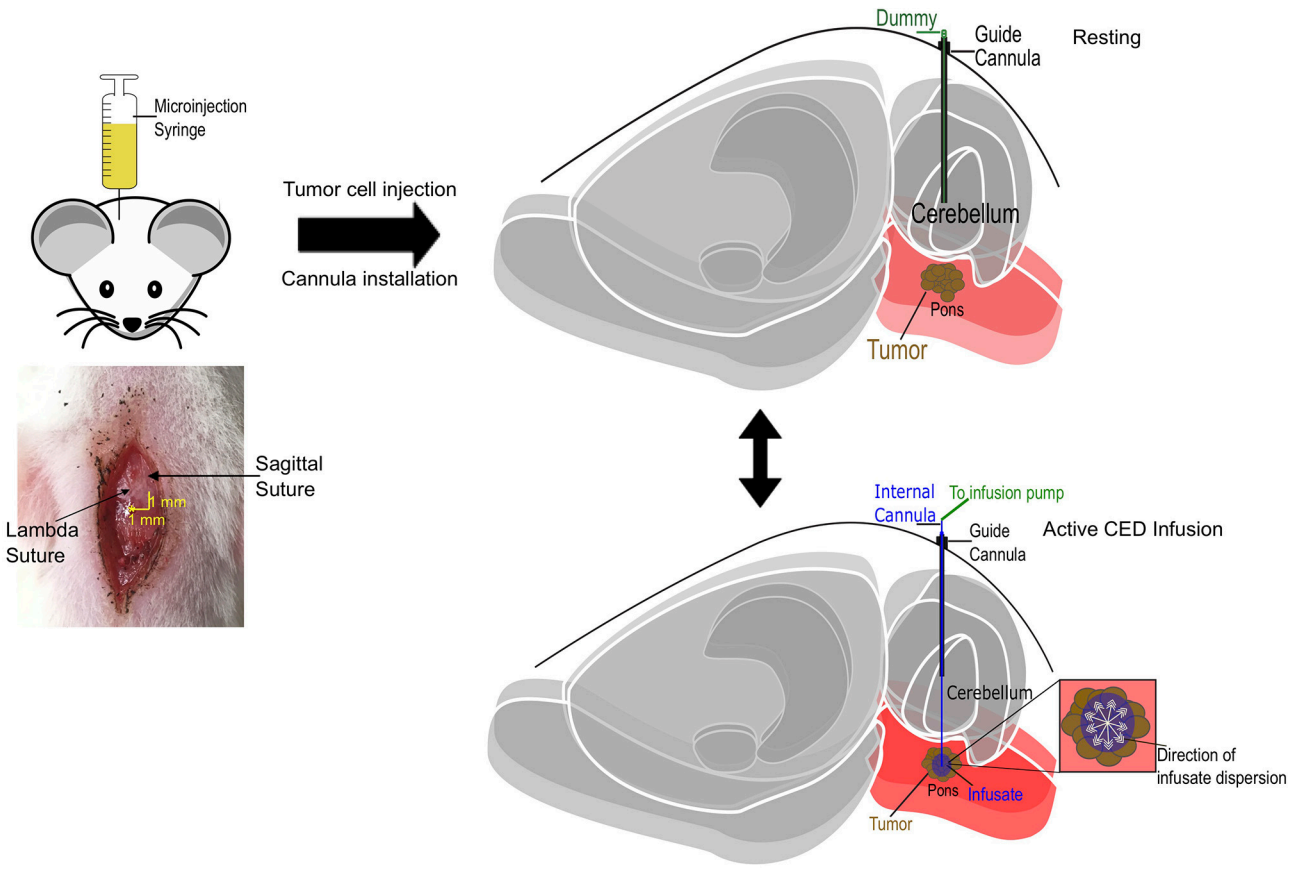

FIGURE 3 | Schematic diagram of cannula-guided convection-enhanced delivery in rat. Cannula and tumor cell implantation coordinates in relation with lambdoid (1 mm lateral) and sagittal (1 mm posterior) sutures. The guide cannula is implanted into the animal post tumor cell implantation at 6 mm below the pedestal. A dummy cannula is inserted into the guide to protect the brain when there is no infusion (resting). During drug delivery, the dummy cannula is replaced with an internal cannula that projects 8 mm into the brain and the input end is connected with a microinjection syringe infusion pump that deliver infusate at a fixed rate.

CURRENT CLINICAL TRIALS FOR DIPG USING CED

^{124}I-8H9

Recent clinical trials in CED for brain tumors have been reviewed extensively by Zhou and colleges and Healey, therefore, a select few trials will be discussed here (46, 53). NCT01502917 is an ongoing phase I dose escalation study, open since 2011, evaluating CED delivery of ^{124}I-8H9, a radionuclide-antibody complex directed against B7-H3, a surface marker expressed on the majority of DIPG tumors (46). This study applies a number of the key principles reviewed thus far, using CED of large molecules (antibodies in this case) to achieve a large volume of distribution, reporting a V_d /V_i ratio of 2.5 to 3.0. Dosimetry is effectively monitored with MRI imaging and V_d confirmed with the use of a radionuclide (46, 71). Thus far, the authors report no dose-limiting toxicities in 20 patients treated (46).

Panobinostat

Panobinostat is a general histone deacetylase (HDAC) inhibitor that has shown good *in vitro* efficacy against DIPG tumors harboring the H3K27M mutation and, interestingly, those tumors without the mutations (22, 64). Orally-administered panobinostat for treatment of DIPG has been attempted, but the drug has known limitations in penetrating the BBB (64, 83, 84). A nanoparticle formulation of the drug, MTX110, as demonstrated a favorable toxicity profile when administered to the brainstem via CED in a rodent model (85). A human Phase I trial for CED of MTX110 opened in humans in May 2018 and is currently enrolling patients with newly diagnosed DIPG with or without biopsy (NCT03566199).

Liposomal Irinotecan

Traditional chemotherapeutic agents are also being trialed for CED delivery to DIPG. Bruce and colleagues reported 2 cases of topotecan delivery via CED to the brainstem in two patients with DIPG (86). Patients underwent stereotactic biopsy of and placement of bilateral CED catheters, with one patient receiving drug treatment prior to radiation therapy and the other patient following completion of radiation. In both cases a modest reduction in tumor size was observed on MRI, and patients experienced worsening neurologic symptoms with high rates of infusion that improved with steroid used and cessation of infusion (86). In one case, infusion was resumed a lower rate following neurologic recovery and the patient tolerated this well (86). However, this study did not have an effective means to monitor the distribution of drug. Currently, a trial is enrolling using nanoliposomal irinotecan with gadolinium infusion for distribution monitoring (NCT03086616). This formulation allows for sustained release of drug over time and has shown some efficacy in rodent models when administered either via CED or intranasal (70).

Multicatheter CED Injections

In an effort to achieve a more maximal and uniform V_d across heterogeneous tumors, Steven Gill et al. have developed a multiple CED catheter system placed with robotic assistance that connect to a single implanted manifold that can be infused intermittently (57). This system has the advantage of improved V_d due to multicatheter placement and the ability to chronically administer drugs of choice, however, the placement of 4 catheters in the brainstem increases the chances for neurological symptoms. They have published several preclinical studies in both small and large animal models, and are now utilizing this system in human patients (57–59). A four-port catheter system was used to treat a patient with recurrent glioblastoma with intermittent carboplatin infusions, with a subsequent reduction of tumor volume (59). The patient in this study ultimately succumbed to her disease 8 months following catheter implantation, but this case illustrates the feasibility of this approach in delivering a therapeutic payload.

Future Directions

Future advancements in CED will come from multiple angles which include further refinements in hardware that have been discussed and an increase in our understanding of optimal drug characteristics for CED delivery which may include the development of CED specific chemotherapies. Robot assisted catheter placement for neurosurgical applications has already become common place for epilepsy procedures, and Renishaw has a robot system already on the market capable of delivering multi-brainstem CED catheters safely (87, 88). Probably more important than hardware technology is increasing our understanding of CED pharmacology. Most drugs that have been utilized for CED delivery have been selected based on anti-tumor efficacy in cell culture or animal models, without an understanding of CED pharmacology or convective kinetics. Studies that define optimal drug size, lipophilicity, status for brain efflux pumps and other important variables are required. In light of the myriad variables in delivering effective CED, developing a CED platform for use in real-world situations is an ongoing challenge and requires further studies. Next generation CED delivery for DIPG tumors will not only optimize the hardware for delivery, but the drugs being used.

CONCLUSION

DIPG remains a devastating disease for which there is no effective treatment. This is due to the nature of the tumor itself and the anatomic location in which it occurs. There is now some promise in the development of targeted therapy, as the majority of these tumors harbor the H3K27M mutation; however, drug delivery remains a large hurdle. CED is an attractive means of delivering therapeutics to DIPG tumors, as it bypasses the BBB and allows for the treatment of a relatively large volume of tissue with small amount of infusate. This presents its own challenges as drug must be specifically formulated for optimal use via CED. There are several ongoing clinical trials investigating CED in DIPG treatment in humans and will hopefully offer hope to patients and families with this devastating disease.

AUTHOR CONTRIBUTIONS

DD provided direction. BH wrote the manuscript. LZ provided the figures. DD made revisions to the manuscript. All authors read and approved the final manuscript.

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Quantitative Evaluation of Intraventricular Delivery of Therapeutic Neural Stem Cells to Orthotopic Glioma

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Neural stem cells (NSCs) are inherently tumor-tropic, which allows them to migrate through normal tissue and selectively localize to invasive tumor sites in the brain. We have engineered a clonal, immortalized allogeneic NSC line (HB1.F3.CD21; CD-NSCs) that maintains its stem-like properties, a normal karyotype and is HLA Class II negative. It is genetically and functionally stable over time and multiple passages, and has demonstrated safety in phase I glioma trials. These properties enable the production of an "off-the-shelf" therapy that can be readily available for patient treatment. There are multiple factors contributing to stem cell tumor-tropism, and much remains to be elucidated. The route of NSC delivery and the distribution of NSCs at tumor sites are key factors in the development of effective cell-based therapies. Stem cells can be engineered to deliver and/or produce many different therapeutic agents, including prodrug activating enzymes (which locally convert systemically administered prodrugs to active chemotherapeutic agents); oncolytic viruses; tumor-targeted antibodies; therapeutic nanoparticles; and extracellular vesicles that contain therapeutic oligonucleotides. By targeting these therapeutics selectively to tumor foci, we aim to minimize toxicity to normal tissues and maximize therapeutic benefits. In this manuscript, we demonstrate that NSCs administered via intracerebral/ventricular (IVEN) routes can migrate efficiently toward single or multiple tumor foci. IVEN delivery will enable repeat administrations for patients through an Ommaya reservoir, potentially resulting in improved therapeutic outcomes. In our preclinical studies using various glioma lines, we have quantified NSC migration and distribution in mouse brains and have found robust migration of our clinically relevant HB1.F3.CD21 NSC line toward invasive tumor foci, irrespective of their origin. These results establish proof-of-concept and demonstrate the potential of developing a multitude of therapeutic options using modified NSCs.

Keywords: glioma, neural stem cells, NSCs, intraventricular administration, therapeutic, drug delivery

INTRODUCTION

Despite aggressive surgery, radiation, and chemotherapy, gliomas remain virtually incurable, with median overall survival of patients with glioblastoma, the most common type of malignant glioma in adults, still measured only in terms of months (1–3). The blood-brain barrier (BBB) imposes a major limitation on the delivery of anti-cancer drugs to treat glioma. Glioma cells disseminate from the primary site to form micro-tumor foci throughout the brain, which often "hide behind" the BBB, through which most chemotherapy agents cannot pass (4). The diffuse and highly infiltrative nature of glioma cells further impedes the success of treating gliomas, as no clear border exists between tumor and normal brain tissue, rendering surgical cures elusive.

Human neural stem cell (NSC)-based therapies have emerged as promising strategies for the treatment of central nervous system (CNS) diseases and injury (5–8). Most current clinical trials aim to use NSCs for regenerative purposes: to replace damaged tissue, stimulate repair, or restore missing enzymes. Our NSC-based anti-cancer strategy, however, harnesses the intrinsic tumor-tropic properties of NSCs (9–15), which permit their use as delivery vehicles to selectively target therapeutic gene products to invasive brain tumor cells (16). By modifying NSCs to express a prodrug-converting enzyme, we can potentially produce higher concentrations of chemotherapy drugs directly at tumor sites while minimizing toxicity to normal regions of the brain (17–21).

We demonstrated the safety of a first-generation NSC-mediated gene therapy that utilized a clonal human NSC line genetically modified to express cytosine deaminase (HB1.F3.CD21; CD-NSCs) in a first-in-human study for recurrent glioma patients (19, 21). Cytosine deaminase is an enzyme that converts the orally administered prodrug fluorocytosine (5-FC) to the chemotherapy agent 5-fluorouracil (5-FU). Results from our study included initial demonstration of safety, non-immunogenicity, and proof-of-concept for brain tumor-localized NSC-mediated 5-FU production (21). We also developed a second-generation NSC-mediated enzyme/prodrug gene therapy by adenovirally transducing CD-NSCs to transiently secrete a highly active modified form of human carboxylesterase (hCE1m6) (22). Carboxylesterase (CE) converts the chemotherapy drug irinotecan (IRN) to the 1,000× more potent topoisomerase-1 inhibitor SN-38. We demonstrated that the CE-secreting NSCs (CE-NSCs) are 70-fold more efficient at converting IRN to SN-38 compared to endogenous hCE1 (<5% conversion in the liver and intestines) (23–25).

Intravenously administered IRN has only modest anti-tumor activity in patients with high-grade gliomas (26–28), likely due to poor CNS penetration of its 1,000-fold more active form, SN-38. Our preclinical data in mice bearing orthotopic human glioma demonstrated that after intracerebral/tumoral (ICT) administration, CE-NSCs migrate to distant tumor sites in the contralateral brain (4, 13). *In vivo* pharmacology studies revealed CE-NSC mediated conversion of IRN to SN-38, resulting in concentrations of SN-38 at the tumor site that are 8–10 times higher than concentrations after treatment with IRN alone (22). Treatment with CE-NSCs and IRN significantly extended the survival of human glioma-bearing mice relative to treatment with IRN alone or no treatment (17). Based on these preclinical data, a phase 1 study (clinicaltrials.gov ID **NCT02192359**) is being conducted at City of Hope in patients with recurrent high-grade gliomas using ICT administration to determine the safety and feasibility of ICT administration of CE-NSCs via a Rickham reservoir/catheter system every 2 weeks, followed by intravenous IRN 2 days later.

IVEN delivery offers five major advantages over ICT delivery: (1) the ability to dose escalate NSCs beyond volume restrictions for ICT administration; (2) improved NSC viability in cerebrospinal fluid (CSF) vs. the hostile environment of the resection cavity; (3) no intratumorally placed catheter tips around which gliosis and scar formation may occur to restrict NSC migration; (4) improved feasibility of performing multi-center studies due to general familiarity with placing Ommaya reservoirs IVEN and using them to administer chemotherapy intrathecally; and (5) potential for CE-NSC mediated gene therapy for treating leptomeningeal metastases from primary and metastatic brain tumors. In this report, we demonstrate that after **intracerebral/ventricular (IVEN)** administration, therapeutic CE-NSCs can migrate to tumors in the brains of mice in three different glioma models: (1) U251 glioma-bearing tumors, (2) patient-derived glioma xenografts (PDXs), and (3) mouse GL261 glioma model (**Figure 1**). Our data demonstrates the distribution of the CE-NSCs to multiple orthotopic glioma sites in mice following **IVEN administration** (**Figure 1**).

MATERIALS AND METHODS

Cell Culture

For all studies, we used the v-*myc*-immortalized, human clonal HB1.F3.CD21 NSC line, which is genetically and functionally stable, non-tumorigenic, and minimally immunogenic (19, 29, 30). Briefly, NSCs were thawed and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum and 2 mM L-glutamine for 3 days (37°C, 6% CO_2) in T-175 tissue culture flasks prior to adenoviral transduction, as previously described (22). NSCs were further engineered for high transient expression of a modified human CE (hCE1m6) by transduction with a replication-deficient adenoviral construct.

In vivo Animal Studies

All animal studies were conducted under a protocol approved by the City of Hope Institutional Animal Care and Use Committee (IACUC #04011). Male and female CE-deficient/severe combined immunodeficiency (*Es1*^{e}/SCID), athymic nude, or C57BL/6 mice (8–12 weeks old) were injected with 2 × 10^5/2 μl U251T.eGFP.FFluc human glioma cells (U251T; n = 6); 2 × 10^5/2 μl patient-derived PBT017.eGFP.FFluc glioma cells passaged in a mouse brain (PBT017; n = 6); or 5 × 10^3/2 μl GL261 mouse glioma cells (n = 5) into the right (U251T and GL261) or both frontal lobes (PBT017). Tumor cells were injected at three different depths 2.25, 2.00, and 1.75 mm. At day 10, post U251 tumor implantation, 2 μl of bolus injection of 4 x10^5 CE-NSC DiI labeled cells were injected into the left lateral

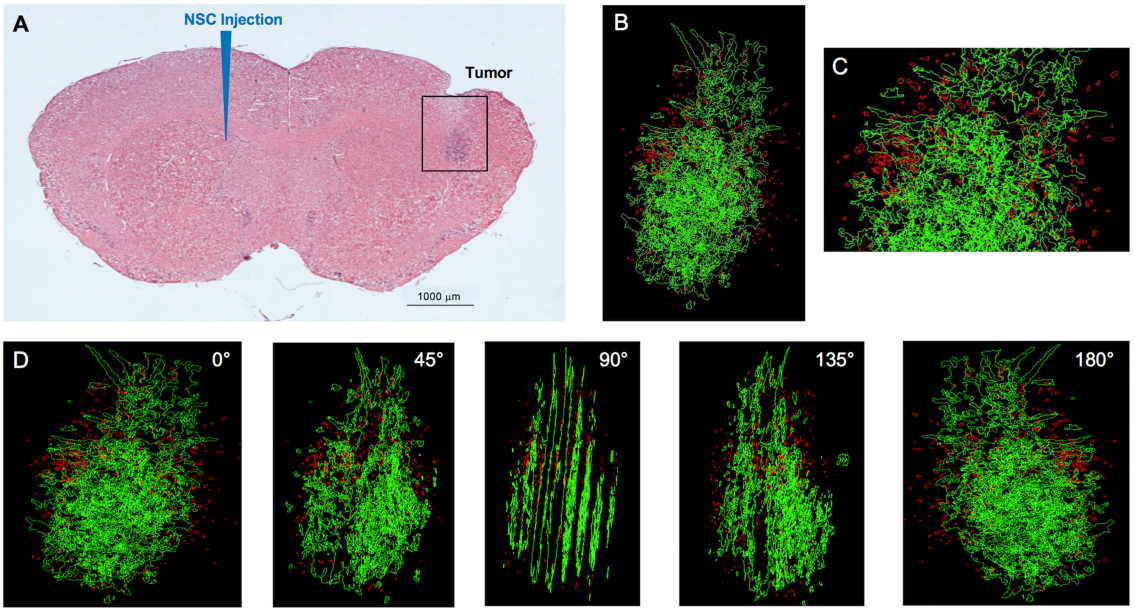

FIGURE 1 | IVEN hCE1m6-NSC distribution in U251 glioma xenografts in *Es1*^{e}/SCID mice. U251T.eGPF.FFluc tumor cells (2 × 10^5/2 μl) were injected into the right frontal lobes of *Es1*^{e}/SCID mice ($n = 4$). At day 10, DiI-labeled CE-NSCs (1.5 × 10^5/2 μl) were administered into the left ventricle. Brains were harvested 3 days after NSC administration, cryosectioned, and stained with Prussian blue to identify NSCs. **(A)** HE-stained brain tissue section (10 μm) with tumor sites on the right and IVEN NSC injection on the left. Scale bar = 1 mm. **(B)** High-power image (scale bar = 0.2 mm) and **(C)** 3D reconstruction of a U251.eGPF.FFluc tumor xenograft (green) and CE-NSCs (red, pseudo-colored) in the right frontal lobe of an *Es1*^{e}/SCID mouse. **(D)** Panels show key still images at various rotations. Scale bars have not been provided with the 3D rendered images due to the distortion associated with viewing 3D image projections at different angles when viewed as a 2D image. For reference, the width of the tumor is roughly 300 μm.

ventricle (+9.0 mm left and −0.3 caudal from bregma) at a depth 2.5 mm. PBT017 (day 14) and GL261 (day 7) tumor bearing mice were given the bolus injection (IVEN) of CE-NSC Molday ion rhodamine B labeled cell at a concentration of 4 × 10^5 per 2 μl (PBT017) and 2 × 10^5 per 2 μl (GL261) using same coordinates.

CE-NSCs (4 × 10^5 cells/2 μl) labeled with Molday ION Rhodamine B were administered into the left lateral ventricle on day 10 post U251 implantation; day 14 post PBT017 implantation; or day 7 post GL261 implantation (each tumor latency and engraftment time was previously determined: Aboody et al., unpublished data). Mice were monitored daily for distress and discomfort in accordance with the recommendations of the Panel of Euthanasia of the American Veterinary Medical Association. Euthanasia was conducted on day 3 after CE-NSCs administration in a CO_2 chamber that enabled visualization of the animals to minimize distress during euthanasia with a gradual increase in the flow of CO_2.

Histopathology and Staining

Brains were fixed in 4% paraformaldehyde (PFA) for 72 h and transferred to 70% EtOH (or 30% sucrose) solution for dehydration for 3–5 days. Frozen brain sections were prepared (10 μm) and every 10th section was stained with hematoxylin eosin (HE) to detect the tumor and Prussian blue staining using an Accustain Iron Stain Kit (Sigma-Aldrich) to identify the presence of CE-NSCs. Tumors and CE-NSCs were visualized by bright field imaging.

3D Reconstruction

Three-dimensional reconstruction was performed using Reconstruct software (SynapseWeb, version 1.1). 9–15 images of serial 10 μm H&E and Prussian blue-stained brain sections were imported into Reconstruct and aligned manually. To produce a 3D image, structures of interest were segmented based on color (Prussian blue for NSCs) and cell density (HE to highlight tumor areas), as described previously (13).

Analysis of CE-NSC Spatial Distribution

CE-NSCs in the mouse brains were identified and quantified to elucidate the patterns of spatial distribution in the brain, especially around the tumors. CE-NSCs stained in Prussian blue were identified on each of the IHC stained slices using the open-source image processing software ImageJ (31). The centers of the clusters of CE-NSCs identified using "color thresholding" and "analyze particles" tools were tabulated. A tumor mask was generated by manually delineating the edges of the tumor using the "polygon selection" tool. The center of this mask was identified as the tumor center of mass and the distance and orientation of the CE-NSC clusters with respect to the tumor center was calculated. The results for all the IHC slices were combined to generate a polar histogram in MATLAB 2018a (Mathworks, Natick, MA) representing the spatial distribution of CE-NSCs with respect to the tumor center for each mouse brain. The size of the bars in the plots indicates the percentage of NSCs found along that radial direction, and the color of

the bars indicates the distance from the tumor center. Because mice implanted with the PBT017 cell line were injected with dual tumors, CE-NSCs identified in the left hemisphere were associated with the tumor in the left hemisphere and CE-NSCs identified in the right hemisphere were associated with the tumor in the right hemisphere. Thus, 2 polar histograms were generated for each mouse.

RESULTS

Migration and Localization of IVEN-Administered CE-NSCs to Brain Tumor Sites *in vivo*

To initiate a xenograft model of glioma, adult *Es1*^{e}/SCID immunodeficient mice were implanted with U251T.eGFP.FFluc cells into the right frontal lobe. On day 10, Molday-labeled CE-NSCs were administered into the left lateral ventricle. When injected IVEN into U251T glioma-bearing mice, CE-NSCs migrated to tumor xenografts established in the opposite hemisphere (**Figures 1**, **2**). These CE-NSCs were visualized in the vicinity of the tumor and were not detected in non-tumor brain parenchyma.

The distribution of Molday-labeled CE-NSCs to U251T tumors was also quantitated in athymic nude mice. CE-NSCs were visualized by Prussian blue staining (**Figures 2A–D**) and migration was quantitated by polar histogram (**Figure 2E**). The polar histogram shows the quantified spatial distribution of CE-NSCs around the tumor, including both the number of CE-NSCs in various directions and their distance from the tumor center. CE-NSCs demonstrated preferential localization around the tumor, with the CE-NSCs closest to the tumor along the superior-medial and inferior sides. CE-NSCs along the medial direction and far from the tumor represent those in the contralateral ventricle.

Migration and Localization of CE-NSCs to Bilateral PDX Tumor Sites Following Injection Into the Left Ventricle

To analyze migration of CE-NSCs to multiple tumor foci within brain parenchyma after IVEN administration, dual tumors were initiated in *Es1*^{e}/SCID mice via bilateral administration of human patient-derived glioma cells PBT017. Fourteen days later, CE-NSCs were administered into the left ventricle, after which the CE-NSCs migrated to both left and right tumor sites (**Figure 3**). Migration of the CE-NSCs was analyzed by 3D reconstruction (**Figure 3**) and polar histogram analysis (**Figure 4**). Since a tumor was inoculated in each hemisphere, a polar histogram was generated for each tumor with CE-NSCs identified in any given hemisphere attributed to the tumor present in that hemisphere. The histological sections show substantial CE-NSC presence around the tumors (**Figures 4A,D**). This observation is reflected in the polar histograms (**Figures 4B,C,E,F**) with nearly all the bars in blue indicating CE-NSCs in the proximity of the tumor. Strong signals indicate preferential localization of CE-NSCs along the superior-medial and inferior directions, indicating possible migration of CE-NSCs into the tumor along these directions. Notably, one mouse (**Figure 4A**) was observed to contain equivalent distributions of CE-NSCs in both hemispheres, whereas another mouse (**Figure 4D**) was observed to contain a significantly higher distribution of CE-NSCs in the tumor in the left (ipsilateral to CE-NSC injection site) hemisphere.

Migration and Localization of CE-NSCs to GI261 Murine Glioma Tumor in C57BL/6 Mice

C57BL/6 mice were injected with GL261 cells (5×10^3 cells/2 μl) into the right frontal lobe, as described above. On day 7 of the study, CE-NSCs were injected into left lateral ventricle. CE-NSCs demonstrated robust migration from the ventricles into the tumors. The histological section presented in **Figure 5B** exhibits what appears to be active migration of CE-NSCs from the ventricles and partially from the subarachnoid space/tumor administration needle track. The invasion of the tumor by CE-NSCs from the superior end is also reflected by the blue bar in the polar histogram (**Figure 5C**) toward the superior direction. CE-NSCs along the medial direction and far from the tumor represent those in the contralateral ventricle. Aggregation of CE-NSCs in the contralateral ventricle was observed.

DISCUSSION

Innately tumor-tropic NSCs are able to penetrate the BBB and migrate through brain parenchyma to efficiently localize to both the primary brain tumor site and invasive foci that often seed recurrent disease. These features provide an unprecedented opportunity to develop an effective, tumor-selective therapy for patients with malignant brain tumors. NSCs can produce increased concentrations of tumor-localized chemotherapy while minimizing toxicity to normal brain tissue. We have demonstrated the efficacy of ICT-administered CE-NSCs + IRN in preclinical studies; however, there are multiple drawbacks to ICT administration of NSCs to brain tumor patients, as described above. Our data demonstrate that IVEN administration of NSCs results in similar distribution and tumor coverage in orthotopic tumors that are both proximal and contralateral to the site of injection. By administering NSCs IVEN rather than ICT, we can optimize therapeutic dosing and potentially increase distribution to tumor sites throughout the brain. Specifically, the major advantages include: (1) the ability to dose escalate NSCs beyond volume restrictions for ICT administration; (2) improved NSC viability in CSF; (3) no intratumorally placed catheter tips; (4) improved feasibility of performing multi-center studies; and (5) potential for CE-NSC-mediated gene therapy for treating leptomeningeal metastases from primary and metastatic brain tumors. Beyond the current NSC-based enzyme/prodrug converting strategies to increase levels of cytotoxic chemotherapy in brain tumors, we envision using our tumor-tropic NSCs as a platform technology can be further modified for tumor-localized delivery of a variety of anti-tumor products, such as apoptotic agents, oncolytic viruses, and antibodies, which could potentially be administered serially or in

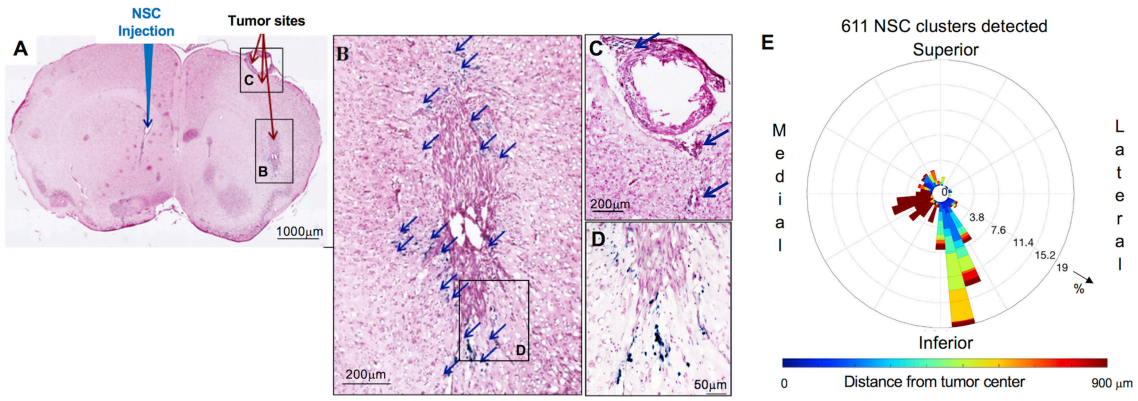

FIGURE 2 | Distribution and spatial analysis of IVEN hCE1m6-NSC migration to U251 glioma xenografts in athymic nude mice. U251T.eGPF.FFluc tumor cells (2×10^5/2 μl) were injected into the right frontal lobe of athymic nude mice ($n = 4$). At day 10, Molday-labeled CE-NSCs (4.0×10^5/2 μl) were injected into the left ventricle. Brains were harvested 3 days after NSC.CD administration, cryosectioned, and stained with Prussian blue to identify NSCs. **(A)** HE-stained brain tissue section (10 μm) with tumor sites on the right and IVEN NSC injection on the left (scale bar is 1 mm). **(B,C,D)** Insets from **(A)**: magnified images of Prussian blue-stained NSCs, indicated with blue arrows (scale bars 200, 200, 50 μm, respectively). **(E)** Polar histogram of CE-NSC distribution demonstrates the spatial distribution of NSC clusters around the tumor center. CE-NSCs close to the tumor (blue bars) were found along the superior-medial, inferior, and inferior-lateral directions of the tumor. NSCs found far away from the tumor (red bars) were found in the contralateral ventricle.

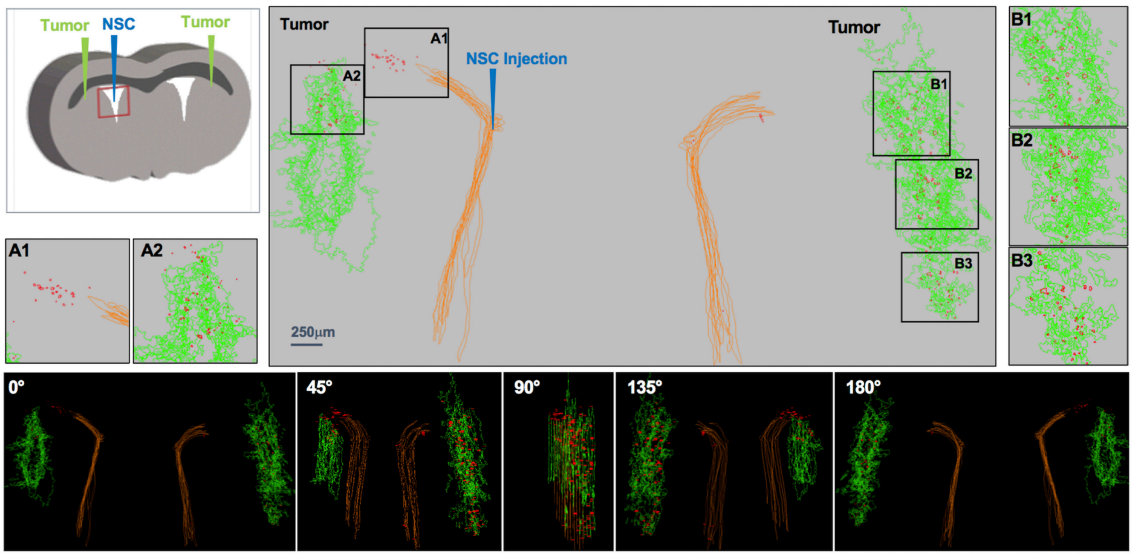

FIGURE 3 | IVEN hCE1m6-NSC distribution in PDX glioma tumors in *Es1*^{e}/SCID mice. PBT017.eGFP.FFluc cells (2×10^5/2 μl) were injected into the right and left frontal lobes of *Es1*^{e}/SCID mice ($n = 6$). At day 14, Molday-labeled CE-NSCs (4.0×10^5/2 μl) were administered into the left lateral ventricle. Brains were harvested and histological sections (10 μm) prepared on day 17. Every 10th section was stained with HE to visualize the tumors and Prussian blue to visualize the NSCs. 3D reconstructions of the right and left PBT017 tumors (green) with right and left lateral ventricles (brown) are shown. Insets A1 and A2 demonstrate CE-NSCs (red) migrating toward left tumor; B1–B3: NSCs migrating to right tumor foci. Also shown are the tumor and the NSCs visualized from different viewing angles.

combination to maximize therapeutic benefit (32). Therefore, the impact of optimizing the delivery of NSCs may be far-reaching.

It should be noted that several challenges remain for therapeutic optimization. This includes determination of dosing and regimen that results in maximal tumor coverage, and more uniform distribution through each tumor mass. Multiple and complex factors can affect NSC tumor tropism including tumor-derived growth factors hepatoxcyte growth factor (HGF), endothelial growth factor (EGF), vascular endothelial growth factor (VEGF), urokinase plasminogen activator (uPA), extracellular matrices (ECM), stromal cell-derived factor 1 (SDF-1), hypoxia inducible factor (HIF-1α, and inflammatory cytokines (e.g., IL-6 and IL-8) (15). Thus, tumor size, location, and heterogeneity likely contribute to the non-uniformity of NSC distribution within a given tumor mass. Clinical correlative studies that include more refined and sophisticated

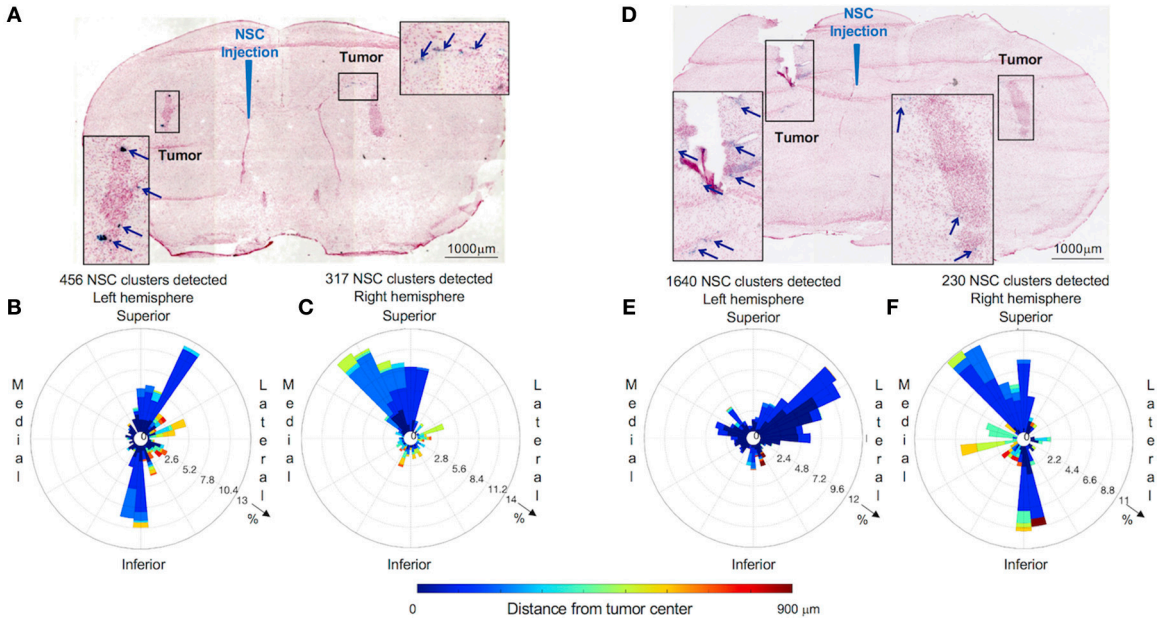

FIGURE 4 | Distribution and spatial analysis of IVEN hCE1m6-NSC migration to bilateral PDX glioma tumors in *Es1*^{e}/SCID mice. PBT017.eGFP.FFluc cells (2 × 10^{5}/2 μl) were injected into the right and left frontal lobes of *Es1*^{e}/SCID mice ($n = 6$). At day 14, Molday-labeled CE-NSCs (4.0 × 10^{5}/2 μl) were administered into the left lateral ventricle. Brains were harvested, cryosectioned, and stained with Prussian blue to identify NSCs. **(A)** Histological section (10 μm) stained with Prussian blue to identify CE-NSCs (scale bars 1,000 μm). Insets show the localization of CE-NSCs near tumors in the left and right hemispheres of the mouse brain. Polar histograms of CE-NSCs identified in the **(B)** left and **(C)** right hemispheres are shown. **(D,E,F)** Same as **(A,B,C)** for a different mouse. Polar histograms show the majority of identified CE-NSCs within 200 μm of the tumor, demonstrating the capability of the cells to migrate to the tumor. Additionally, these data show preferential accumulation of CE-NSCs along the superior-medial and inferior directions of the tumors. Notably, the number of identified CE-NSC clusters were similar in the left and right hemispheres of the first mouse. In the second mouse, CE-NSCs were preferentially found in the left hemisphere.

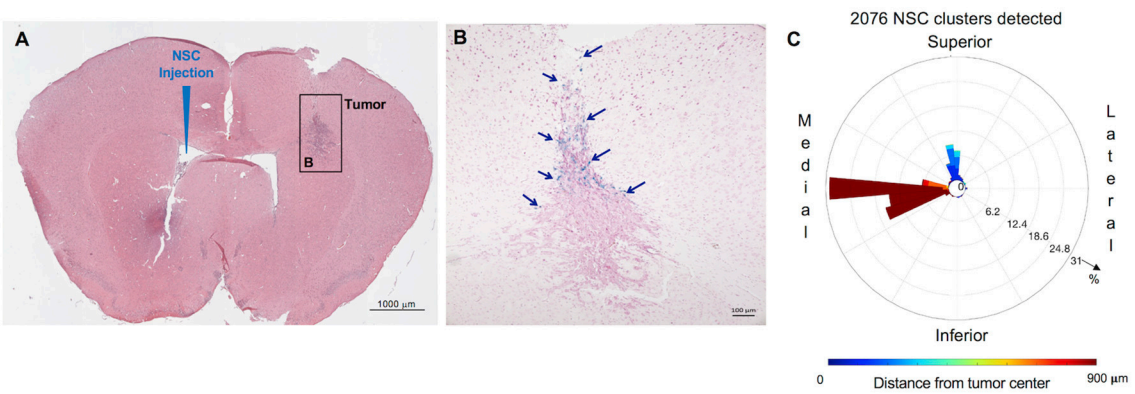

FIGURE 5 | Distribution and spatial analysis of IVEN hCE1m6-NSC migration to GL261 murine glioma tumors in C57BL/6 mice. GL261 tumor cells (5 × 10^{3}/2 μl) were in injected into the right frontal lobe of C57BL/6 mice ($n = 5$). At day 7, Molday-labeled CE-NSCs (2.0 × 10^{5}/2 μl) were injected into the left ventricle. Brains were harvested 2 days after CE-NSC administration, cryosectioned, and stained with Prussian blue to identify NSCs. **(A,B)** HE-stained brain tissue section (10 μm) with tumor sites on the right and IVEN NSC injection on the left (scale bars 1,000 and 100 μm, respectively). **(C)** Polar histogram of NSC distribution around the tumor. NSCs can be observed to invade the tumor from the superior direction (blue bars), consistent with NSCs visualized in the tumor. Distant clumping of the CE-NSCs in the contralateral ventricle can also be observed (red bars seen medially).

imaging analysis, in addition to intracerebral microdialysis and histopathology, may shed more light on this subject.

In our first-in-human study, we documented NSC migration to distant tumor foci in the human brain at the time of autopsy. Permission for brain autopsy was obtained from the families of two study participants. The autopsied brains were extensively sampled, including areas adjacent to and distant from the CD-NSC injection site and ipsilateral and contralateral areas of obvious tumor involvement, as well as deep nuclei, periventricular areas, long axonal tracts, cortical

gray matter, and subcortical white matter. All of the samples areas were assessed for the presence of CD-NSCs by nested PCR for the v-*myc* gene. In both brains, v-*myc*-positive areas of single cells were detected distant from the primary injection sites (including the opposite hemisphere) in areas of tumor cells.

We observed aggregation of CE-NSCs within the left ventricle (injection site) of a mouse bearing GL261 murine glioma cells (**Figure 5**). Although the migration of CE-NSCs to the tumor site was evident, such aggregation of CE-NSCs at the injection site might result in a loss of therapeutic efficiency. We suspect that the aggregation was caused by rapid injection of the CE-NSCs. Thus, the rate and number of NSCs injected must be adjusted and properly monitored to achieve optimal therapeutic efficiency. However, human ventricles are much larger than mouse ventricles; therefore, we do not expect such clumping when CE-NSCs are used as therapeutics clinically.

We observed that CE-NSCs localized along the superior-medial and inferior directions around the tumors established from U251 and PBT017 cell lines. The superior-medial localization of CE-NSCs can be attributed to invasion of CE-NSCs from the ventricle into the tumor. In mice bearing GL261 tumors, CE-NSCs localized along the superior direction around the tumors, indicating invasion from the subarachnoid space along the tumor cell injection track. We previously demonstrated migration of NSCs along white matter tracts (33). However, it was also documented that the NSCs migrated with CSF flow through the third and fourth ventricles and subarachnoid space and entered the tumor site through the tumor injection needle track, consistent with our observations. The utilization of such diverse migration routes to tumor sites strongly supports the use of IVEN-delivered CE-NSCs as delivery vehicles for a variety of anti-cancer therapeutics. A potential limitation is the immune mediated impact of delivery of NSCs via IVEN, this will need to be carefully assessed prior to translation to the clinic.

DATA AVAILABILITY

All datasets for this study are included in the manuscript files. The raw data supporting the conclusions of this manuscript will be made available by the authors to any qualified researcher.

AUTHOR CONTRIBUTIONS

LF, LT, RT, SA, MM, JG, and AA performed experiments. VA performed quantitative analysis and produced figures. RR and VA designed the quantitative analyses. AA, TS, and JP were involved in discussion and data analysis. MG, KA, and RR led the project, contributed to experimental design, analyses design, review and discussion. All authors reviewed the final manuscript.

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REVIEW
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Harnessing Radiation Biology to Augment Immunotherapy for Glioblastoma

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Glioblastoma is the most common adult primary brain tumor and carries a dismal prognosis. Radiation is a standard first-line therapy, typically deployed following maximal safe surgical debulking, when possible, in combination with cytotoxic chemotherapy. For other systemic cancers, standard of care is being transformed by immunotherapies, including checkpoint-blocking antibodies targeting CTLA-4 and PD-1/PD-L1, with potential for long-term remission. Ongoing studies are evaluating the role of immunotherapies for GBM. Despite dramatic responses in some cases, randomized trials to date have not met primary outcomes. Challenges have been attributed in part to the immunologically "cold" nature of glioblastoma relative to other malignancies successfully treated with immunotherapy. Radiation may serve as a mechanism to improve tumor immunogenicity. In this review, we critically evaluate current evidence regarding radiation as a synergistic facilitator of immunotherapies through modulation of both the innate and adaptive immune milieu. Although current preclinical data encourage efforts to harness synergistic biology between radiation and immunotherapy, several practical and scientific challenges remain. Moreover, insights from radiation biology may unveil additional novel opportunities to help mobilize immunity against GBM.

Keywords: radiation, glioblastoma, GBM, PD-1/PD-L1, CTLA-4, immunotherapies, innate and adaptive immune responses

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INTRODUCTION

Glioblastoma (GBM) is a deadly and highly infiltrative tumor. It is the most common primary brain tumor in adults, causing about 3–4% of all cancer-related deaths (1). Surgery followed by fractionated radiotherapy (RT) and temozolomide (TMZ) has been standard of care for newly diagnosed GBM since 2005 (2). To date, scientific advances in genomics and immunotherapy have failed to translate into effective therapies for GBM, with median survival of just over a year from diagnosis. Once recurrence has occurred, prognosis is extremely guarded with a minority of patients responding meaningfully to second-line therapies or surviving >6 months from time of recurrence (3). Novel approaches to treat GBM are urgently needed and much effort has sought to determine whether immunotherapy may provide a useful adjunct.

Immunotherapies, epitomized by successful trials with checkpoint blockade, have been widely hailed as a breakthrough in cancer therapy over the past decade. Seminal work from the Allison laboratory in 1996 showed that the antibody-blocking cytotoxic T-lymphocyte

antigen-4 (CTLA-4) could elicit regression of murine colon carcinoma and fibrosarcoma (4). Since then, several other preclinical models have further validated the effectiveness of blocking CTLA-4 and supported the clinical development of anti-CTLA-4 therapy. The first human phase III study of anti-human CTLA-4 (Ipilimumab) demonstrated improved survival in patients with advanced melanoma (5). Subsequent successes followed with antibodies against programmed cell death-1 (PD-1) and programmed death ligand-1 (PD-L1) (6, 7), confirming the broad utility of blocking inhibitory pathways that interfere with anti-tumor T cell responses.

There is a strong correlation between high somatic mutation burden and the clinical response to immune checkpoint monotherapies (8). Non-synonymous somatic mutations lead to an altered amino acid sequence and give rise to neoepitopes that can serve as neoantigens recognized by the immune system (9, 10), triggering an anticancer immune response. In contrast, GBM has a relatively low burden of neoantigens (11), yielding "cold tumors" for which clinical response immune checkpoint monotherapy is infrequently observed. The "cold" phenotype of GBM is also attributed to recruitment of immunosuppressive immune cell types and secretion of immune suppressive cytokines (12–14). Much work has sought to convert the "cold" GBM phenotype into a "hot" phenotype more responsive to immune checkpoint blockade. To this end, radiation and radiation-induced immune processes have demonstrated particular promise.

Immune infiltration is a doubled sword. Despite the benefit of immune infiltrate for a successful immune therapy response, more aggressive tumors, such as mesenchymal subtype GBM, are typically heavily infiltrated by immune cells (15). In this setting, immune cells are believed to be reprogrammed by the tumor to perform pro-tumorigenic functions. However, whether the presence of robust immune infiltrate is a cause or effect of GBM aggressiveness has been controversial. Mutations in the gene isocitrate dehydrogenase (IDH) are very common in World Health Organization classification of Grade II and III gliomas and in 10% of GBM that have evolved from lower-grade tumors (16, 17). Overproduction of oncometabolite 2-hydroxyglutarate (2HD) in the D-enantiomer is a major hallmark of these glioma subtypes (18). IDH mutation status is an important classifier in stratifying glial tumors. Patients with IDH-mutant gliomas have a substantial survival benefit following chemotherapy and radiation compared to patients with IDH wild type tumors (19). A study by Amankulor et al. used this model to help shed light on the role of immune cells in tumor aggressiveness (20). It is known that IDH-mutant gliomas have fewer tumor-infiltrating immune cells, including T cells, microglia, and macrophages, compared to IDH wild-type tumors; thus IDH-mutant tumors typically exemplify "cold tumors" and may not respond to immunotherapies. The authors generated genetically engineered mice that were identical, except for the presence or absence of IDH mutation, with concomitant increase in 2-HG levels. Decreased leukocyte chemotaxis and prolonged survival was seen in the IDH-mutant tumors supporting the concept of immune infiltration as causatively pathologic in more aggressive gliomas. Whether IDH-mutant gliomas or tumors with inherently lower immune infiltration (e.g., proneural) are inherently less responsive to immunotherapy due to their "cold" phenotype is hypothesized, but remains to be demonstrated clinically. Nevertheless, since radiation is currently standard of care for all subtypes of infiltrative glioma, potential synergy between immunotherapy and radiation is an opportunity to be exploited therapeutically. In such work, the goal will be to promote and maintain an anti-tumorigenic rather than pro-tumorigenic phenotype of recruited leukocytes, even long after completion of radiotherapy.

Preclinical data have provided robust proof of principle that radiation can boost both the local and systemic antitumor immune response to augment tumor control even at sites distant from radiation—eliciting the so-called "abscopal effect." Although radiation and immunotherapy are both currently employed in early clinical trials of immunotherapy, it is less certain whether their potentially synergistic biology is optimally harnessed using current protocols. Emerging preclinical data suggest that established standards of care for GBM—including radiotherapy fractionation regimens, use of systemically immunosuppressing TMZ, and frequent use of steroids—may need to be revisited before the potential of immunotherapy is fully realized for GBM. This review begins by addressing the current understanding of immune-modulatory effects of radiation and highlights the salient features of the highly immunosuppressive microenvironment of GBM. We then discuss preclinical data supporting the synergistic combination of radiotherapy with immunotherapies targeting both innate and adaptive immune modulators and explore important challenges yet to be overcome in search of a clinically optimal regimen.

GBM AND THE ADAPTIVE IMMUNE SYSTEM

Brain: No Longer an Immune-Privileged Organ

The central nervous system (CNS) has long been considered immune privileged due in part to the presence of the blood brain barrier, a unique structural feature that restricts entry of molecules and immune cells into the brain. This view was further supported by relatively low numbers of antigen presenting cells (APCs) and T cells in the brain parenchyma, as well as the historically perceived lack of lymphatic vessels to drain APC and antigen to regional lymph nodes (21). Findings in recent years have challenged long-standing thinking by demonstrating that even the healthy brain is in fact under constant immune surveillance. Brain-derived antigens can entrain peripherally-derived immune cells that in turn penetrate the blood brain barrier (22, 23). Identification of a novel CNS glymphatic system, wherein most APCs could travel from the brain into the cervical lymph nodes and prime T lymphocytes (24, 25), forced reconsideration of the supposedly immune privileged status of the CNS. The revised model is in line with empiric findings of tumor-infiltrating lymphocytes detected in human GBM after vaccination with autologous tumor lysate-pulsed dendritic cells (DCs) (26, 27). It is within this dynamic scientific era that insights

are sought from the brain and tumor microenvironments to optimally harness immunotherapy for GBM.

Immune-Suppressive Microenvironment of GBM

Tumors subvert systemic and local immune mechanisms to establish an immune tolerant microenvironment permissive to infiltration and proliferation. The following sections outline several of the immunosuppressive mechanisms defined to date; the extent to which radiation may help attenuate the immunosuppressive microenvironment of GBM is discussed in section *Radiation and GBM*.

Like many tumors, GBM express relatively low levels of histocompatibility complex (MHC) class I and II molecules, thereby minimizing display of tumor-associated antigens (28). GBM also secrete immunosuppressive cytokines, such as IL-10 and TGF-β (29). TGF-β is a pleiotropic cytokine that blocks the cytotoxic T cell response and promotes the activity of CD4+ regulatory T cells (Tregs).

Tregs express CD25+ and the transcription factor FoxP3+ (30) and may derive from the periphery (pTregs) from conventional T cells or from the thymus (tTregs) (31). Tregs can be recruited to the tumor or generated via proliferation of pre-existing Tregs in the tumor microenvironment and *de novo* conversion of tumor-infiltrating CD4+ lymphocytes (TIL) into pTregs (32, 33). Tregs exert their suppressive activity through cell surface molecules such as CTLA-4, perforin, and CD73. These inhibit maturation of APCs and block B7-CD28 co-stimulatory signals. ATP released from dying cells is pro-immunogenic, but is degraded by Tregs. In addition, Tregs can also mediate their suppressive activity via contact-independent mechanisms, secreting inhibitory cytokines that suppress effector T cell function (34).

The enzyme indoleamine 2,3 dioxygenase (IDO) can be produced by both tumor and tumor APCs, including DCs and macrophages (35), to induce immune suppression. IDO contributes to immune tolerance by catabolizing tryptophan to catabolites, such as kynurenine (36). Deprivation of the critical amino acid tryptophan and exposure to metabolites inhibits the proliferation of cytotoxic CD4+ and CD8+ T cells (37), as well as natural killer (NK) cells (38). Preclinical work by Wainwright et al. has demonstrated that GBM tumor-derived IDO increased the recruitment of Tregs and decreased survival of mice with intra-cranial tumors (39). Of note, IDO expression levels tends to positively correlate with glioma grade (40).

Although GBM is confined to the brain, patients with GBM may be profoundly immunosuppressed systemically with decreased numbers (41) and function (42) of circulating lymphocytes. GBM accumulate robust numbers of intra-tumoral activated Tregs that impede the proliferation of, and cytokine secretion by, autologous lymphocytes (43, 44). Furthermore, depletion of Tregs using anti-CD25 antibodies augmented anti-tumor CD4+ and CD8+ T cell responses (45, 46). These studies emphasize the role of GBM-associated Tregs in maintaining a systemic tolerogenic environment that impedes anti-tumor immunity.

T Cell Exhaustion in GBM

Viruses have evolved highly effective strategies for establishing chronic infection and avoiding clearance by the immune response (47, 48). During chronic viral infections, persistent antigen exposure drives CD8+ T cells to increase the expression of inhibitory receptors, dampening their ability to clear the infection (49). This state of decreased proliferation and decreased effector function, including reduced cytokine secretion accompanied by metabolic and transcriptional changes, has been termed "exhaustion" and is also induced by cancers to avoid immune clearance (50, 51). Targeting such T cell exhaustion may be more complex in cancer due to intra-tumoral heterogeneity, resulting from stochastic tumor evolution and spatial gradients within the tumor microenvironment (51). The exhausted T cell phenotype is characterized by upregulation of multiple inhibitory immune checkpoint receptors, such as PD-1 (52), CTLA-4 (4), T cell immunoglobulin 3 (TIM-3) (53), lymphocyte-activation gene 3 (LAG-3), T cell immunoreceptor with immunoglobulin and ITIM domains (TIGIT), V-domain Ig Suppressor of T cell Activation (VISTA), and CD39 (54–56). These molecules are prominently expressed on CD8+ TILs from human GBM (57) with stably elevated checkpoint expression restricted TCR repertoire clonality throughout the stages of GBM progression (58). Under normal homeostasis, these molecules play critical immune regulatory roles in mediating tolerance to self-antigens and preventing auto-immunity (59, 60). While it has been known that multiple tumors induce T cell exhaustion to promote survival (61), the degree of T cell exhaustion in patients with GBM was recently determined to be particularly severe (57). To date, the predominant strategy investigated to attenuate T cell exhaustion has included one or more immune checkpoint inhibitors (62). However, modulating metabolic and stromal components in the tumor microenvironment may prove synergistic (51). The potential role of radiation to facilitate such modulation is discussed below.

Role of Immune Checkpoints in GBM

Numerous preclinical studies have demonstrated efficacy of antibodies targeting CTLA-4 or the PD-1/PD-L1 axis (4, 63, 64). Subsequently, these antibodies have also demonstrated clinical benefit in multiple tumor types, particularly including "hot" tumors with innately high immunogenicity. Monotherapy with ipilimumab, an anti-CTLA-4 antibody, yielded a durable response in ∼10% of patients with advanced metastatic melanoma (5). Additionally, lambrolizumab (anti-PD-1) yielded a robust and durable response in about 35% of patients with advanced melanoma (65). Based on numerous such encouraging trials, several immune checkpoint inhibitors have now been FDA approved for multiple cancers. Examples include inhibitors targeting CTLA-4 (ipilimumab), PD-1 (pembrolizumab and nivolumab), and PD-L1 (atezolizumab and avelumab), that have collectively yielded profound impacts on the management of multiple systemic malignancies.

The dysregulation of immune-checkpoint pathways in GBM has provided ample proof of principle suggesting checkpoint inhibitors could also offer a therapeutic avenue for GBM (66). Indeed, in addition to upregulation of inhibitory checkpoint

molecules, such as PD-L1 on T-regs and exhausted T-cells, these are also expressed on tumor-associated macrophages and microglia (TAMs) isolated from human GBM (67). Moreover, immunosuppressive cytokines in the GBM microenvironment, including IL-10, promote expression of checkpoint inhibitor expression on GBM itself (67). Despite promising responses in a subset of patients (68), benefits of checkpoint inhibition have yet to be observed in any phase III clinical trial for GBM.

Nevertheless, immune checkpoint dysregulation alone in GBM may be insufficient to portend reliable responses via checkpoint blockade. Increasing data suggest that an elevated tumor mutational burden (69, 70) and a robust lymphocytic infiltrate within the tumor microenvironment ("hot tumors") correlate with improved clinical response to checkpoint blockade (69, 71, 72). Indeed, consistent with the relatively immunologically "cold" nature of GBM, including modest levels of tumor neoantigens and lymphocytic infiltrate, several late stage clinical trials have failed to demonstrate clinical benefit (see **Supplementary Table 1**). Nevertheless, promising responses in a subset of patients continue to foster enthusiasm for harnessing checkpoint inhibitors in GBM. The portfolio of checkpoint inhibitors is continuing to expand with preclinical and efficacy data in targeting LAG-3 (73), TIM-3 (74), and TIGIT (75), each showing particular promise in combination with PD-1 inhibition. Moreover, harnessing the use of immunostimulatory strategies, such as radiation, to augment checkpoint responses has generated particularly promising preclinical data (76). The following sections offer additional details regarding the more thoroughly studied checkpoint molecules CTLA-4 and PD1/PDL1 that have provided a foundation for GBM immunotherapy efforts to date.

Cytotoxic T-Lymphocyte Antigen-4 (CTLA-4)

T cells are typically activated when an MHC-bearing APC presents an antigenic peptide and engages a T cell receptor (TCR). Full activation of T cells requires engagement of the co-stimulatory T cell receptor, CD28, with its ligands, CD80 and CD86, expressed on APC (77). CTLA-4 primarily regulates the early stages of T cell activation. CTLA-4 begins as an intracellular protein, but upon T cell activation translocates to the immunological synapse and co-localizes with TCRs (78, 79). CTLA-4 outcompetes the co-stimulatory TCR CD28 by binding with higher affinity to the ligands CD80 and CD86 expressed on APCs (80). CTLA-4 can also limit conjugation times between T cells and APCs, limit T cell proliferation, and reduce cytokine production (81). CTLA-4 inhibits Akt phosphorylation by activating protein serine/threonine phosphatase PP2A, but does not alter phosphatidylinositol3-kinase (PI3K) activity (62, 82). The intracellular domain of CTLA-4 can recruit the protein phosphatase 2 A to decrease phosphorylation of proteins in the TCR signaling cascade (83). CTLA-4 plays a key role in maintaining immune-regulated homeostasis by enhancing suppressive functions of Tregs (84) and impeding the function of CD4+ helper T cells (85). Anti-CTLA-4 antibodies can mitigate T cell exhaustion by attenuating the inhibitory functions of CTLA-4 and suppressive actions of Tregs. Ipilimumab and tremelimumab were the first anti-CTLA-4 antibodies to enter clinical trials in patients with advanced cancer. Ipilimumab is currently FDA approved for metastatic melanoma and renal cell carcinoma.

Programmed Cell Death-1 (PD-1) and Programmed Death Ligand-1 (PD-L1)

In contrast to CTLA-4, which largely regulates T cell activation, PD-1 plays a prominent role in inhibiting proliferation and functions of effector T cell responses. PD-1 is absent on resting naïve and memory T cells, but expressed on tumor infiltrating lymphocytes (TILs) (86). PD-1 is upregulated on activated T cells upon TCR engagement and mediates T cell suppression (87) upon binding PD-L1 (52) or PD-L2 (88). PD-L1, also known as CD274 and B7-H1, is largely undetectable in most normal tissues, but is expressed on macrophages and APCs, particularly in the context of classical (M1) activation (89). PD-L1 is elevated in tumors—not only on APCs, but also tumor cells themselves, promoting tumor cell survival (90, 91). PD-L2 expression is limited to certain immune cell types, mostly DCs, mast cells, and macrophages (87). Both PD-1 and PD-L1 are expressed on Tregs (92). Binding of PD-1 on activated T cells to PD-L1 decreases TCR-mediated signaling by antagonizing PI3K, leading to decreased Akt phosphorylation and thus decreased levels of activation, including decreased IL-2 production and decreased T cell proliferation (62). Engagement of PD-L1 on macrophages to PD-1 promotes IL-10 production, which further promotes immune suppression (93). Currently FDA-approved drugs targeting PD1/PD-L1 for other cancers include the anti-PD1 drug Nivolumab and the anti-PD-L1 drugs pembrolizumab, atezolizumab, and avelumab. No immunotherapeutic drug has been approved to date for glioma.

TIM-3 and Other Candidates for Adaptive Immune Regulation

As exemplified by exhausted T cells, several additional checkpoint molecules exist besides CTLA-4 and PD-1/PD-L1 that regulate T cell activation and are being assessed as targets for immunotherapy (94). Among these, TIM-3 is expressed by IFNγ-secreting T-helper 1 (Th1) cells, DCs, monocytes, CD8+ T cells, and other lymphocyte subsets (95, 96). TIM-3 is expressed on dysfunctional CD8+ T cells in preclinical models of both solid and hematological malignancies (74, 97). Upregulation of TIM-3 is associated with exhaustion of tumor antigen-specific CD8+ T cells in human melanoma and tumor-induced T cell exhaustion is reversed by administration of anti-TIM-3 antibodies (98, 99). TIM-3 is also expressed on Tregs, with TIM-3+ Tregs identified in solid tumors, such as ovarian, colon, and hepatocellular carcinomas (100). As with other checkpoint molecules, including LAG-3 (73) and TIGIT (75), combination therapies blocking TIM-3 in combination with PD-1 exhibited synergistic effects in preclinical tumor models (74, 101). Kim et al. demonstrated that combination therapy of anti-TIM-3 and anti-PD-1 improved survival in mice with GL261 intra-cranial tumors with optimal outcomes observed using both

in combination with stereotactic radiosurgery (76). Several of these checkpoint inhibitors are in clinical trials for GBM (see **Supplementary Table 1**). Available preclinical data suggest a combined strategy of multiple checkpoint inhibitors with pro-immunogenic interventions, such as stereotactic radiosurgery or oncolytic therapy, may yield optimal outcomes. Much work lies ahead to critically and mechanistically evaluate such combinatorial approaches in clinical trials.

GBM AND THE INNATE IMMUNE SYSTEM

Roles of Innate Immune System in GBM

The innate immune system, comprising CNS-derived microglia, peripherally-derived neutrophils, macrophages, and lymphoid-derived NK cells, has a central role in both glioma and radiation biology (15). In response to CNS inflammation, activated microglia proliferate, secrete cytokines and chemokines, and upregulate cell surface markers such as CD80, CD86, and MHC-II. Microglia also express pattern recognition receptors and cross-present antigens to activate T cells within the CNS (102, 103). Normally absent from the healthy brain, peripherally-derived macrophages are recruited into the GBM microenvironment where they facilitate antigen presentation, immune induction, and removal of cellular debris. Microglia-derived and infiltrating TAMs can comprise up to half the cells in GBM and play a prominent role in tumor growth and invasion (104). Two distinct polarization states of activated macrophages have been frequently described: classically activated "pro-inflammatory" (M1) and alternatively activated "anti-inflammatory" or "chronic inflammatory" (M2) macrophages (105). M1 macrophages serve an important role in phagocytosis of neoplastic cells (106, 107). However, glioma cells can secrete suppressive immune cytokines, such as IL-10 (108), and TGF-β (109), that promote M2 polarization and suppress the M1 phenotype (110). Characterization of TAMs within human GBM has revealed impaired production of pro-inflammatory cytokines, defective antigen-presentation, and poor induction of T cell proliferation (104). Similarly, the GBM microenvironment can also directly render TAMs tolerogenic. GBM cells can induce downregulation of TNF-alpha production, concomitant with induction of anti-inflammatory cytokine IL-10 from microglia through upregulation of STAT 3 and 5 (108).

Another population of peripherally-derived monocytes within GBM are myeloid-derived suppressor cells (MDSCs) that also act to suppress adaptive immunity (111). MDSCs accumulate in GBM, express PD-L1, and impair CD4+ T cell memory function (112). MDSCs lack macrophage-specific markers, such CD68, CD16, and S100A9 (113), and secrete suppressive cytokines, such as TGF-β (114). Though originally described as pleiotropic cells simultaneously expressing both M1 and M2 polarization markers, more recent work has suggested that MDSC are malleable in their polarization phenotype with M1-polarized MDSCs exhibiting tumoricidal properties (115).

Collectively, these studies illustrate the substantial cross-talk between the multiple constituents of the GBM ecosystem in maintaining a milieu conducive to GBM. The therapeutic potential to reprogram TAMs and MDSCs from pro-tumorigenic to tumoricidal polarization states is an area of intense interest. The following sections provide example mechanisms of innate immune system regulation that could be harnessed to anti-tumor effect. To date, radiotherapy has provided a relatively blunt instrument via which to activate the innate immune system. However, limitations include CNS including CNS toxicity and potential for inadvertent activation of pro-tumorigenic sequelae (15). Improved understanding of innate immune mechanisms may provide opportunities to more effectively attack the tumor, whilst protecting against cognitively deleterious effects of radiation.

Toll-Like Receptor Agonists

Toll-like receptors (TLRs) are pattern recognition receptors (PRRs) expressed by a variety of cell types comprising the innate immune system. The primary function of TLRs is to sense damage and mediate response to pathogens and tumors. TLRs bind to pathogen associated molecular patterns (PAMPs), conserved structures expressed by pathogens, and danger-associated molecular patterns (DAMPs), such as high mobility group box 1 (HMGB1) and fatty acids. TLR 2, 3, 4, and 9 are expressed on human microglia and TAMs (116). DCs also play a prominent role in the development of anti-glioma immunity and anti-tumor response (117). Dead glioma cells release HMGB1 which can activate TLR 2 on DCs, promoting expansion of T cells (118). Preclinical studies with intra-cranial tumors have shown that administration of TLR 3 agonist poly(I:C) attenuated tumor growth in mice (119). Additionally, CpG, in combination with tumor lysate, effectively induced maturation of DCs to control tumor growth (120). Recent work from the Lim laboratory found that that mice treated with poly(I:C) and anti-PD-1 in combination demonstrated increased DC activation, T cell proliferation, and improved tumor control (76). In a phase I clinical study, concomitant administration of DC vaccine, together with adjuvants comprising the TLR7 agonist imiquimod or poly(I:C), appeared safe and increased serum levels of TNF alpha and IL-6 (26). Clinical trials evaluating the safety and efficacy of TLR9 agonist CpG oligodeoxynucleotides demonstrated safety, but no improvement in survival when combined with standard care radiotherapy and TMZ (121–123).

CD47-SIRP1α Axis

CD47 is a transmembrane immunoglobulin that binds to integrins and serves as a receptor to signal regulatory protein alpha (SIRP1α) and Thrombosponin-1 (TP-1). Expressed on most tumor cells, including GBM (124), CD47 signals "don't eat me" to macrophages. CD47 binding by SIRP1a initiates a signaling cascade that promotes phosphorylation of intracellular ITIMs and activates inhibitory phosphatases SHP-1 and SHP2 (125). These phosphatases dephosphorylate immunoreceptor tyrosine-based activation motifs inhibit pro-phagocytic signals and disrupt cytoskeleton rearrangements necessary for macrophage phagocytosis (125, 126). Antibodies blocking CD47 have been investigated in multiple tumor types to help promote macrophage tumor phagocytosis with efficacy observed in numerous preclinical models, including GBM (124, 127). Clinical trials are underway for both hematologic

and solid malignancies (128, 129). Used in combination with radiation, CD47 inhibition has been shown to improve tumor radiosensitivity (130). Anti-CD47 therapy has also been shown to boost antigen presentation (131, 132) and augment cytotoxic CD8+ T cell activity (133). As an adjuvant to radiation therapy, CD47 blockade has the unique advantage of mitigating radiation-induced TSP-1 signaling, which promotes resistance to radiation injury due to decreased inhibition of nitric oxide signaling in normal tissues. As such, whereas most radiation sensitizers increase damage to both tumor and normal tissues alike, the unique biology of CD47 blockade may concurrently enable improved tumor radiosensitivity (via improved phagocytosis) (134), whilst enhancing radioresistance of healthy tissues via increased nitric oxide signaling (130).

Repolarizing Macrophages

Chemokines, such as colony stimulating factor 1 (CSF-1) and its receptor CSF1R, regulate macrophage homeostasis by regulating proliferation, differentiation, migration, and survival. The intra-tumoral presence of CSF1R-expressing macrophages correlates with poor survival of patients with solid tumors (135). Secretion of CSF-1 by GBM impacts tumor progression through CSF1R signaling. Treatment of GBM with the CSF-1R inhibitor, BLZ945, in transgenic mouse and human xenograft models suppressed tumor growth and improved survival. Although the number of TAMs was not affected, the expression of M2 markers was decreased, consistent with a reduced tumor-supportive phenotype (136). TAMs support tumor progression by blocking anti-tumor immunity and secreting factors to promote angiogenesis (137). TAMs secrete cytokines, such as TGF-β and IL-10, which augment Treg populations while inhibiting effector T cell activity (138). TAMs have been shown to reversibly change their functional phenotype upon exposure to the tumor microenvironment (139). Therefore, strategies that alter the microenvironment to facilitate the repolarization of M2-like TAMs to a M1-tumor-suppressive phenotype are a potential clinical strategy (140).

RADIATION AND GBM

Impact of Radiation on Tumor Immunity

Radiotherapy is a cornerstone of management for GBM with radiation typically delivered to the enhancing tumor and infiltrative margin via 30 fractions of 2.0 Gy, using IMRT or 3D-conformal therapy. Shorter courses have been considered in elderly patients or as a salvage therapy in recurrent disease. Fractionated radiosurgery has been explored on a trial basis without obviously worse outcomes than standard therapies (141), but has not been adopted in standard management protocols. Radiation acts to ablate dividing cells, induce senescence within non-ablated cells (142). Radiation also stimulates local tumor immunity, promoting anti-tumor immune responses via a host of molecular mechanisms (**Figure 1**).

MHC class I molecules present intracellular peptide fragments to T cells and are expressed on the surface of all nucleated cells, albeit with reduced expression in tumor and stem cells. MHC class 1 molecules are highly expressed on APCs where they may present phagocytosed peptides from tumors. After activation of APCs, such as DCs, antigens are cross-presented to CD8+ T cells. In the healthy brain parenchyma, microglial cells are the main resident antigen-presenting innate immune cell (143). DCs are also present in the choroid plexus (144). After radiation, the extracellular presence of danger-associated molecular patterns (DAMPs) and cytokines, such as MCP1, contribute to rapid microglial activation (145, 146). We have previously shown that radiation induces a unique polarization state in microglia, which is more closely related to M1 than M2, but distinct from both (147). How the transcriptional responses of human microglia and mouse microglia compare following radiation remains to be determined, though persistent microglial activation has been reported in humans even decades following brain radiation (148). Few lymphocytes are typically found in the healthy brain, despite the role of memory CD4+ memory cells in CNS immunosurveillance (21). Murine brain radiation induces a delayed CNS recruitment of T cells, even in the absence of tumor (149).

NK cells are present in relatively low numbers within the GBM microenvironment, when compared to other tumor types (150). Moreover, these NK cells express relatively low levels of the activating receptor natural killer group 2D (NKG2D) (151). Even within the periphery, patients with GBM demonstrate relatively low numbers of circulating NK cells (152), a number that, like T cells (153), falls further after standard radiation and TMZ (152). NKG2D ligands are potent mediators of both the innate and adaptive immune system (154). Radiation upregulates NKG2D ligands in multiple tumor cell lines, which sensitizes them to NK cell mediated cytotoxicity (110). At present, the impact of radiation on NK cell infiltration into GBM is unclear, though may vary as a function of concomitant TMZ and radiation fractionation schemes.

Although GBM display relatively low levels of surface MHC class I (155), radiation increases MHC class I levels, enhancing cross-presentation of tumor associated antigens in the draining lymph nodes and facilitating recognition of antigenic peptides by CD8+ T cells (156–158). Thus, radiation-induced changes can facilitate activation and proliferation of T cell populations to augment anti-tumor immune response.

Interferon (IFN) levels are robustly elevated following radiation and augment systemic anti-tumor immune response. Of the three distinct types of IFN, types I and II play an important role in sculpting anti-viral and anti-microbial defenses. DNA released from irradiated tumor cells is sensed by stimulator of interferon genes (STING) molecules present on DCs to produce type I IFN. Activation of STING pathway and IFN signaling is required for efficient radiation-induced adaptive immune response (116). IFN-γ, a type II interferon, can upregulate MHC class I and NKG2D expression to increase tumor recognition, inhibit development of Tregs, and increase the induction of cytotoxic T cells (159). Radiation-induced production of IFN-γ by CD8+ T cells augments the immunostimulatory anti-tumor effects of radiation (160).

Interestingly, not all of the pro-inflammatory impacts of radiotherapy necessarily serve to enhance anti-tumoral immunity, illustrating the complexity of regulating immune

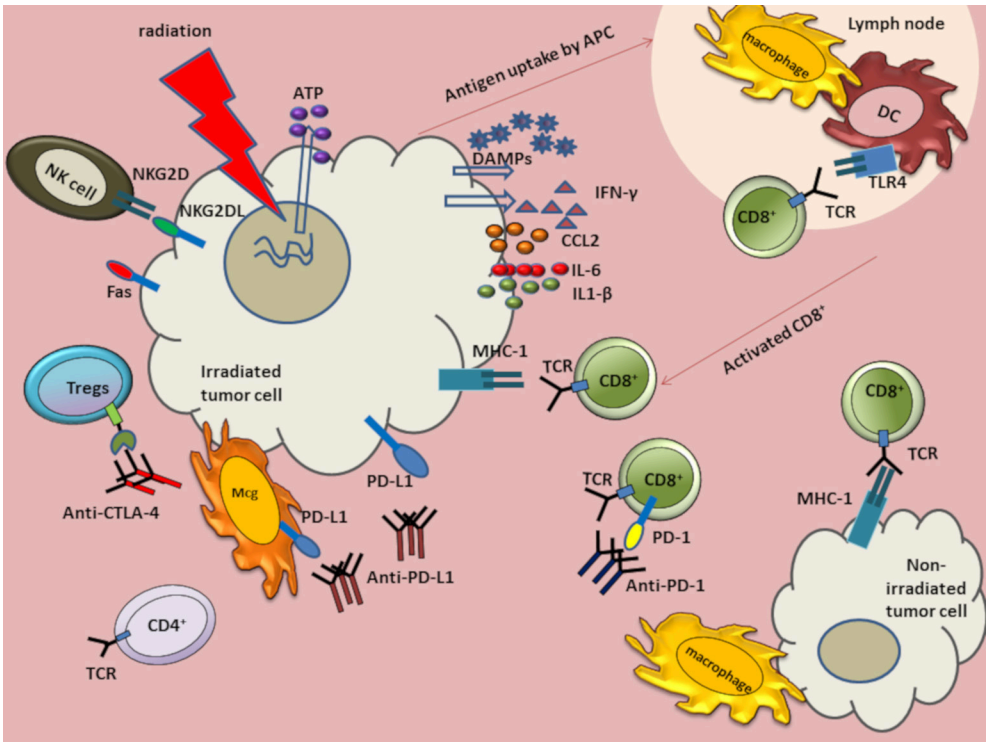

FIGURE 1 | Anti-tumor immune response augmented by the abscopal effect of radiation in combination with immunotherapies. Radiation induces DNA damage and cell death. The dying cells release ATP and DAMPs such as HMGB1 and calreticulin. Although HMGB1 binds TLR4, ATP and calreticulin modulate TLR4 signaling without directly binding to TLR4. Radiation also induces release of tumor antigens to antigen presenting cells (APCs), such as macrophages and dendritic cells (DCs). Antigens are then processed and presented on major histocompatibility complex (MHC) Class I molecules to activate and induce proliferation of CD8+ T cells. The activated cytotoxic CD8+ T cells migrate to tumor sites to induce cell death. Radiation can also induce release of cytokines IL-6 and interferon-gamma (IFN-γ). Radiation also increases tumor cell expression of programmed cell death-1 ligand (PD-L1) and MHC class I molecules. Radiation upregulates immunomodulatory surface proteins, such as Fas and NKG2D ligands on tumor cells. The NKG2D upregulation facilitates NK-mediated tumor cell death. Antibodies, such as α-CTLA-4, α-PD-L1, and α-PD-1 have been used as cancer immunotherapies. When combined with radiation, these antibodies can augment anti-tumor responses in GBM. Anti-CTLA-4 can bind CTLA-4 on Tregs and downregulate suppressive activity. Anti-PDL1 can interact with PD-L1 on tumor cells and on myeloid derived suppressor cells (MDSCs) to curtail suppressive activity induced by MDSCs. Anti-PD-1 antibody can bind to programmed cell death-1 (PD-1) expressed on exhausted T cells.

responses. For example, INF-γ and hypoxia—both of which are induced by radiation—upregulate PD-L1 expression on tumor and tumor-associated immune cells (161, 162). Consistent with this finding, anti-PD-L1 therapy has demonstrated synergistic impacts with radiation to promote anti-tumor immunity (161, 163); results that have been found in metastatic melanoma to be further enhanced by deploying radiation in combination with dual checkpoint blockade (164). Recent data in preclinical models indicate the same may likely hold true in GBM (76).

Abscopal Effect—Proof of Principle for Radiation-Induced Immunity

Single tumor radiation has occasionally been clinically reported to decrease growth of tumors at distant sites—a previously poorly-understood phenomenon termed the abscopal (ab: "away from;" scopos: "target") effect (165). In 2004, Demaria et al. used the growth factor Flt3-Ligand to experimentally enhance numbers of antigen presenting cells providing direct evidence that the abscopal effect is immune mediated and tumor-type specific (166). Numerous studies of metastatic cancers have since demonstrated that radiation in combination with checkpoint inhibitors augment the abscopal effect (167–169). Unlike metastatic cancers for which the abscopal effects may be harnessed to attenuate growth of metastatic lesions elsewhere in the body, GBM is typically restricted to a single (occasionally multifocal) lesion within the CNS. Theoretical limitations of a modest neoantigen repertoire, as well as historically regarded modest CNS immune surveillance, could further confound efforts to elicit an abscopal effect for GBM. Nevertheless, the infiltrative nature of GBM, making it refractory to resection, together with known dose-limiting toxicity of brain radiation, increase motivation to harness abscopal biology against infiltrative tumor cells. Multiple studies have reported that systemic immune status may dictate therapeutic efficacy of radiation (170, 171), providing further impetus to optimize radiation by augmenting immune responsiveness.

Radiation-Induced Cell Death and Immune Activation

Although radiation alone has proven unable to cure glioma, radiation does kill a subset of tumor cells—particularly those that are rapidly dividing. Such cell death facilitates antigen

release, as required for adaptive immunity, and stimulates innate immune responses (**Figure 1**). Radiation induces several types of DNA damage, including simple and complex double stranded breaks (172) with cytotoxic effects (173). Mechanisms of radiation-induced cell death can include necroptosis (174) and p53-dependent apoptosis (175). Radiation-induced mitotic catastrophe may result from radiation, as characterized by aberrant nuclear morphology, multiple nuclei or micronuclei, typically leading to cell death when cells subsequently attempt to divide. However, a small subset of cells may survive with aneuploid or poplyloid karyotypes (176).

Immune activation, as augmented by radiation-induced cell death, facilitates subsequent activation of both the innate and adaptive immune systems against the tumor (177). Immunogenic cell death is mediated by the release of DAMPs directly by tumors or by inflammatory cells present in the microenvironment. Radiation may promote immune activation and immunogenic cell death via at least three mechanisms.

(1) **Translocation of Calreticulin (CRT):** CRT is a DAMP that is typically restricted to the endoplasmic reticulum. Translocation of CRT to the cell surface of dying cells stimulates DCs to cross-present antigens to cytotoxic T cells (178).
(2) **Extracellular release of HMGB1 and ATP:** Extracellular HMGB1 induces DC activation through TLR-4. TLRs play an essential role in activation of APCs (179) and microglia (180), as well as release of pro-inflammatory signals, including IFN-γ (156, 160). The physical interaction between HMGB1 and TLR4 further prompts optimal cross-presentation of antigens derived from tumor cells by DCs to T cells (181). ATP release from dying cells can also trigger IL-1- β production and priming of CD8+ T cells by activating P2RX7 and PR2Y2 receptors on DCs and macrophages, respectively (182).
(3) **Translocation of heat shock proteins:** Cell surface expression of heat shock proteins HSP70 and HSP90 on dying cells induces NK cell activation and promotes cross-presentation of tumor antigens to facilitate DC maturation. Given tumor cell death releases tumor-specific antigens to APCs, including DCs, such cross-presentation of antigens to cytotoxic CD8+ T cells facilitates an anti-tumor T cell response (177, 183).
(4) **Upregulated Fas expression:** Garnett et al. have demonstrated radiation increases surface expression of Fas on tumor cells, which augments their destruction by antigen-specific immune effector cells via Fas-dependent mechanisms (184). Binding of Fas, a plasma membrane death receptor protein, to its extracellular ligand, Fas-L, activates caspase 3 and triggers apoptosis. The Fas-FasL axis is integral to maintenance of regulation of immune homeostasis (185, 186) and CD8+ T cell-mediated cytotoxicity (187). CD8+ T cell cytotoxicity is a multi-step process in which the effector cells act to induce cell death by forming cell–cell contacts with potential target cells expressing cell death triggering ligands. Following MHC-antigen recognition, CD8+ T cells lyse target cells via secretion of granzyme and perforin and by the engagement of FasL on T cells with Fas expressed on target cells. Both pathways lead to apoptotic cell death (188).

Preclinical Data Supporting Combined Radiation and Immunotherapy for GBM

Multiple preclinical studies provide robust proof of principle supporting the combined role of radiation and immunotherapy for GBM (64, 76, 189). In an orthotopic (intracranial) GL261 mouse model, median survival doubled from 27 days with anti-PD1 antibody alone and 28 days in radiation alone, to 53 days when the two modalities were combined. Immunohistochemistry confirmed increased tumor infiltration of cytotoxic CD8+ T cells and decreased regulatory CD4+ T cells in the combination group (64). Similarly, combined radiation and use of an agonist antibody for the co-stimulatory molecule glucocorticoid-induced TNF receptor (GITR) expressed on both regulatory and cytotoxic T cells yielded a cure rate of 24%, compared to 0% for radiation or anti-GITR therapy alone (190).

GL261 is a widely employed mouse GBM line that permits studies in immunocompetent animals (191, 192). As such, many of the seminal studies of immunotherapy with or without radiation have utilized this model. Nevertheless, some have criticized the GL261 model as more highly immunogenic than the immunologically "cold" GBM, thereby potentially over-estimating the clinical potential of immunotherapies for GBM. Numerous genetically engineered models of GBM have been developed, several of which have been well described as "transplantable GEM models" and provide important immunocompetent alternatives to GL261.

As noted in earlier sections, multiple immune checkpoints and other immmunosuppressive strategies are harnessed by GBM to avert immune detection (193). Accordingly, as with preclinical models of metastatic disease, preclinical GBM models have similarly demonstrated improved outcomes with multimodal immune therapy in combination with radiation. Combined use of CTLA-4 blocking antibodies and pro-cytotoxic function CD137 (4-1BB) agonist antibodies with RT yielded 50% survival at 100 days in a GL261 orthotopic model, compared to 20% without RT, and 0% with radiotherapy alone (189). Radiotherapy plus dual checkpoint antibodies against PD-1 and TIM-3 yielded 100% survival of GL261-bearing mice at 100 days, compared to 60% with the best combination of only two of the three treatment modalities (76). Both of these radiation plus dual immunotherapy studies documented elevated CD8+ and CD4+ T cells within the tumor of combined therapy-treated animals (76, 189). Belcaid et al. further performed depletion studies to find that CD4+ but not CD8+ T cells were required for the survival benefit of combined therapy (189).

Optimizing Radiotherapy for Immune Stimulation

Most clinical trials of immunotherapy, to date, have enrolled patients with recurrent disease following prior standard therapy. As such, patients would have previously undergone radiation and chemotherapy, though would not typically receive further

radiation as part of the trial protocol. As such, it is important to note that Belcaid et al. found a trend toward improved outcomes with concurrent, rather than sequential, administration of radiation and immunotherapy (189). Additionally, prior exposure to TMZ attenuates the immune response to checkpoint inhibitors (194).

Current standard therapy for GBM includes chemotherapy and fractionated radiation, frequently also with administration of corticosteroids, which collectively induce lymphopenia and immune suppression (195–198). Importantly, mathematical modeling indicates that even the fractionated radiation to the tumor itself accounts for lymphotoxic doses of radiation to the entire circulating blood pool, even independent of immunosuppressive chemotherapy and steroids (199). As such, stereotactic radiosurgery has been evaluated as an alternative to standard fractionated radiation with a goal of decreasing immunosuppression and increasing tumor ablation and immune activation. Also of note, traumatic brain injury leads to immune suppression via ill-defined mechanisms (200). Whether additional such mechanisms may further impede immune function following brain radiation, independent of lymphodepletion, remains similarly ill-defined. Most GBMs in humans exceed the size limit (∼3 cm) considered acceptable for single fraction radiosurgery, though fractionated radiosurgery has been explored with demonstration of feasibility in a preliminary dose-escalation study (141).

With the increasing clinical prevalence and importance of immune-based strategies, attention has focused on how best to harness immune-activating impacts of radiation. The linear quadratic equation is used to determine which fractionated radiation regimens yield equivalent biologically effective doses (201). Importantly, recent data have revealed that too much radiation in a single fraction may inhibit the very immune mechanism one is attempting to activate through radiation-induced immune activation. In an OVA murine melanoma model, 7.5 Gy/fraction yielded best tumor immunity while minimizing numbers of Tregs (202). Radiation doses above 12 Gy were recently found to activate DNA exonuclease Trex1, which decreases DNA from the cytosol and thereby reduces immunogenicity (203). Current efforts to optimize fractionation schemes to optimize RT-mediated immune activation were recently reviewed elsewhere (204). Importantly, optimal parameters appear tumor-dependent. Few studies to date have addressed this question for GBM, though dedicated clinical trials may be needed to elucidate optimal parameters for human patients. A dose escalation study (25–40 Gy) using 5 Gy/fraction with 5 mm margins revealed a maximum tolerated dose of 40 Gy in 8 fractions and an overall survival of 15 months–similar to standard therapy. Further work would be needed to assess relative efficacy of immunotherapies in such novel paradigms compared to that seen with conventional therapy.

IMMUNOTHERAPY FOR LOW-GRADE GLIOMAS

The role of immunotherapy for low grade infiltrative gliomas remains poorly characterized. Preclinical efforts in this domain are hampered by the paucity of available animal models. Low-grade gliomas are ultimately fatal due to transformation into high-grade gliomas. Clinical application of immunotherapies for low-grade gliomas are hampered by the lack of biomarkers for efficacy and prolonged periods of relative clinical stability with existing therapies. Low-grade gliomas demonstrate less immunosuppressive phenotypes compared to high-grade gliomas (196, 205–208). This could portend an improved capacity for inducing an immune response, particularly in the context of a more indolent lesion that affords more time to achieve a therapeutic response before the patient would otherwise succumb to disease (209, 210). Conversely, most low-grade gliomas are IDH-mutant and overproduce 2-hydroxyglutarate, which has been found to be immunosuppressive (211). Nevertheless, the specific IDH1 (R132H) mutation itself could serve as a potential vaccine target (212). Preliminary safety trials of vaccines have been performed in pediatric patients with low-grade glioma. A Poly-IC-containing synthetic peptide-based vaccine against the glioma-associated antigens EphA2, IL-13Rα2, and survivin yielded notable immunologic and radiologic responses in a subset of patients (209, 210, 213). Further work is needed to elucidate prospectively which patients and tumor subtypes could benefit from immunotherapy and how favorable responses can be made more consistent.

IS RADIATION AND IMMUNOTHERAPY RELEVANT TO TARGETING GLIOMA STEM CELLS?

Cancer stem cells (CSC) have been identified in numerous tumors and play a role in development, invasion, and metastasis. Glioma stem cells (GSC) (82, 214), represent tumor-initiating cells notable for markers of neural stem cell markers, such as CD133 (214) and Nestin (215). Upregulated markers of pluripotent stem cells, including nanog and Oct4, have also been reported (216). GCS demonstrate therapeutic resistance in part through upregulation of DNA damage checkpoint responses and enhanced DNA repair (217). Radiation can induce de-differentiation of GBM cells into a stem cell-like phenotype with increased self-renewal and tumorigenesis capacity in a survivin-dependent manner (218).

GSCs are primarily enriched in the perivascular niche (219). Both microglia and TAMs are found in the perivascular niche and GSC play a prominent role in immunomodulation by recruiting microglia and TAMs. For example, GSCs secrete periostin to recruit TAMs that largely exhibit an M2 phenotype (220). GSCs have also been shown to activate TLR4 on microglia to induce IL-6 secretion (221). Immune therapies against GSCs have included peptide and DC vaccines. Cantini et al. reported in a GL261that vaccination with GLAST, a CNS protein enriched on radial glial cells, promoted tumor immunity without evidence of autoimmunity (222). DC-based vaccines have been explored using tumor lysate or GSC-associated peptides to stimulate *ex vivo* DCs. Administration of loaded DCs in human patients induces prolonged anti-tumor immunity against a potentially broad range of antigens (223). In a GL261-murine model, Pellegatta et al. demonstrated that

vaccination using CSC antigens yielded improved anti-tumor effects of DC vaccination when compared with vaccination using regular tumor antigens (224). Similarly, in a rat model, Xu et al. showed that rats vaccinated with GSC-enriched lysates from neurospheres survived longer than rats vaccinated with non-GSC-enriched lysates (225). In recent years, such strategies to target GSCs have been extended to clinical trials.

A Word of Caution

Immune targeting of GSCs ideally seeks to promote immune responses against antigens uniquely expressed on GSCs, but not healthy tissues. However, care may be needed to ensure that rare endogenous tissue stem cells (neural stem cells or oligodendrocyte progenitor cells) are not inadvertently targeted. Currently, this question is complicated in part by controversy surrounding the presence and identity of adult human endogenous neural stem cells (226). Since GSCs likely reactivate more primitive developmental programs than adult CNS or other tissue progenitor populations, targeting these most primitive markers may help minimize depletion of adult progenitor populations. Since the phenotypes of certain human endogenous progenitor populations remains ill-defined, vigilance for cognitive or other toxicities should be maintained in any therapies potentially inducing auto-immunity against non-mutant endogenous peptides.

ADJUNCTIVE TOOLS TO PROMOTE TUMOR IMMUNITY

DC Vaccines

DCs are one of the most important APCs and have prompted several groups to develop DC-based vaccines for GBM (27, 227–230). DCs have a high capacity to detect maturation signals and process antigens as peptides to generate an efficient and sustained T cell response (231, 232). In an early clinical study of standard chemoradiotherapy followed by GSC-pulsed DC vaccine, 7/11 enrolled patients completed treatment with a median survival of 694 days (233). Currently, it is unclear which factors impact the efficacy of DC vaccination. However, a pre-clinical study by Mitchell et al. showed that DC migration to tumor draining lymphnodes could be enhanced by exogenous administration of the chemokine CCL3 (234). In addition, the authors demonstrated that modulation of CMV-specific DCs with a potent tetanus/diphtheria antigen increased the migratory capacity of DCs and improved the clinical outcomes in patients with GBM (234). A DC vaccine (ICT-107) loaded with six synthetically processed GBM associated peptides (tumor stem cell antigen MAGE-1, her-2, AIM-2, Trp-2, gp100, and IL-13 Rα2) yielded improved progression-free survival and a trend toward improved survival in a randomized, double-blind, placebo-controlled phase II clinical trial for newly diagnosed GBM; however, the study did not meet the primary endpoint of improved overall survival (235). A phase III study was begun, but suspended due to insufficient funding. An initial report demonstrated a median overall survival of 23.1 months in the intention-to-treat population (236). To date, clinical trials have deployed DC therapies following completion of standard chemoradiation therapy. Whether or not modifications to standard therapy could further augment DC-mediated responses remains to be investigated.

Targeted Immunotherapy

Epidermal Growth Factor Receptor Variant III Vaccines

Epidermal growth factor receptor (EGFR) variant III (vIII) is expressed in 20–30% of GBM (237). EGFRvIII is absent in normal tissues and selective activation of PI3K/Akt pathway contributes to GBM resistance to radiotherapy (238). Work by Heimberger et al. demonstrated that immunization of DCs mixed with a tumor-specific peptide of EGFRvIII, PEP-3 conjugated to the immune adjuvant keyhole limpet hemocyanin (KLH), resulted in long-term survival of mice with intracranial melanomas (239). The vaccine Rindopepimut, which targets EGFRvIII, has shown efficacy in phase I/II clinical trials, but demonstrated no survival benefit in a phase III trial (see **Supplementary Table 1**) (240, 241).

Survivin

Survivin, a regulator of both mitosis and programmed cell death (242), is a tumor associated antigen, making it an attractive candidate for targeted cancer therapy and immunotherapy (242–244). Normal glial cells do not express survivin, whereas survivin is highly expressed in GBM and is associated with poorer prognosis (245). Epitopes of survivin are immunogenic and are presented by MHC Class I complexes. Anti-survivin antibodies have been identified in patients with GBM (246). In an effort to identify a survivin peptide mimic that could elicit a potent T cell response, Ciesielski et al. created SVN53-67/M57, a peptide vaccine derived from survivin. SVN53-67/M57 produced cytotoxic T cell-mediated killing of human glioma cells *in vitro* and, in combination with GM-CSF, was able to control tumor burden in mice bearing GL-261 glioma tumors (247). A phase II trial of SVN53-67/M57-KLH (SurVaxM) and TMZ is currently recruiting patients with malignant glioma and the therapy has shown to be well tolerated and generates anti-survivin antibody and survivin specific CD8+ T cells (248).

Oncolytic Viruses

While this review focuses particularly on the facilitating role of radiation in checkpoint blockade, oncolytic viruses may serve a similar role by means of immune activation in GBM (249). Although oncolytic viruses are selected or engineered for their propensity to replicate or selectively kill tumors cells, complete viral-induced lysis of all tumor cells is not observed with the relatively attenuated viral constructs clinically deployed, to date. Instead, the lysis of a subset of tumor cells may serve to promote both anti-viral and anti-tumor immune responses (250). The combination of measles virus-expressing carcinoembryonic antigen with radiation has been shown to improve tumor control (251). Similarly, the combination of radiation with oncolytic DNA viruses, such as herpes-simplex virus-1 and conditionally replicating adenovirus, has demonstrated longer survival and improved outcomes in pre-clinical GBM models (252–254).

Despite some case reports of remarkable responses, clinical trials of oncolytic therapies for GBM have proven disappointing, to date, with only marginal therapeutic efficacy reported (249) (ClinicalTrials.gov, Unique Identifier: NCT01280552)[1]. These findings have prompted ongoing efforts to both better predict which patient populations may respond favorably and how responses may be further augmented.

CLINICAL TRANSLATION: CHALLENGES AND PRACTICAL CONSIDERATIONS

Translating immunotherapy for GBM has proven challenging. Optimally harnessing radiation to augment the efficacy of immunotherapy is a promising avenue, but is not without its own unique challenges (**Figure 2**). While many patients seeking clinical trials have recurrent disease, prior radiation may preclude further radiation due to risk of toxicity and may impact immune responses in ways that are difficult to predict. While TMZ may attenuate bone marrow immune responses, TMZ-induced mutations may provide important neoantigens to catalyze immune recognition of the tumor.

Tumor heterogeneity remains a challenge, both within and between patients. Furthermore, human immune responses are complex and will likely require molecular and genetic subtyping to identify potential subclasses and individual "responders" or "partial responders." For example, the phase II ICT-107 autologous DC vaccine trial suggested clinical responses only in subjects who were HLA-A2 positive (a phase III trial was suspended for financial reasons). Several immunotherapy clinical trials are ongoing for GBM, which is routinely treated with radiation, including DC vaccines, EGFRvIII vaccines, and checkpoint inhibitors, among others. However, few studies, to date, have specifically focused on optimizing synergy between radiation and immunotherapy.

GBM is transcriptionally subclassified into proneural, neural, classical, and mesenchymal based on genomic profiling (255). However, single cell transcriptome data suggest variable representations of each transcriptional cell type within each tumor, challenging selective targeting of the tumor phenotype. Moreover, radiation has been shown to induce a mesenchymal phenotype, notable for its poorest prognosis; likely due in part to radiation-induced upregulation of treatment-resistant stem-like properties (256). Data from other tumor types suggest that cytokines from local tissue in response to immunotherapies may offer an important source of more reliable biomarkers, including biomarkers of therapeutic responsiveness (257). If also true in glioma, this may create impetus to identify technologies for *in vivo* evaluation of such biomarkers locally within the tumor microenvironment in response to therapy—an avenue our group is currently exploring.

The paucity of prompt biological feedback regarding efficacy remains a challenge. While systemic immune cell populations can be serially accessed to monitor leukocyte numbers and phenotypes, these data are at best an indirect and imperfect indicator of therapeutic efficacy within the tumor. Imaging criteria to interpret immunotherapy responses, despite interpretations challenges of radiation- and immunotherapy-induced pseudoprogression, have been drafted (iRANO). The lack of definitive imaging biomarkers of responsiveness is underscored by the need to follow the trajectory of imaging changes over months to interpret findings (258).

Finally, it is increasingly appreciated that standard management strategies aside from radiation likely inhibit the efficacy of immunotherapy, including immuosuppressive corticosteroids and systemic chemotherapy. Corticosteroids, such as dexamethasone, are used to control vasogenic edema due to infiltrative tumor, surgery, and radiotherapy (259). Pre-clinical models and retrospective data from clinical studies indicate that

[1]Pembrolizumab and Standard Therapy in Treating Patients With Glioblastoma. Available online at: https://clinicaltrials.gov/ct2/show/NCT03197506.

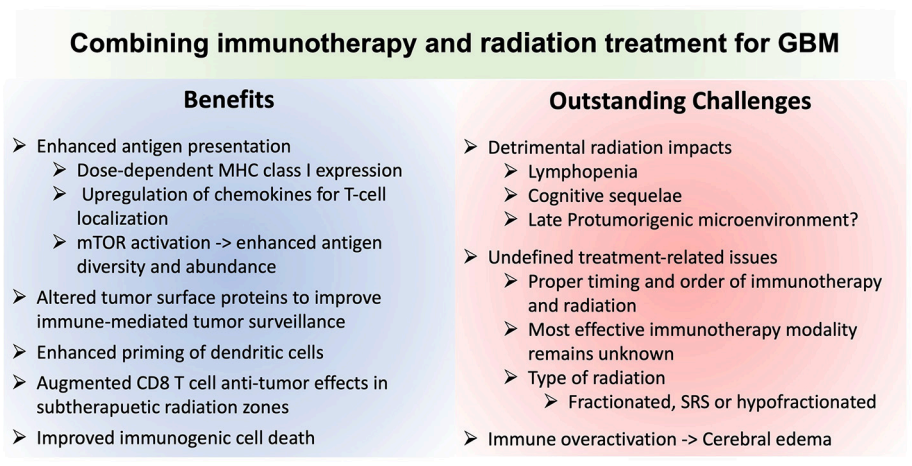

FIGURE 2 | Comparison of the advantages and potential challenges of combining immunotherapy and radiation for glioblastoma treatment. MHCI, Major histocompatibility complex class I molecule; mTOR, mechanistic target of rapamycin; SRS, stereotactic radiosurgery.

dexamethasone treatment attenuates the efficacy of radiotherapy, presumably by impeding normal radiation-induced immune responses (260). While TMZ is the cornerstone of the standard STUPP regimen for GBM, experimental data demonstrate that systemic chemotherapy impedes the anti-tumor effects of anti-PD-1, despite the potential for local chemotherapy to augment immunotherapeutic responses (194). These studies highlight practical challenges of optimizing the therapeutic impacts of immunotherapy. Until methods can better predict responses or evaluate therapeutic impact in real time, forgoing the established standard of care (TMZ) to theoretically augment an unproven experimental therapy may prove challenging. Our group recently initiated a clinical trial providing anti-PD-1 in biopsy-proven GBM prior to definitive surgical resection and subsequent chemo/radiation. Insights from early histological analysis of tissue from patients treated with anti-PD-1 may help identify biomarkers and selection criteria for future single and combination immunotherapy trials (ClinicalTrials.gov, Unique Identifier: NCT03197506). As increasing evidence emerges about untoward chronic impacts of radiation on the CNS microenvironment for tumor aggressiveness, could future paradigms replace standard fractionated radiation with combination immunotherapy and hypofractionated SRS applied to just a portion of the tumor? Alternatively, perhaps residual tumor cells after chemo/radiation may be best eliminated with combined immunotherapy and senolytic therapy? Finally, strategies are needed to optimally titrate the immune response to avert potentially severe or fatal toxicities. These may vary in a tumor- and patient-specific manner based on biomarkers of susceptibility and responses that have yet to be identified. We posit that dedicated efforts to understand the human biology of CNS radiation and therapeutic responses may reveal opportunities to optimize safety and efficacy of combined radiation and immunotherapy for glioma.

CONCLUSIONS

The dramatic anti-tumor clinical responses observed in certain tumors treated with anti-CTLA-4 and anti-PD-1 antibodies have ushered in a new era for effective cancer therapies. Radiation modulates the tumor microenvironment and offers a potential immune adjuvant to enhance the anti-tumor response in combination with immunotherapies. Preclinical models of GBM illustrate potent opportunities to harness combination immunotherapy with brain radiation. However, several questions remain unanswered regarding the optimal paradigms of combination immunotherapy, timing in relation to radiation, and the potential to mitigate adverse impacts of currently standard treatments, such as fractionated radiotherapy-induced lymphopenia and chemotherapy- and corticosteroid-induced immunosuppression. Preclinical evidence suggests robust opportunities to add optimized strategies of immunotherapy into standard-of-care for GBM. Much work lies ahead to improve translational paradigms that could increase mechanistic insights gleaned from each treated patient and enable iterative improvements in protocols within the life-times of individual patients.

AUTHOR CONTRIBUTIONS

KR, LC, and TB conceived the aim of the review, performed the literature search, and wrote the manuscript with additional intellectual contributions from IP, AJ and AW. KR drafted **Figure 1**, LC drafted the **Supplementary Table 1** and **Figure 2**.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2018.00656/full#supplementary-material

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REVIEW
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Repurposed Biguanide Drugs in Glioblastoma Exert Antiproliferative Effects via the Inhibition of Intracellular Chloride Channel 1 Activity

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The lack of in-depth knowledge about the molecular determinants of glioblastoma (GBM) occurrence and progression, combined with few effective and BBB crossing-targeted compounds represents a major challenge for the discovery of novel and efficacious drugs for GBM. Among relevant molecular factors controlling the aggressive behavior of GBM, chloride intracellular channel 1 (CLIC1) represents an emerging prognostic and predictive biomarker, as well as a promising therapeutic target. CLIC1 is a metamorphic protein, co-existing as both soluble cytoplasmic and membrane-associated conformers, with the latter acting as chloride selective ion channel. CLIC1 is involved in several physiological cell functions and its abnormal expression triggers tumor development, favoring tumor cell proliferation, invasion, and metastasis. CLIC1 overexpression is associated with aggressive features of various human solid tumors, including GBM, in which its expression level is correlated with poor prognosis. Moreover, increasing evidence shows that modification of microglia ion channel activity, and CLIC1 in particular, contributes to the development of different neuropathological states and brain tumors. Intriguingly, CLIC1 is constitutively active within cancer stem cells (CSCs), while it seems less relevant for the survival of non-CSC GBM subpopulations and for normal cells. CSCs represent GBM development and progression driving force, being endowed with stem cell-like properties (self-renewal and differentiation), ability to survive therapies, to expand and differentiate, causing tumor recurrence. Downregulation of CLIC1 results in drastic inhibition of GBM CSC proliferation *in vitro* and *in vivo*, making the control of the activity this of channel a possible innovative pharmacological target. Recently, drugs belonging to the biguanide class (including metformin) were reported to selectively inhibit CLIC1 activity in CSCs, impairing their viability and invasiveness, but sparing normal stem cells, thus representing potential novel antitumor drugs with a safe toxicological profile. On these premises, we review the most recent insights into

the biological role of CLIC1 as a potential selective pharmacological target in GBM. Moreover, we examine old and new drugs able to functionally target CLIC1 activity, discussing the challenges and potential development of CLIC1-targeted therapies.

Keywords: glioblastoma, cancer stem cells, CLIC1, biguanide, metformin

AN INTRODUCTION TO CANCER STEM CELLS IN HUMAN GLIOBLASTOMA

Glioblastoma (GBM) is the most aggressive and prevalent primary brain cancer in adults, characterized by morphological, cellular, and molecular heterogeneity leading to invasive growth and resistance to therapy (1). Despite the use of aggressive multimodal treatments, GBM invariably recurs, and the median overall survival time of patients is extremely poor (∼15 months after diagnosis) (2). The high drug resistance and recurrence rate of GBM is mainly ascribed to a sub-population of cancer stem cells (CSCs) within the tumor mass (3). GBM CSCs (GSCs) share functional features with neural stem cells, including self-renewal and multipotency, as well as the over-activation of biochemical signaling pathways (i.e., Sonic hedgehog, Akt, and Wnt/β-catenin). On the other hand, GSCs possess distinct genetic and epigenetic alterations which sustain their *in vivo* tumorigenic potential: through asymmetric division GSCs give rise to all the differentiated non-tumorigenic cells forming the bulk of the tumor mass, while their stem cell-like properties provide them with inherent resistance and evasion of apoptosis (4–6).

Phenotypically, GSCs are characterized by the expression of a combination of stem cell markers (e.g., CD133, Olig2, Sox2, Nanog), although different GSC populations exist, and a unique tumor-related phenotype has not been yet identified. Several proteins contribute to the maintenance of the stem-like phenotype, the aggressiveness, and the white matter invasiveness of GSCs, including CD44, sprouty2, Notch, tGLI1, and PrP (7–11). Moreover, the microenvironment in which GSCs develop is extremely complex, harboring non-neoplastic stromal cells, mesenchymal stem cells (MSCs), endothelial cells, immune cells, and other glial cell types, organized to compose the tumor niches (12). A dynamic and reciprocal crosstalk between GSCs, GBM bulk cells and the microenvironment cells occurs in the niches, via paracrine signals, mainly mediated by chemokine systems (for ex. CXCR4/7-CXCL12) (13) or direct cell-cell interactions. This microenvironment contributes tumor progression, invasion, angiogenesis, escape from immune surveillance, drug resistance, as well as to GSC maintenance, favoring the retaining of the stem-like properties (14, 15).

GSCs sustain neovascularization via the release of pro-angiogenic factors and vascular transdifferentiation (16), and are able to secrete cytokines inducing immune suppression (17, 18). Moreover, alteration of metabolic programs (i.e., the Warburg effect) drives the aggressive phenotype of GSCs providing them biosynthetic molecules useful for rapid growth (19).

Cytotoxic drugs, such as temozolomide, might favor a mutagenic selection of treatment-resistant GSC clones, further increasing GSC genetic heterogeneity, which represents a relevant mechanism for tumor recurrence (20). In addition, GSC and non-GSC populations retain dynamic interconversion through self-differentiation and de-differentiation, respectively (21, 22). Given the capacity of GSCs to generate all the different tumor cell populations composing the tumor mass, GSC targeting agents should be used in combination with existing therapies to arrest tumor growth and improve the clinical outcome.

Overall the complex nature of GSCs makes their eradication the main therapeutic goal for GBM, but a still unsolved challenge (23). In fact, conventional antitumor drugs spare GSCs, allowing tumor re-growth. Potential innovative strategies to eradicate GSCs from tumors are directed to: (i) impair specific pathways crucial for GSC survival and functioning (i.e., Notch, Wnt, Sonic hedgehog); (ii) targeting GSC perivascular or hypoxic niches; (iii) block metabolic and/or epigenetic modifications providing GSCs with stem-like properties. However, GSCs frequently activate multiple compensatory signaling pathways, change phenotype along tumor progression, displaying genetic heterogeneity, high plasticity and diversity of stemness markers, nullifying potential effective therapies (24). The identification of the distinctive GSC Achilles heel is an urgent goal for GBM treatment, since innovative therapeutic approaches identified for other cancer types left the survival of GBM patients practically unchanged over the past decades.

ION CHANNELS IN CANCER: CLIC1 FUNCTIONAL EXPRESSION AND THERAPEUTIC POTENTIAL

Ion channels are integral membrane proteins that form pores through which enable the passage of ions between cell compartments, regulating electrical excitation, cell proliferation, motility, survival, and maintaining tissue homeostasis. Structural defects or dysregulated functioning of ion channels play a pathogenic role in several human diseases including cancer. In particular, alterations of ion channel activity contribute to malignant transformation, inducing aberrant cell cycle rate, inability to activate the apoptotic program, and increased migration and invasion abilities (25). Genes encoding ion channels involved in oncogenic transformation (26) are differentially expressed in cancer and normal cells, in breast cancer (27), lung adenocarcinoma (28), and GBM (29).

While the role of plasma membrane channels has been extensively studied, less is known about intracellular ion channels. These molecules, inactive in the cytoplasm, are able to

auto-insert into membranes where they act as functional integral ion channels, and have been recently recognized to regulate cell cycle, apoptosis, cell adhesion and motility (30). In this scenario, pharmacological modulation of intracellular ion channels would represent a potential innovative therapeutic option.

Among the ion channels whose aberrant expression and activity is relevant for neoplastic transformation, the chloride intracellular channel (CLIC) family recently gained particular attention. The six members of CLIC group (CLIC1-6), are present in both soluble and membrane-associated forms, displaying cell-specific expression and biological functions in mammalian tissues, not functioning as conventional chloride channels but possessing peculiar physiological roles in each different cell type (31). CLIC1 is the most widely expressed and studied channel of this family, in both physiological and pathological conditions, including brain functioning and cancer cell proliferation (32).

Overview of the Mechanisms of CLIC1 Activation and Related Physiological Functions

CLIC1 is a metamorphic protein (33) able to switch from a soluble cytoplasmic conformation to a transmembrane isoform (tmCLIC1) (34). Thus, CLIC1 exists in three different states: a monomeric soluble form, an oxidized soluble dimeric intermediate form, and an integral membrane form. The soluble monomer contains a thioredoxin-like N-domain with a glutathione binding site. The formation of the dimer is stabilized through a disulfide bond which connects two conserved cysteine residues, Cys-24 and Cys-59, which are essential for channel assembly, as the mutation of each one of them prevents the channel formation (34). Cys-24 residue is also required for the protein redox regulation, rising the hypothesis that CLIC1 membrane insertion could be controlled by reactive oxygen species (ROS) signaling (35, 36). Membrane association implies the formation of oligomeric CLIC1 complexes (37).

The ability to form the channel pore was confirmed in artificial lipid bilayers by Littler et al. (38). Membrane-associated CLIC1 exposes the N-terminal region to the extracellular space, determining the ability to activate a selective chloride conductance. Both oxidizing conditions and changes in pH levels control CLIC1 membrane insertion. In fact, CLIC1 membrane insertion is not only dependent on the level of cellular oxidation, as suggested by the observation that the dimeric intermediate form is reversible under reducing conditions, but its assembling within lipid bilayers and channel activity are also dependent on pH, being minimal at pH 7 and reaching the maximum rate at ± 2 pH units (39, 40). Mutation of two histidine residues, His-74 and His-185, impairs CLIC1 pH sensitivity, preventing membrane insertion at acidic pH 5.5 (41).

CLIC1 Signaling in Brain Function

CLIC1 is almost ubiquitously expressed in human tissues, including the central nervous system (CNS) where it is expressed in both excitable and non-excitable cells. CLIC1 is also present, in cytoplasmic conformation, in microglia, the brain intrinsic immune system (42, 43). Being chronic inflammation of the CNS during neurodegenerative disorders sustained by activated glia, tmCLIC1 was involved in the pathophysiology of Alzheimer's disease, considering that the neurodegenerative process implies an overproduction of ROS mediated by activated microglia (42, 44). This phenomenon can be reproduced *in vitro* stimulating microglial cells with amyloid β (Aβ) peptides: Aβ-activated microglia is characterized by high proliferative rate, production of large amount of ROS, and sustained by tmCLIC1 activity (42). Though tmCLIC1 rapidly increases in response to Aβ stimulation, it is rarely detectable in quiescent microglia cells (45). Indeed, CLIC1 downregulation in microglia by small interfering RNA or its inhibition using the channel blocker IAA94 and/or specific antibodies, prevents Aβ-induced neurotoxicity (45). Analogously, CLIC1 activity is a pre-requisite for ROS overproduction in β-amyloid-activated microglia (42). All together these findings indicate that tmCLIC1 plays a crucial role in the microglial inflammatory state characterizing the neurodegenerative processes, and support therapeutic targeting for neuroprotective strategies (44).

CLIC1 in Cancer

In the last years, growing evidence highlighted the role of CLIC1 as key factor in tumor development and progression. Working as a chloride channel, tmCLIC1 plays an essential role in tumorigenesis, controlling cell volume regulation (46), cell migration and invasion (47–49), and neoangiogenesis (50). CLIC1 is overexpressed in several human solid tumors, as compared to the surrounding normal tissue. For example, CLIC1 gene expression is significantly increased in bladder (51), *in situ* breast ductal (52) and ovarian (53) carcinomas, and it has been linked to oncogenic functions and poor prognosis in colorectal (48), gastric (49), hepatocellular (54), gallbladder (55), pancreatic (56), and lung carcinomas (57), and sarcomas (58). Bioinformatic analysis (cBioPortal/TCGA datasets) of CLIC1 mRNA levels in several human aggressive carcinomas (breast, colorectal, esophagus, liver, ovarian, stomach, prostate, thyroid, uterine, head & neck, and pancreas) shows that this channel is expressed at similar levels in all the different types of neoplasia, with a small increment only in colorectal, head & neck and pancreatic cancers (**Figure 1**). These data suggest the relevance of this channel in the development and progression of most malignant neoplasms. Moreover, CLIC1 gene is highly conserved among tumors in the various districts, with only 2% of patients carrying missense or non-sense mutations, clearly indicating that its role in tumorigenesis is more related to membrane localization and activity than to mutations.

The expression and function of several ion channels are altered in GBM cells, and, within chloride channels, changes in CLIC1 gene expression have been frequently detected (51). CLIC1 mRNA and protein levels are up-regulated in GBM as compared to normal brain parenchyma. The analysis of TCGA database identified a weak correlation with tumor stage, displaying lower expression in low grade gliomas than in GBM (**Figure 1**), suggesting a potential role for this channel in the malignant behavior of this tumor. Similarly, CLIC1 expression levels directly correlated to GBM aggressiveness in experimental models (30). Beyond

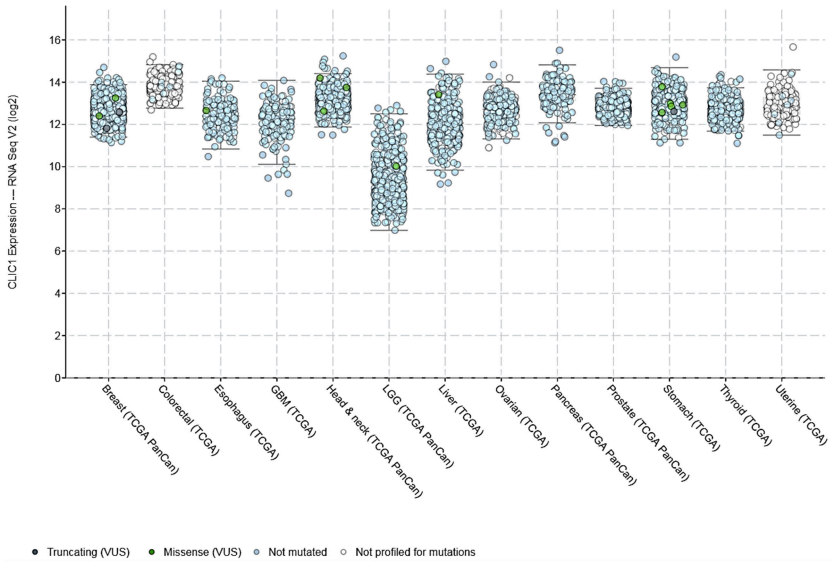

FIGURE 1 | CLIC1 mRNA expression levels in various human carcinomas and mutations found according to the cBioPortal/TCGA datasets.

expression levels, *in vitro* and preclinical *in vivo* studies analyzing CLIC1 channel function in malignant transformation and progression, shed new light on its biological and clinical significance in tumors. CLIC1 channel activity is involved in invasion and migration through ROS-mediated MAPK pathway in colon cancer cells (46, 48), and gastric cancer cells (49, 59). Also the metastatic process has been associated to CLIC1 functioning in gallbladder and hepatocellular carcinomas (55, 60).

The abundance of tmCLIC1 expression in cancer and its high activity in all the cells with sustained proliferation rate, raised the hypothesis of an oncogenic role of CLIC1 (43, 56).

CLIC1 Role in Cell Cycle Progression of Cancer Cells

Different chloride channels are involved in cell division and specifically in the regulation of cell cycle progression, showing a functional activity restricted to a specific cell cycle phase, the G1/S transition (61, 62). In physiological conditions, CLIC1 is mostly cytoplasmic and, upon an oxidative burst, it transiently inserts into the plasma membrane. However, after persistent oxidative stress and/or alkaline cytoplasmic pH, the integral membrane channel form becomes constitutive. Oxidation and cytoplasm alkalization are hallmarks of cancer cells (63, 64) and both conditions promote G1/S cell cycle progression (65, 66). Intriguingly, high ROS production, cytoplasm alkalization, and the subsequent G1/S transition occur in the same time-window in which CLIC1 is active as ion channel (43, 67). Indeed, tmCLIC1 functional expression undergoes a well-defined timing, as shown by electrophysiology measurements, demonstrating that chloride current increases along G1/S phase progression, reaching a peak just before G1/S transition (68) (**Figure 2**). Fluorescence intensity analysis of tmCLIC1 by TIRF microscopy supports these results, demonstrating a different localization of the protein during the different phases of the cell cycle (67). Moreover, the inhibition of CLIC1 activity with the specific blocker IAA94 (69), or using an antibody targeting the NH2 extracellular portion of the channel, induces the accumulation of cancer cells, including GSCs, in the G1 phase with a consequent delay of cell cycle progression (68). Elevated ROS levels and alkaline pH can result from the overexpression and/or hyperactivation of NADPH oxidase and Na^+/H^+ exchanger 1 (NHE1), respectively, and both NADPH oxidase and NHE1 activities are impaired by targeting CLIC1 function (67). In this scenario, functional expression and activation of tmCLIC1 trigger a feed-forward mechanism which involves the activity of NAPDH oxidase and NHE1 establishing a vicious loop which generates a cellular microenvironment that favors the abnormal proliferative rate of tumor cells (67).

Role of CLIC1 in Cancer Stem Cell Proliferation

The relevance of CLIC1 in tumor biology, and for GBM in particular, is further supported by the observation that tmCLIC1 is highly expressed in patient-derived GSC sub-populations (30, 68). Moreover, CLIC1-mediated chloride current is higher in GSCs isolated from neurospheres and expressing stemness markers (nestin, Sox2, Olig2), than in the differentiated GBM cell counterpart (68). Inhibition of CLIC1 activity using IAA94 (69), anti-CLIC1 N-terminus antibodies, or CLIC1 downregulation using small interfering RNA, causes a reduction of self-renewal, proliferation and *in vivo* tumorigenic ability of patients-derived GSCs (30, 68, 70).

This evidence strengthens the idea that CLIC1 plays a critical role in the tumorigenic potential of GBM-derived stem/progenitor cells. The differential functional expression

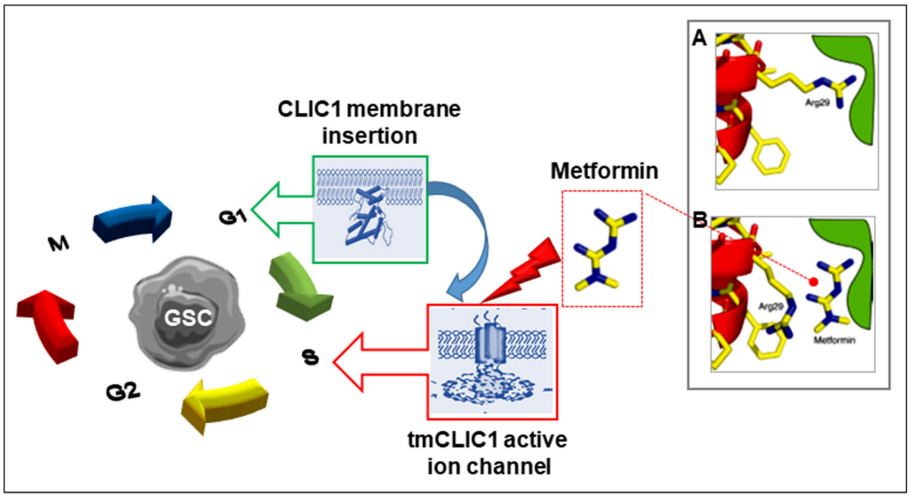

FIGURE 2 | Scheme of the proposed mechanism by which metformin and other biguanides interacts with CLIC1 in glioblastoma stem cells. CLIC1 is a main regulator of GSC functioning once expressed into the plasma membrane and acting as chloride ion channel (tmCLIC1). CLIC1 activity promotes cell cycle progression and cell division. While in normal cells this functional expression transiently occurs during G1/S phase transition, it is constitutive in cancer cells leading to accelerated growth rate. Metformin (and other biguanides) directly interact with the extracellular portion of the active tmCLIC1, interfering with its activity and inhibiting cell cycle progression with high specificity toward GSCs, due to the high activity of CLIC1 in these cells. The insert shows a schematic representation of the putative molecular site of CLIC1 blockade by metformin: **(A)** In the closed state of CLIC1, the side chain of Arg29 makes an interaction that destabilizes the closed state. This facilitates the opening of the channel; **(B)** Metformin (and possibly other biguanides) interacts with the amino terminus of the channel, presumably in the vicinity of Arg29 side chain responsible for pore opening, stabilizing the closed state and blocking the channel activity [modified from Gritti et al. (68)].

of CLIC1 between GSCs and their differentiated counterpart could represent a possible strategy to selectively recognize and hit the tumor stem cell subset. Thus, tmCLIC1 represents a potential ideal target for antineoplastic treatments, also as chemo-sensitizing approach which, hitting CSC subpopulations, may increase tumor responsiveness to conventional anticancer therapies.

THERAPEUTIC POTENTIAL OF CLIC1 PHARMACOLOGICAL TARGETING IN GLIOBLASTOMA

Rationale for Targeting CLIC1

To date, GBM represents the biggest challenge for cancer therapy. The main reason for the failure of GBM treatments is represented by tumor occurrence in one of the most critical area of human body, physically shielded by the skull and pharmacologically isolated by the BBB. Although GBM, as every tumor, represents a detriment from a clinical point of view, cancer cells may be considered an evolutionary successful model, being able to dynamically adapt the changing microenvironment and reprogram their own physiology setting in a new steady state. Tumor cells are in a chronic hyper-activated (allostatic) state (71) that supports their abnormal proliferative rate. A novel strategy for GBM treatment could be to hit one or more components that promote the assessment of the chronic allostatic state, restoring the physiological homeostasis (67). Several proteins, including NADPH oxidase and NHE1 exchanger, involved in the establishment of the allostatic condition (72, 73) are crucial for survival of both cancerous and normal cells, therefore limiting the possibility of their pharmacologic or genetic targeting. In this scenario, the peculiar ability of CLIC1 to change its functional localization depending on the activation state of the cells could be a compelling strategy to impair tumor cell proliferation and/or survival with a higher efficacy in CSCs (67). The possibility to selectively hit the CSC fraction could be instrumental for a more efficient activity of standard antineoplastic drugs, also considering that CLIC1 inhibition-dependent delay of G1/S phase transition might also favor microglia activity toward tumor cells, and potentiate conventional cytotoxic therapies.

Cellular and molecular steps through which ion channels, including CLIC1, support malignant cell phenotype and specifically CSC features (enhanced survival and proliferation rate, self-renewal, migration ability, and resistance to apoptosis and chemo- or radio-therapy) are still not completely defined. However, a growing bulk of evidence is currently available to be exploited in pre-clinical investigation or in medicinal chemistry studies for the identification of novel compounds able to target ion channels involved in cancer cell proliferation.

CLIC1 displays several peculiar features which render this channel an ideal pharmacological target in cancer cells. First, CLIC1 is overexpressed in several cancer types as compared to non-cancer cell counterparts; second, its activity is pivotal for cancer cell functioning; third, although in physiological conditions it is ubiquitously expressed, its chloride channel activity, absolutely dependent on its membrane insertion, is constitutive only in tumor cells and, in particular, in the CSC compartment (32). In fact, the translocation of CLIC1 to the membrane is reversible and the channel activity is transient

in normal cells, with only few channels active at a given time. On the contrary, in cancer cells the specific intracellular microenvironment generated by the high production of ROS and low pH levels (74, 75) enhances CLIC1 functional expression promoting its constitutive membrane localization as an active ion channel (67). Given that tmCLIC1 is largely more abundant in GSCs than in healthy tissues (30), the possibility to specifically hit the transmembrane isoform could be a promising novel therapeutic approach for GBM. Indeed, a successful therapy should slow-down the proliferation of GBM cells, preventing relapses by inhibiting GSCs with the minimum possible systemic toxicity. However, IAA94, the only known compound able to block CLIC1 activity *in vitro* (69), can't be used as a potential drug for GBM due to its off-target toxicity *in vivo*.

Importantly, recent studies identified the well-known antidiabetic drug metformin as a compound able to impair tmCLIC1 activity (68) (**Figure 2**). Metformin is a generally very well tolerated type 2 diabetes (T2D) first line drug, which displays antineoplastic effects, although the molecular mechanism at the basis of this effect is still debated. Thus, understanding at a molecular level how metformin interferes with cancer cell proliferation and the role of CLIC1 in such effect might improve its repositioning as antitumor agent or, alternatively, allow the development of structural-related molecules showing higher efficacy and potency against tmCLIC1.

DEVELOPMENT OF PHARMACOLOGICAL TOOLS TO TARGET CLIC1 ACTIVITY TO COUNTERACT GLIOBLASTOMA CANCER STEM CELL TUMORIGENESIS

The low clinical outcome of the available therapeutic approaches for GBM urges the identification of novel molecular targets and new molecules able to hit them. In this respect, as detailed in the previous paragraphs, CLIC1 represents a potential ideal candidate, for its relevance in GSC biology and its functional irrelevance in normal cells. If these premises are confirmed, a selective CLIC1 inhibitor should have high efficacy against tumor cells and low toxicity on the normal cell counterparts.

Unfortunately, the introduction of new molecular entities in clinics is becoming more and more difficult, due to the outraged increased costs of development and the tighter regulatory rules. Therefore, in the last years the approval of novel chemotherapeutics, with the only exception of biologicals, faces a significant slow-down. Drug repositioning, a strategy based on the identification of new disease indications and/or molecular targets for existing compounds, represents a drug discovery strategy which bypasses all the preclinical and early phase clinical trials and allows a faster, more efficient and less expensive way to bring molecules from bench to bedside. This is especially true if the studied drug has already proven good safety and tolerability profile in humans (76). Interestingly, a CLIC1 inhibitory activity was reported in some Chinese traditional medicine molecules, identified by bioinformatic strategies (77), although most attention has been dedicated to the effects of metformin a biguanide antidiabetic drug. In particular, it was shown that metformin is a powerful CLIC1 inhibitor in GSCs (78), and its repositioning as GBM drug could have a significant impact for the treatment of these patients. However, metformin represents the most studied repurposed drug in oncology in almost all human tumors and several intracellular mechanisms were proposed to mediate these effects. Thus, to show that CLIC1 inhibition is a primary target for this drug in GBM a pharmacological class effect should be demonstrated, showing that also structurally related drugs (i.e., containing a biguanide moiety) have the same biochemical mechanism (CLIC1 inhibition) and clinical effects (antitumor activity).

In the next sections we will discuss the general pharmacology of metformin (and of other biguanides), the evidence of their antiproliferative effects, and data showing that CLIC1 is one of the main molecular targets involved in the inhibition of GSC proliferation and invasiveness induced by this class of drugs, highlighting the pros and cons of their possible use for treatment of GBM.

Pharmacology of Biguanides

Biguanides are a class of drugs whose functional group consists of two guanidines linked by a common nitrogen (**Figure 3**); biguanides have a broad range of medical indications spanning from the first line pharmacological approach for T2D, by metformin and its derivatives phenformin and buformin (although the latter compounds are no longer used in therapy), to antimalarial prophylaxis and therapy by proguanil, and antiviral and antimicrobial activity by moroxydine, chlorophenylbiguanide, and chlorhexidine.

Original interest in biguanides derived from their potential antimalarial effects, particularly by proguanil the first compound of this class used in 1950, and still a current antimalarial drug. Proguanil is a synthetic arylbiguanide acting as oral prodrug and is considered the safest antimalarial compound; *in vivo* it is metabolized to the active derivative cycloguanil, which contains a cyclized biguanide moiety and acts as dihydrofolate reductase and folate synthesis inhibitor within malaria parasites (79). Proguanil is used for both malaria prophylaxis and treatment, in combination with atovaquone or cloroquine (80). The observation that this drug may cause hypoglycemia as side-effect, triggered the development of the dimethylbiguanide metformin (81).

Metformin was licensed as anti-diabetic agent in the UK in 1958, but only in 1995 in the USA, due to concerns about lactic acidosis and cardiac mortality, which, however, are now considered as very rare occurrences. Among the different biguanides introduced for diabetes therapy in late 1950s, metformin shows the better safety profile and tolerability (82). Two other biguanides, phenformin (phenethyl biguanide) and buformin (N-butyl biguaninde), although more potent than metformin as hypoglycemizing agents, were discontinued in 1970s due to the same adverse events (lactic acidosis and cardiac mortality) but occurring at higher rate than observed with metformin (83). In T2D patients, glucose-lowering effect of metformin is attributed to the reduction of hepatic glycogenolysis and gluconeogenesis, enhancement of insulin receptor tyrosine kinase activity, improvement of insulin

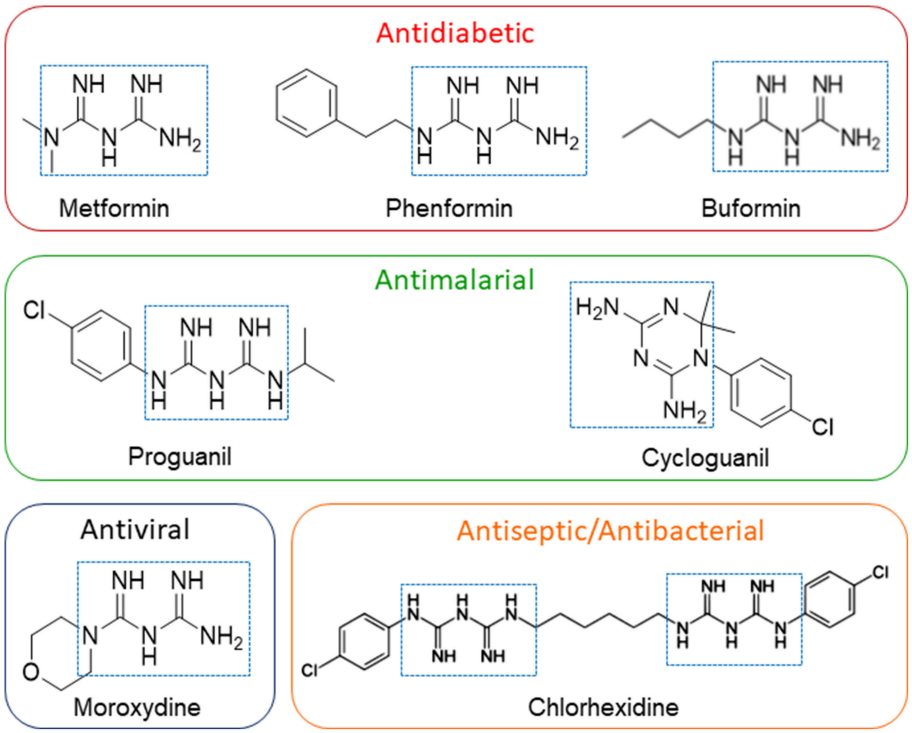

FIGURE 3 | Structures of drugs possessing a biguanide moiety as pharmacophore. The presence of the biguanide moiety in clinically-relevant compounds, highlighting their actual clinical applications, is evidenced by the dotted squares. Figure includes compounds affecting CLIC1 activity to inhibit GSC proliferation and self-renewal.

sensitivity, and reduction of enteric glucose absorption (84). Metformin also induces peripheral glucose uptake, increasing glucose transporter GLUT4 activity, and glycogen synthesis, stimulates glucagon-like-peptide-1 (GLP-1) release, and reduces lipolysis and triglyceride levels.

Moroxydine, is another heterocyclic biguanide proposed in the 1950s as anti-influenza agent. Moroxydine exhibits anti-viral activity against RNA and DNA viruses, and was originally used for prophylaxis or therapy of viral infections. Moroxydine has negligible side effects, but very little information exists on its mechanism of action (85). Despite its favorable pharmacological profile, moroxydine has been scanty investigated, and only recently it has gained new interest as potential anti-hepatitis C agent (86).

Repositioning of Metformin and Other Biguanides as Antitumor Agents

Repositioning of Metformin as Anti-tumor Agent

On the basis of several epidemiological and preclinical observations, several biguanides have been proposed to possess anti-neoplastic activity; to date, metformin is the most promising application of repositioning of a non-oncological drug as anti-cancer agent. Epidemiologic studies suggested a correlation between chronic use of metformin in T2D patients and the reduction of incidence and related mortality of several solid tumors, when compared to T2D patients treated with other classes of hypoglycemic drugs (87–89). These observations triggered a series of pre-clinical and clinical investigations in several tumor types to detail the antitumor mechanism of action of metformin and its potential efficacy as adjuvant agent in clinics (90, 91). In diabetic patients, metformin use was correlated to a reduced risk of development of different cancer types, including pancreatic cancer (92), and lung and hepatocellular carcinomas also increasing survival time (93–95). However, to date non-univocal results were reported in the many studies published. Some meta-analyses confirmed a significant association between metformin use and lower incidence of pancreatic, liver, renal, endometrial, prostate, breast, colorectal, and ovarian carcinomas, while no correlation was found in other studies (96–103). Metformin use in T2D patients with HER2-positive breast cancer was associated with a better outcome (104) and a meta-analysis in breast cancer patients reported a significant association between metformin therapy and the reduction of all-cause mortality without observing a reduction of breast cancer incidence in these subjects (105).

Repositioning of Other Biguanides

Although less investigated than metformin, also other biguanides were shown to possess anti-cancer activity. Phenformin exerts antitumor activity in preclinical models *in vitro* and *in vivo*, using ovarian cancer (106) NSCLC (107), and hepatocellular carcinoma (108) cells, and pancreatic cancer patient-derived xenografts (109); moreover, phenformin was also reported

to selectively affect the CSC compartment (110). Other studies showed that the antitumor activity of phenformin in mammary cancer was dependent on the inhibition of angiogenesis, apoptosis, and epithelial-mesenchymal transition (EMT) (111–113). The anti-cancer activity of buformin in rat mammary breast cancer carcinogenesis was also reported (114, 115).

However, compared to the other biguanides, the evidence of a potential broad antitumor activity, associated with the overall safety and low cost, has opened a new horizon for repurposing of metformin in oncology (87, 116, 117).

Repurposing of Biguanides for Targeting of CSCs

Several preclinical studies reported that metformin is effective against CSC subpopulations, the key target for all antitumor pharmacological approaches, at odd with most conventional anti-neoplastic drugs which have little or no effect on CSCs (118). Selective anti-cancer properties of metformin have been described in CSC-like cultures derived from colorectal (119), gastric (120), breast (121, 122), prostate (123), pancreatic (124, 125), and ovarian (126) carcinomas and osteosarcoma (127, 128). Metformin effects on CSCs include the impairment of self-renewal and survival, decreased expression of stemness markers, the slow-down of cell cycle progression and inhibition of invasiveness. Moreover, a chemo-sensitizing activity of metformin, helping to overcome refractory CSCs to radiotherapy (129), and chemotherapeutic agents (130) has been also described. As far as GBM, metformin was reported to synergize with temozolomide (131) and reduce the acquired resistance to this alkylating agent (132).

While most of the human tumors, at least at preclinical level, are affected by metformin, this drug is almost completely harmless for normal cells. The low toxicity observed in T2D patients after chronic treatment already suggested this eventuality, but it was directly demonstrated by *in vitro* experiments, in which metformin concentrations able to reduce CSC viability were ineffective in normal cells, including MSCs (68, 70, 133). These data clearly suggest that a tumor-specific target should mediate metformin antitumor effects.

To date, mainly pharmaco-epidemiologic and preclinical data were at the basis of the assumption that metformin may be useful in cancer prevention or treatment. However, hundreds of clinical trials are in progress to validate this hypothesis (see www.clinicaltrials.gov). Translation from retrospective to prospective trials however, is not easy-going also in light of several biases often present in retrospective studies (134). Some preoperative or neo-adjuvant window of opportunity studies reported a decrease in the expression of Ki-67, a marker of cell proliferation, after metformin treatment in breast, prostate, and endometrial cancers (135–137), although another study found no effects in breast cancer (138). An unpublished study (NCT01620593) found a significant decrease of prostate-specific antigen (PSA) after treatment of prostate cancer patients with metformin, while, in ovarian cancer (NCT01579812) no changes in progression-free and overall survival were reported (www.clinicaltrials.gov). Only few prospective studies have been to date published, reporting that metformin provided benefit in patients in colorectal adenoma and, in association with paclitaxel, in non-small cell lung cancer patients (139, 140).

Phenformin has been evaluated in a clinical trial (NCT03026517) in combination with dabrafenib and trametinib (RAF and MEK inhibitors, respectively) in patients with BRAF-mutated melanoma, but, till now, no results are available.

Overall the available literature data about the clinical antitumor efficacy of metformin are not conclusive, possibly due to the heterogeneous composition of patient cohorts, the study design, pharmacokinetics and posology discrepancies, as well as variable responses in different cancer types (141). Thus, repositioning of metformin and, potentially, other biguanide derivatives, in oncology is still a controversial topic, and results from clinical trials that are going to be concluded in the next years in different cancer types, mainly investigating the adjuvant efficacy of metformin in association with chemo- and radiotherapy, will provide a clearer picture of its clinical impact.

Notwithstanding these unsolved problems, a huge amount of data has been produced to detail the molecular mechanism(s) of the antiproliferative activity of metformin.

Molecular Mechanisms of Metformin Antitumor Effect

Although numerous experimental studies analyzed the antiproliferative, pro-apoptotic, and anti-invasive activity of metformin, at present, the exact molecular mechanisms through which this drug exerts its antitumor activity is only partially known. In fact, most of the possible intracellular pathways involved in tumor cell proliferation have been reported to be affected by metformin treatment in different cancers. Consequently, not only metformin seems to not display tumor specificity but also its activity seems to involve a wide plethora of intracellular signaling pathways.

The classical intracellular pathway proposed as molecular target for metformin antitumor effects has been derived by the mechanism activated in the liver to control glucose release (142). Metformin affects the energetic balance interfering with the complex I enzymes within mitochondrial respiration, reducing ATP content and the ATP/ADP ratio (143). This alteration, altogether with a direct regulation via liver kinase B1 (LKB1), causes the activation of the cellular energy sensor AMP-activated protein kinase (AMPK), which in turn leads to the inhibition of mTOR (144, 145), a kinase acting as crucial mediator of tumor cell metabolism (146). AMPK, activated after metformin treatment, was reported to directly phosphorylate PD-L1 causing its endoplasmic reticulum (ER) accumulation and ER-associated protein degradation. In fact, breast cancers from metformin-treated patients exhibit reduced PD-L1 levels, which enhances cytotoxic cell immunity against cancer cells (147). In addition, metformin, lowering the plasma levels of insulin and insulin-like growth factors, might indirectly inhibit the PI3K/Akt/mTOR pathway (148). AMPK activation following metformin treatment has been described in several human cancer cell types including breast (149, 150), endometrial (151), ovarian (152), pancreatic (153, 154), lung (155), prostate (123), head and neck (156), and colon carcinomas (157), often correlating with antiproliferative effects.

However, AMPK-independent pathways have gained increasing attention. Metformin was reported to directly inhibit mTOR signaling by inactivating Rag GTPases (158), or inducing cell cycle arrest through REDD1, a negative regulator of mTOR (159). Furthermore, several other intracellular effectors were reported to be modulated by metformin treatment to reduce cell proliferation, including, among others, the VEGF/PI3K/Akt pathway (160) in prostate cancer cells, Sonic hedgehog (Shh) signaling pathway in gastric cancer cells (161), inactivation of p38 MAPK and activation of ERK3 (both effects leading to inhibition of mTORC1, in which AMPK was only partially involved) in intrahepatic cholangiocarcinoma cells (162), reversal of the activation of ERK1/2 in ovarian cancer cells (163), inhibition of CLIC1 in gallbladder cancer cells (164); metformin also counterbalanced the overactivation of Notch1/Hes1 signaling observed in colorectal cancer patients (165), and induced apoptosis via the up-regulation of adenosine A1 receptor in human colorectal cancer cells (166). Other putative mechanisms of metformin anti-tumor activity involve the reduced RANKL (167) or caveolin 1 (168) expression in breast CSCs, and HIF-1α gene expression in oral squamous cell carcinoma cell lines, which caused inhibition of cell proliferation and migration (169). Moreover, metformin antiproliferative activity was also ascribed to enhanced autophagy in cancer cells, which causes cell cycle arrest or apoptosis (170, 171), and the modulation of miRNA activity (172, 173).

In addition, metformin downregulates ROS production of through inhibition of mitochondrial complex I (174, 175), and possesses anti-inflammatory and immunomodulatory activity, affecting energy metabolism of immune cells and stimulating $CD8^+$ tumor infiltrating lymphocytes leading to a cytotoxic response against cancer cells (176); moreover, metformin enhances immune response *in vivo* in mouse melanoma model (177), and inhibits NF-κB nuclear localization and Stat3 activity in breast cancer CSCs (178).

Metformin disrupts TGFβ-mediated oncogenesis and invasiveness (179) either by direct binding (180) or by blocking autocrine TGFβ1 signaling (181). TGFβ1-dependent metastasization and invasive effects are mainly mediated by epithelial-to-mesenchymal transition (EMT). In this context metformin acts as EMT suppressor in different epithelial tumors (e.g., melanoma, colon, breast, lung, prostate, and thyroid cancer cells) (182–185). In prostate cancer, metformin represses EMT and metastasis by targeting the COX2/PGE2/STAT3 axis (186), while in breast cancer the AKT/mTOR/ZEB1 pathway was involved (187). Metformin also directly affects cancer cell metabolism interfering with glycolysis and the tricarboxylic acid cycle, decreases the production of ATP, NADH-linked respiration in cells and mitochondria, and the aspartate biosynthesis (188, 189), while induces indirect antiproliferative effects reducing hormones, cytokines and growth factor production (144, 190, 191).

Altogether, these preclinical studies, reporting metformin ability to modulate multiple, apparently unrelated mechanisms, strongly support its antitumor activity. However, the unexpected and unprecedented high number of different intracellular mechanisms regulated by a single drug in such different tumor cell types, suggests that most of these intracellular pathways could be indirectly modulated, being downstream from a common tumor-specific target directly affected by metformin.

However, it is worth to note that several unsolved issues are present in these studies. First, metformin concentrations used to cause antitumor effects in all *in vitro* studies here reported, largely exceed those obtained by the antidiabetic doses, and are difficult to be reached in patients. A second issue puzzling the anti-cancer use of metformin is its hydrophilic nature which limits its passive diffusion into cells (192), making necessary organic cationic transporters (OCT1, OCT2, and OCT3) for its internalization within cells (193, 194) and to cross the blood-brain barrier (195). The overexpression of these transporters is considered at the basis of the observation that metformin concentration in tissues is much higher than in plasma, and in tumors higher than in normal cells. Intratumor accumulation of metformin, induced by OCTs, has been involved in the direct antineoplastic activity of this biguanide (196). For example, in mammary tumor-bearing rats and in ovarian tumor biopsies form metformin-treated patients, metformin effects were dependent on high intratumor concentrations, which in the mammary cancer model were related to OCT2 expression (197, 198). Thus, it was suggested the possibility to potentiate metformin antiproliferative activity, obtaining clinically relevant concentrations due to the specific drug accumulation within tumors. This opportunity was demonstrated using pharmaceutic preparations and routes of administration different from the oral way (i.e., subcutaneous) allowing a topical tumor treatment (199–201).

Potential Role of Metformin and Other Biguanides as Antiproliferative Agents for Glioblastoma Stem Cells

Although *in vitro* studies reported the antiproliferative and proapoptotic efficacy of several drugs on GSC cultures (202–204), the same activity in patients was never reported. Thus, the lack of effective antitumoral drugs for GBM patients, pushed the testing of metformin repositioning in *in vitro* and *in vivo* GBM models (78).

Metformin reduces survival and proliferation rate not only of GBM cell lines (131, 205–207) but also of patient-derived GSC cultures (68, 70, 208–211) suggesting its efficacy to impair mechanisms involved in cancer cell stemness. This effect is time-dependent, since prolonged treatment caused significant antiproliferative effects also for relatively low concentrations (68). Importantly, metformin interference with GSC activity was further supported by the observation that, beside proliferation, several distinctive stemness features were also impaired in metformin-treated cultures, including self-renewal ability, as shown by colony-forming and spherogenesis assays (70, 132, 210, 211), migration and invasiveness (132, 210, 211) (**Figure 4**), and EMT (187), which is activated in GSCs to sustain GBM aggressiveness (212).

Metformin was also tested in combination therapy with classical anti-cancer drugs, mainly the alkylating agent temozolomide, the GBM standard of care. *In vitro* and *in vivo* studies describe a potential synergism between the

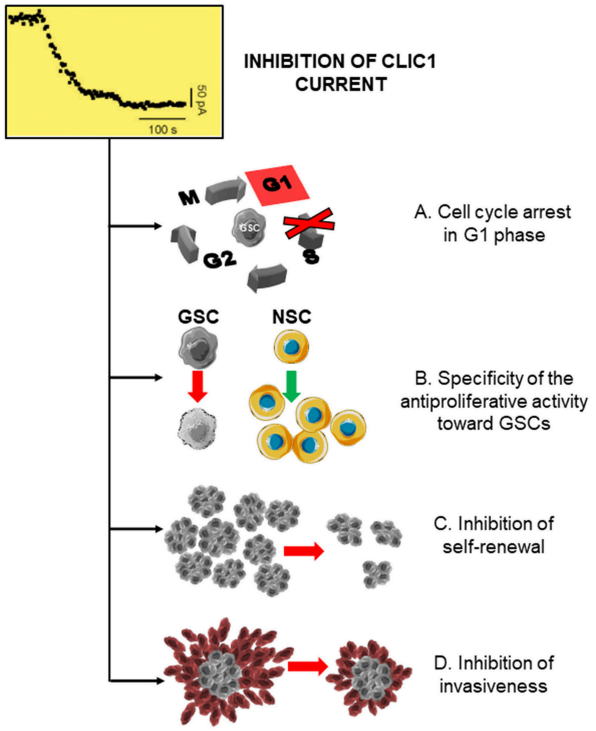

FIGURE 4 | CLIC1 inhibition leads to the inhibition of glioblastoma stem cell proliferation, self-renewal, and invasiveness**.** Inhibition of CLIC1 activity by metformin and other biguanides causing a decrease in the chloride current induces several inhibitory effects in GSCs, including: **(A)** cell cycle arrest and cell accumulation in G1 phase, acting **(B)** selectively in GSCs, while sparing normal stem cells (NSC); **(C)** impairment of GSC self-renewal ability; and **(D)** inhibition of the invasive behavior.

antiproliferative effects of metformin and temozolomide in GBM cell lines and GSCs (131, 132, 206, 207, 213). Moreover, a strong synergism between the antitumor effects of metformin and *in vitro* cell irradiation (5Gy), in the presence or absence of temozolomide, was also reported in GBM U87, U251, LN18, and SF767 cells (206).

Also in GBM several intracellular mechanisms mediating the antiproliferative and anti-invasive activity of metformin were reported. These include the inhibition of STAT3 (214) and Akt (70), the induction of apoptosis by increasing Bax/Bcl-2 ratio, reduced ROS production when co administered with temozolomide (213), or the downregulation of AKT-mTOR signaling pathway (207). Although some studies proposed an AMPK-dependent mechanism for the antitumor activity of metformin (208, 209, 215, 216) in other studies the inhibition of proliferation and self-renewal occurred in the absence of AMPK activation (70). Moreover, comparing the effects of metformin with a "pure" AMPK activator, the peptide A769662, which was unable to inhibit mTOR and GBM cell proliferation, it was shown that metformin suppresses GBM proliferation enhancing PRAS40–RAPTOR association to inhibit mTOR, independently of AMPK (217). Although this issue is still debated, recent data seem to confirm that the activation of AMPK and the inhibition of mTOR are not the main targets in GBM. In fact, on one hand a randomized phase II study assessing the efficacy of everolimus in combination with chemoradiation showed that mTOR inhibition does not improve GBM patients PFS (218), and, on the other, AMPK was shown to be chronically activated under cancer-associated stress conditions, to increase proliferation and survival. Moreover, AMPK inhibition reduces viability of patient-derived GBM stem cells (GSCs) (219), clearly indicating that, at least in GBM, AMPK activation cannot justify metformin antiproliferative effects and different molecular targets have to be found.

Furthermore, several studies showed that metformin activity was selectively directed against GSCs rather than differentiated glioma cells (68, 70, 211), indicating that a CSC-specific target mediates its activity.

In vivo, metformin significantly impairs GBM growth either after subcutaneous (206–208, 217) or intracranial grafting (210, 213, 220) in immunocompromised mice. These effects were obtained mainly after i.p. injection, although in one study (207) metformin was administered *per os* by gavage. In the cited studies, metformin induced a slow-down in tumor growth and prolonged mice survival, mainly acting in synergy with temozolomide or 2-deoxyglucose, inducing a significant effect also in temozolomide-resistant cells (213). However, it is worth to note that most studies were carried out on human GBM cell lines (mainly U87, U251) and only in few cases patient-derived GSCs were used (208, 210).

Phenformin exerts antitumor effects in GSCs overcoming resistance to temozolomide, suppressing GSC self-renewal via the reduction of the expression of stemness and mesenchymal markers, and the increase of miR-124, miR-137 and let-7 expression (221). Phenformin activity has been also analyzed as potential way to disrupt energetic/metabolic pathways sustaining GSC survival and proliferation (222, 223). *In vivo*, phenformin added to the drinking water, caused a significant inhibition of the growth of GBM, in an orthotopic model in which patient-derived GSCs were grafted in nude mice (221), confirming that beside metformin also other biguanides are active against GBM.

In fact, biguanides, unrelated to the antidiabetic drugs (i.e., moroxydine, and cycloguanil) were also reported to significantly reduce proliferation, self-renewal and invasiveness of GSCs, showing higher *in vitro* potency than metformin (211). This observation suggests that antitumor activity against the GBM stem cell-like compartment is a common feature of all biguanides. However, if this is true, all the biguanide moiety-containing molecules have to act through a common intracellular mechanism and all the other pathways proposed for metformin antitumor activity should represent tumor-specific downstream effectors dependent on a common effector which represents the direct biguanide target.

CLIC1 as Preferential Molecular Target Mediating Metformin, and Other Biguanides, Antitumor Effects in Glioblastoma Stem Cells

The controversies about the anticancer mechanisms of metformin led to overemphasize the role of AMPK in GSC

antiproliferative effects, since the liver anti-hyperglycemic activity of this drug is mediated through the activation of this kinase (142). However, a growing bulk of evidence reported, in different cancer models, and in CSCs in particular, that (i) several AMPK-independent pathways are activated by metformin (70, 217); (ii) contrarily to what initially hypothesized, AMPK agonists enhance cancer cell proliferation and metabolism under metabolic stress (i.e., A-769662), while metformin and phenformin inhibit these cellular functions in an AMPK-independent manner (224); (iii) other compounds with a biguanide structure (i.e., moroxydine and cycloguanil), used with different clinical indications, and devoid of AMPK-related effects in the liver, induce the same anti-proliferative and anti-invasive activity in GSCs (211). The latter evidence strongly supports the possibility that, in GSCs, a common molecular target can be hit by all the biguanide-based compounds representing a new pharmacological class effect.

In this line of research, the observation that metformin and related compounds exert their activity on CSCs, not only in GBM (70, 208, 210, 211, 221) but also in different tumor types, such as breast cancer (121), while differentiated cells composing the tumor mass are relatively spared, clearly indicates that biguanides should interact with a CSC-specific target. In recent years, among the possible cancer-specific molecular targets for metformin, CLIC1 has been proposed to represent the main transducer of the biguanide effects in GSCs (68, 211). As detailed in the previous paragraphs, CLIC1 behaves as CSC-specific target because, although expressed in most normal and differentiated (non-stem) tumor cells, it is mainly present as inactive cytosolic monomer, with a very low activation rate (68, 211). This activation kinetics renders non-CSC subpopulations (and normal cells) relatively independent from CLIC1 for proliferation and survival. Conversely, CLIC1 is functionally expressed in GSCs, where it shows a constitutive activity with a peak at the G1-S transition (67), and its activity is absolutely necessary for GSC proliferation (**Figure 2**). Metformin treatment causes a significant inhibition of CLIC1 activity, measured by voltage-clamp electrophysiology experiments (**Figure 4**), reaching at high concentrations (5–10 mM) the same efficacy observed using IAA94. Electrophysiology experiments showed that metformin perfusion decreases the whole cell current that cannot be further reduced by the perfusion of IAA94. Current/voltage relationships show that the current amplitudes, at different membrane potentials, are superimposed, suggesting that the two drugs converge on the same molecular target (68). By single amino acid mutation experiments, metformin was also shown to directly interact with tmCLIC1 through Arg29 located within the inner side of the pore structure of the channel (68) (**Figure 2**). Interestingly this binding site is different from that of IAA94 identified as the external Cys24 (35) allowing a possible discrimination between the effects of the two drugs.

CLIC1 blockade directly correlates with the antiproliferative effects of metformin causing GSC arrest in the G1 phase of the cell cycle. Conversely, metformin, used at the same concentrations, was harmless for cells in which CLIC1 activity was negligible (i.e., MSCs or differentiated GBM cells) confirming the specificity of these effects for GCSs (**Figure 4**). Moreover, the down-regulation of CLIC1, while reducing the growth rate of GSCs (30) also diminished the antiproliferative activity of metformin, corroborating the hypothesis that, at least in these cells, CLIC1 is the main target of this biguanide (68, 211). This evidence implies that, although metformin directly or indirectly modulates different intracellular signaling, the inhibition of CLIC1 activity is sufficient and necessary to induce antiproliferative activity, at least in GSCs. Moreover, it is important to remark that the inhibition of a GSC-specific molecular target (i.e., tmCLIC1) confers metformin with high selectivity against tumor cells, while sparing normal cells, as also confirmed by the known very low toxicity observed when metformin is used as antidiabetic agent. This observation provides the molecular basis for metformin repositioning as promising novel antitumor agent, being at the same time highly effective toward tumor cells and causing low systemic toxicity (78).

A main issue in metformin-induced tmCLIC1 blockade (and in its antitumor activity, in all the tumor models analyzed) is the high drug concentration (up to 10 mM) required to induce an effect.

Thus, a potential new a therapy could be really successful if retains the efficacy and the discrimination capability among healthy and cancer cells, provided by CLIC1 localization, and the ability to block tmCLIC1, as metformin, but acting at lower doses. The search for novel, more potent tmCLIC1 inhibitors can have a big advantage whether the channel targeting ability can be shared by different structurally-related molecules representing a pharmacological class effect for biguanides. In this line, it is relevant that also phenformin and buformin, former antidiabetic biguanides, were reported to behave as anti-tumor agents (114).

The possible role of CLIC1 as molecular determinant of biguanide antitumor class effect has been recently analyzed in several patient-derived GSC cultures (211). In this study, metformin and phenformin, representative of the antidiabetic biguanides for which antitumoral activity was already proposed, were compared with two antimalarial compounds, proguanil and cycloguanil, and the antiviral compound moroxydine. All these molecules inhibited GSC proliferation, self-renewal, migration and invasion, showing a much higher potency that metformin (up to 50 fold lower IC_{50} than metformin observed with cycloguanil). However, proguanil effects were not specific since it was similarly toxic for GSC and normal stem cells. The direct effects of these molecules on CLIC1 activity were measured by electrophysiology experiments. All the compounds, but proguanil, exerted a significant inhibition of CLIC1-dependent ion current, acting at potency and efficacy related to the respective antiproliferative activity. The lack of efficacy of proguanil as far as CLIC1 inhibition, was proposed to be dependent on the simultaneous presence of the 1-(4-chlorophenyl) ring and the bulky 5-isopropyl group on the rigid biguanide skeleton, thus preventing the access to CLIC1 pore region.

Evidence from this study strengthen the hypothesis that molecules containing a biguanide moiety are potent CLIC1 inhibitors and, consequently, drugs able to selectively interfere with GSC proliferation, migration and self-renewal. Importantly,

higher potency than metformin on both cell proliferation and CLIC1 activity inhibition was also demonstrated by these biguanides, suggesting that more easily reachable concentrations of these drugs could be similarly active as the high doses of metformin. Although all these drugs have known limitation for chronic use in patients with GBM, these data demonstrated that CLIC1 inhibition is not only a pharmacological property of metformin, but it may represent a class effect endowed of all the compounds containing a biguanide structure. The relevance of this information resides in the possibility to develop novel biguanide containing drugs, which retain the safety profile of metformin but endowed with increased efficacy and potency toward GSCs.

CONCLUSIONS AND FUTURE PRESEPECTIVES

At odd with most malignant tumors, therapeutic perspective for GBM did not significantly progress in the last decades. In this context, GSCs play a central role in drug resistance, being still extremely elusive as far biological features and pharmacological sensitivity. However, the recent identification that CLIC1 activity is necessary for GSC proliferation, self-renewal and invasiveness, while it is dispensable for most non-transformed normal cell populations, opened new perspectives in the potential development of new therapeutics for this still incurable tumor. This observation found new strength after the recent report that metformin is an efficient inhibitor of CLIC1 activity, although with low potency (IC_{50}: 10–30 mM) (211). These data are extremely relevant due to the strong interest in metformin repositioning as antitumoral agent. Several epidemiological, preclinical, and, more recently, some clinical trials are addressing the efficacy of this biguanide in basically all the human tumor types. Conversely, pharmacokinetic and even pharmacodynamic issues are still unsolved to better translate this information in a clinical setting. In particular, as far as GBM is concerned, the main intracellular mechanism associated to metformin antiproliferative activity, the activation of AMPK and the consequent mTOR inhibition, had to be discarded since AMPK was discovered to promote GSC proliferation. Thus, metformin antiproliferative activity has to depend on a completely different mechanism from its glucose-lowering effects. Among all the reported intracellular pathways affected by metformin in tumor cells, the inhibition of CLIC1 activity is of particular interest since it is GSC-specific (thus its targeting does not affect normal cell viability), in line with the low toxicity of the drug when chronically used in T2D patients. Moreover, this effect was directly evaluated by electrophysiology measurement preventing the possibility of effects mediated indirectly by other biochemical regulators. This observation supports that, in GSCs, the inhibition of CLIC1 is a common effect different drugs containing a biguanide structure.

In conclusion, the inhibition of CLIC1 is a novel and unexpected biguanide class effect, which could be used to develop novel drugs with a strong efficacy against GSCs. In fact, although all the biguanides to date tested as inhibitory of CLIC1 activity in GSCs are not completely satisfactory as far as pharmacokinetics and long term tolerability, we believe that this information might pave the way for the identification of novel structurally-related molecules, which in future will provide a better clinical outcome for GBM.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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