Targeting Glioblastoma: Advances in Drug Delivery and Novel Therapeutic Approaches

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oligodendroglia. Further sub classification relies on molecular profiles and gene alterations. For instance, mutation in isocitrate dehydrogenase (IDH1) is more often found in secondary GB and is associated with better prognosis. Other genomic alterations commonly found in GB include loss of chromosomal arms 1p and 19q and mutations in EGFR, TP53, and PTEN genes. These genetic alternations can be targeted for precision medicine and drug delivery. Overexpressed and mutated cell surface proteins such as EGFR and Transferrin receptors can be used as targets for directing and binding moieties. Mutations which regulate tumorigenicity can be modulated by delivery of oligonucleotides to the tumor. GB heterogeneity represents a big challenge for GB therapy and requires multiple precise and targeted approaches. Since GB is a highly angiogenic tumor, patients often receive anti-vascular endothelial growth factor (VEGF) treatment. Bevacizumab is a monoclonal antibody which neutralizes VEGF function, thus normalizing blood vessels and delaying angiogenesis. Treatment with Bevacizumab does not improve overall survival but prolongs progression-free survival (PFS). In contrast, the angiogenic properties of GB, including hyperpermeability and a compromised blood brain barrier (BBB), can be exploited for drug delivery. One of the biggest challenges in GB treatment is the BBB, which prevents extravasation of drugs from the bloodstream into the brain tissue. The BBB is composed of specialized endothelial cells forming tight junctions and controlling the intake and outtake of different substances from the brain using efflux pumps such as permeability-glycoprotein 1 (Pgp; also known as ATP-binding cassette sub-family B member 1 (ABC1), multidrug resistance protein 1 (MDR1) or cluster of differentiation 243 (CD243)) and other ABC transporters. An additional important component of the BBB are astrocytes, which physically support endothelial tight junctions and secrete factors that regulate the BBB integrity.

Therefore, large efforts are dedicated to the development of a nanomedicine which will be able to pass through the BBB, accumulate in the tumor site and be efficacious against such a heterogeneous, invasive and resistant tumor. Drug delivering nanoparticles may cross the BBB following adjustment of their chemical properties (i.e., hydrodynamic diameter size, zeta potential, hydrophobicity) or addition of targeting moieties binding to specific receptors and entering the brain by transcytosis mechanism. Different approaches for transit breakdown of the BBB are under development as well as drugs and treatments to be delivered locally into the tumor area in the brain. The brain microenvironment also plays a crucial role in cancer cell invasion and blood vessel formation. Microglia which are the brain resident immune cells, and astrocytes which are the most abundant glial cells in the brain, have been shown to facilitate tumor progression and immune suppression. Current immunotherapies have yet to show benefit for GB therapy. However, extensive research focuses on finding new targets in the brain microenvironment and stimulating the immune response against the tumor.

As there are many factors limiting the therapeutic options for GB patients, the use of nanomedicine and targeted approaches may improve the delivery, specificity and efficacy of current and new therapies. Here, we will discuss in detail the latest achievements and advances in GB therapy and the potential use of nanotechnology and nanoscience to improve patient outcome.

### 1.1. Extravasation-Dependent Nanoparticles

#### 1.1.1. Chemotherapeutic Delivery

Several nanoparticles (NPs) have been developed in an effort to overcome the main obstacles in GB therapy, such as liposome-based NPs, amphiphilic micelles, dendrimers, inorganic NPs, and polymeric NPs. A suitable nanocarrier for cancer therapy may protect the drug from degradation, improve its solubility, prolong plasma half-time, increase tumor accumulation, provide sustained drug release, and consequently increase the therapeutic efficacy and safety. Nanocarriers can passively target solid tumors taking advantage of the enhanced permeability and retention (EPR) effect. The leaky vasculature and deficient lymphatic drainage in the tumor allows nanocarrier extravasation and accumulation in the tumor site due to its unique physico-chemical features. Nanostructures smaller than 10 nm suffer from fast kidney clearance. In contrast, structures larger than 200 nm might suffer from hepatic filtration and recognition by the Reticuloendothelial System (RES) reducing their circulation time in the bloodstream. Surface charge and hydrophobicity play an important role in order to avoid aggregation, opsonization and immunogenicity. Cationic NPs tend to internalize more than anionic ones but might trigger immunogenicity by activation of the complement system. Thus, nanocarriers with neutral charge and hydrophilic surface will accumulate in the tumor site more efficiently exploiting the EPR effect. Several strategies have been applied to increase nanocarriers circulation time by coating with hydrophilic polymers such as chitosan, polyethylene glycol (PEG), dextran, or polyvinylpyrrolidone (PVP).

Up to date, several liposomal nanoformulations have been established for the treatment of different types of cancer, some of which have reached the market (Lipoxal, Lipoplatin, DaunoXome, Caelyx). Lipid-based nanocarriers, such as liposomes or lipid nanocapsules (LNCs), show high biocompatibility, biodegradability, protect drugs from the surrounding environment, and can encapsulate both hydrophilic and hydrophobic drugs. Few nanocarriers loaded with chemotherapeutic drugs have been developed for GB therapy and some are being evaluated in clinical trials (Table 1). Lipid-based nanomedicines are being and some have already been tested in clinical trials for GB treatment. These nanoparticles have been shown to reduce the systemic toxicity of chemotherapeutic agents, to improve their pharmacokinetics and enhance their selective accumulation in the tumor site. Polymeric nanoparticles (PNPs) are considered to be the most promising alternative to lipid-based carriers for the delivery of chemotherapeutic agents to the brain. PNPs include poly-(alkyl cyanoacrylate) (PACA), poly-(l-glutamic acid) poly (lactide-co-glycolide, PLGA), polyactide (PLA), polyethyleneimine (PEI), N-(2-hydroxypropyl)methacrylamide (HPMA) copolymer, poly(ethylene glycol) (PEG), dextran, and chitosan. Therapeutic agents can be either chemically conjugated or encapsulated in the PNPs, offering high loading efficiency and scalability. Compared to liposomes, the PNPs might be more stable during circulation in the blood and provide a better control over the drug release profile. For example, PLGA nanoparticles loaded with camptothecin (CPT) have been already shown to prolong survival in a GL261 GB mouse model, although they have failed to encapsulate efficiently...
The permeability and retention in brain tumors is highly heterogeneous and changes during tumor development, thus it may not be as relevant as in other cancer tissues. Moreover, there is a gap between preclinical animal models and the EPR effect presented in humans. This challenges the translation of EPR-based NPs from the bench to the bedside. The main differences rely on the amount and size of fenestrations in the tumor endothelium, the pericyte distribution, the presence of hypoxic and acidic areas as well as the differences in the interstitial fluid pressure (IFP) induced by the ECM. Several delivery systems have been developed in order to enable treatment of GB with additional cytotoxic agents beyond TMZ (Table 1). Only a few have reached clinical trials. This highlights the need for advanced targeting and/or therapeutic strategies.

### 1.1.2. Oligonucleotides Delivery

Oligonucleotides (ONTs) are short, synthetic polymers (13–30 nucleotides long) that bind to RNA or DNA and specifically modulate gene expression. ONTs developed for therapeutics include antisense oligonucleotides (ASO), small interference RNA (siRNA), and microRNA-modulatory oligonucleotides (antago-miRs and miR-mimics). ONT-based therapeutics have several advantages, such as high specificity and capability to regulate several cellular pathways, therefore avoiding the development of resistance. However, ONTs are not stable, and their size and charge prevent them from crossing the BBB. Therefore, ONTs must be modified to improve their nuclease resistance, tissue distribution and cell uptake.

Recent developments that enable ONTs delivery to gliomas are reviewed herein.

Neuroactive lipids were proven to improve ONT uptake and distribution in GB. Imetelstat (GRN163L), a lipidated 13-mer-thiophosphoramidate ONT, shows high affinity for the telomerase RNA (hTR or hTERC). Imetelstat serves as a competitive telomerase inhibitor that showed promising results in preclinical GB models. It is highly resistant to nucleases due to the thiophosphoramidate modification, and the palmityl modification confers enhanced penetration and retention properties. Imetelstat showed high toxicity in children with ependymoma, medulloblastoma and high-grade glioma. However, it achieved significant inhibition on both peripheral blood mononuclear cells and intratumoral response.

Cationic Nanoemulsions (NE): Cationic NE have emerged lately as promising systems for brain delivery of ONTs, forming neutral complexes that facilitate cell uptake. CD73, an enzyme responsible for adenosine (ADO) production, is overexpressed in several types of cancer cells including GB cells. Braganhol and his group have recently developed a cationic NE to deliver CD73 siRNA (NE-siRNA CD73R) via nasal route to deliver CD73 siRNA (NE-siRNA CD73R) via nasal route to deliver CD73 siRNA (NE-siRNA CD73R) via nasal route.

### Table 1. Nanoparticles for chemotherapeutics delivery in preclinical and clinical studies for GB therapy.

| Carrier    | Drug             | Mechanism of action          | Status             | Major Findings                                                                 | Reference |
|------------|------------------|------------------------------|--------------------|---------------------------------------------------------------------------------|-----------|
| Liposome   | Oxaliplatin (Lipoxal) | Alkylating agent             | Preclinical in vivo | Reduced systemic toxicity and prolonged survival in rats F98 tumor models      | [46]      |
| Liposome   | Cisplatin (Lipoplatin) | Alkylating agent             | Preclinical in vivo | Reduced systemic toxicity and prolonged survival in rats F98 tumor models      | [46]      |
| Liposome   | DN (DaunoXome)   | DNA Intercalating agent      | Clinical Trial Phase I | The efficacy in high-grade glioma pediatric patients remains to be undetermined | [47]      |
| Liposome   | DOX (Myocet)     | DNA Intercalating agent      | Clinical Trial Phase I | The efficacy in high-grade glioma pediatric patients remains to be undetermined | [48]      |
| PEG-Liposome | DOX (Doxil)     | DNA Intercalating agent      | Clinical Trial Phase II | Did not add significant clinical benefit                                       | [49]      |
| PEG-Liposome | DOX (Caelyx)    | DNA Intercalating agent      | Clinical Trial Phase II | Well tolerated and modest clinical benefit                                       | [50]      |
| PEG-Liposome | TMZ             | Alkylating agent             | Preclinical in vivo | Enhanced pharmacokinetics in naïve rat                                           | [51]      |
| PEG-grafted LNC | Fc-diOH     | ER-independent toxicity      | Preclinical in vivo | Reduction of systemic toxicity and tumor volume in 9L GB in rat model          | [65]      |
| PEG-grafted LNC | Fc-diAC or Fc-diPal | ER-independent toxicity      | Preclinical in vivo | Prolonged survival in 9L GB rat model                                            | [66]      |
| PEG-grafted LNC | ansa-Fc-diOH  | ER-independent toxicity      | Preclinical in vivo | Reduction of systemic toxicity and tumor volume in 9L GB in rat model          | [67]      |
| C-LNCs     | Curcumin        | Sp-1 deactivation            | Preclinical in vivo | Prolonged survival in C6 GB rat model                                            | [68]      |
| CBSA-MPEG/PLA NPs | Aclarubicin ACL | DNA Intercalating agent      | Preclinical in vivo | Reduced systemic toxicity and prolonged survival in C6 GB model                | [69]      |
| PLGA NPs   | TMZ             | Alkylating agent             | Preclinical in vivo | Poor nanoparticle loading and sustained release                                | [81]      |
| PLGA NPs   | CPT             | Topoisomerase inhibitor      | Preclinical in vivo | Prolonged survival in GL261 GB mice model                                       | [82]      |
| PB2 NPs    | DOX             | DNA Intercalating agent      | Preclinical in vivo | Enhanced pharmacokinetics in naïve rat                                           | [64]      |
| PB2A NPs   | TMZ             | Alkylating agent             | Preclinical in vivo | Prolonged survival in 101/8 GB rat model                                         | [70]      |
| PH2A NPs   | DOX             | DNA Intercalating agent      | Preclinical in vivo | Prolonged survival in 101/8 GB rat model                                         | [70]      |
| PEG-grafted CMC NP | DOX | DNA Intercalating agent      | Preclinical in vivo | Higher cellular uptake and proliferation inhibition in C6 cells                | [71]      |
Stimuli-responsive nanoparticles for GB therapy. A) NPs coated with enzyme-responsive linkers and cell penetrating peptides (CPP). Upon enzymatic cleavage (primarily by MMPs), CPP are exposed and facilitate cellular uptake and cargo release. B) Redox-responsive NPs internalize into the cell via endocytosis and release their cargo following exposure to intracellular glutathione. C) Gold NPs sensitize tumor cells to radiation therapy following photo-activation. Gold NPs can be conjugated with therapeutic agents and targeting moieties and therefore can be exploited as a delivery system. D) Iron NPs generate local hyperthermia in response to magnetic fields.

Treatment with NE-siRNA CD73R complexes of glioma-bearing Wistar rats reduced tumor growth by 60% and decreased ADO levels by 95% in vivo.\textsuperscript{[80]} Notably, treatment with NE-siRNA CD73R complexes of glioma-bearing Wistar rats reduced tumor growth by 60% and decreased ADO levels by 95% in vivo.\textsuperscript{[80]}

**Modified Biodegradable Poly(\(\beta\)-Amino Ester)s (PBAE):** NPs have been reported as effective nanocarriers for systemic siRNA delivery to patient-derived GB cells.\textsuperscript{[81,82]} The engineered nanoparticles crossed the BBB via a vesicular mechanism in an orthotopic GB mouse model. siRNA was successfully delivered by the NPs to the cytosol of human brain cancer cells, and gene silencing was achieved both in vitro and in vivo.\textsuperscript{[81]}

**Cell-Derived Extracellular Vesicles (EVs) or Exosomes:** contain endogenous nucleic acids, that are efficiently internalized by several cells.\textsuperscript{[84]} EVs can be loaded with synthetic ONTs and therefore may be the basis for an alternative ONT delivery approach.\textsuperscript{[85]} However, the safety and efficacy of this new targeting method remain to be investigated.

**Spherical Nucleic Acid Nanoparticles (SNAs):** consist of densely packed siRNA covalently bound to gold NPs (13 nm AuNPs). SNAs targeting Bcl2|ike12 (Bcl2L12)-a p53 inhibitor and effector caspase highly expressed in GB, successfully knocked down its endogenous levels, and induced apoptosis in glioma cells.\textsuperscript{[86]} The particles crossing of the BBB is mediated via class A scavenger receptors.\textsuperscript{[87]} The first human safety trial administering intravenous (IV) siRNA to GB patients utilizes SNAs (NCT03020017).

**Aptamers:** Aptamers or chemical antibodies, assume a specific and stable 3D conformation as single stranded DNA or RNA, that enable them to specifically bind protein targets.\textsuperscript{[88]} AS1411, a nucleolin-specific ONT, is the first aptamer that reached phase II clinical trials for a number of cancers. Nucleolin is widely expressed on the surface of several cancer cells, including glioma. AS1411 inhibited the proliferation of glioma cells in vitro and prolonged survival of U87 glioma-bearing mice.\textsuperscript{[88,89]} Additional studies have developed aptamers for several glioma receptors (e.g., PDGFRb, EGFRvIII) and evaluated their conjugation to a number of ONTs.\textsuperscript{[90-92]} Recent publications show aptamers’ accumulation in brain tumors, suggesting their potential as new therapeutic carriers for GB.\textsuperscript{[90]} Addition of targeting moieties may improve the delivery of NPs into the brain and specifically to GB tissues. Several such targeting moieties have been evaluated for ONT delivery and will be reviewed in the following sections.
Table 2. Stimuli responsive NPs designed for GB therapy.

| Delivery system                                      | Mechanism of action                          | Therapeutic agent | Type of study                                      | Reference   |
|------------------------------------------------------|----------------------------------------------|-------------------|---------------------------------------------------|-------------|
| PEG-PCL block copolymer                              | MMP-2/9-dependent, activatable low           | PTX               | Preclinical, C6 GB bearing mice                    | [98]        |
|                                                      | molecular weight protamine (ALMWP)           |                   |                                                   |             |
| PEG-PCL block copolymer conjugated to angiopep-2     | MMP-2 dependent ACPP                         | AnACNPs           | Preclinical, U87 GB mouse model                    | [100]       |
| Crosslinked iron oxide NPs                           | MMP-14 cleavable linker                      | Azademethylcolchicine | Preclinical, pcGBM2 tumor bearing mice               | [101]       |
| Poly-l-lysine dendrigraft (DGL)                      | PH-MMP2 dependent ACPP                       | shVEGF and DOX    | Preclinical, U87 GB mouse model                    | [102]       |
| PEGylated PLA NPs                                    | uPA dependent ACPP                           | PTX               | Preclinical, C6 GB bearing mice                    | [99]        |
| Human serum albumin NPs with PEG                    | Payload release under GSH-reductive          | PTX               | Preclinical, U87 GB mouse model                    | [112, 113]  |
|                                                      | conditions                                   |                   |                                                   |             |
| Angiopep-2 functionalized, virus-mimicking polymerosomes | Redox-responsive, cross-linked,              | saporin           | Preclinical, U87 GB mouse model                    | [104]       |
|                                                      | Chitosan-PEG copolymer, covalently modified  |                   |                                                   |             |
|                                                      | with chlorotoxin (targeting MMP2 and Annexin A2) |                   |                                                   |             |
| Iron oxide NPs                                       | Alternating magnetic field induced           | N/A               | Preclinical and phase I clinical trial             | [118]       |
|                                                      | hyperthermia                                 |                   |                                                   |             |
| Magnetoosomes                                         |                                              | N/A               | Preclinical, GL261 GB mouse model                   | [119]       |
|                                                      |                                              |                   |                                                   |             |
| Gold NPs                                             | Photoactivated radio-sensitization           | N/A               | Preclinical, F98 glioma rat model                   | [121]       |
|                                                      |                                              |                   |                                                   |             |

1.2. Stimuli-Responsive Nanoparticles

Stimuli-responsive NPs are designed to exploit specific features of the tumor tissue, such as enzyme expression and physiological parameters (i.e., pH and redox potential) and enable selective drug-release by external stimuli (Figure 1). Therefore, these NPs may improve the specificity and efficacy of anticancer treatments. Table 2 summarizes the stimuli-responsive approaches tested for GB therapy.

1.2.1. Enzyme Responsive Nanocarriers

Many biological and metabolic processes are tightly controlled by enzymes. In pathological conditions, including several cancer types such as GB, expression and activity of enzymes are dysregulated compared to healthy tissue or physiological conditions.[30,93,94] Therefore, nanocarriers can be designed exploiting overexpression of certain enzymes, taking advantage of their selective catalytic activity to trigger drug release in the tumor site. Among these potential overexpressed enzymes in GB, are the matrix metalloproteinases (MMPs) which their expression and function are key factors in tumor invasion and metastasis.[93,95] These enzymes are overexpressed at angiogenic sites within the tumor and can be easily accessed by nanocarriers presenting enzyme-responsive elements.[96] In fact, a few CNS-targeting nanocarriers that are responsive to the activity of specific enzymes have been already developed.[94,97-99] For instance, Gu et al. took advantage of the overexpression of MMP-2 and MMP-9 in GB and low molecular weight protamine (LMWP) to design “smart” PEG-PCL block copolymer NPs. The NPs were conjugated to MMP-2/9-dependent, activatable LMWP (ALMWP), that can deliver paclitaxel (PTX) selectively to the tumor site.[98] The ALMWP-PEG-PCL NPs loaded with PTX exhibited significantly high accumulation in C6 tumor spheroids and decreased toxicity compared to the free PTX in vitro. Moreover, nanoparticles accumulation in C6 glioma-bearing nude mice, resulted in prolonged survival. Taking in consideration the overexpression of MMP-2 and low-density lipoprotein receptor-related protein 1 (LRP-1), Gao et al. designed a targeted enzyme-responsive nanocarrier capable to cross the BBB and to target and treat GB.[100] The nanocarrier consisted of PEG-PCL block copolymer NPs previously exploited by Gu et al. with 110 nm in diameter, linked to a MMP-2 activatable cell penetrating peptide (ACPP) and angiopep-2, a ligand of LRP-1. Due to their high LRP-1 and MMP-2 expression, angiopep-2 and ACPP modifications could both enhance cell uptake. Furthermore, the double targeted nanoparticles showed a superior targeting efficiency and distribution within the tumor. Mohanty et al. went one-step further and applied this concept to develop a GB-targeted theranostic nanocarrier. It consisted of crosslinked iron oxide nanoparticles conjugated to azademethylcolchicine, an MMP-14 cleavable
linker and a potent vascular-disrupting agent. The iron core of these NPs allowed in vivo drug tracking by MRI, showing significant tumor accumulation in pGBM2 tumor-bearing mice. In addition, the treatment with the NPs in combination with TMZ treatment resulted in tumor remission and significantly increased mouse survival compared to the single TMZ treatment. Huang et al. exploited the overexpression of MMP-2 in GB as well, and the tumor differential extracellular pH by designing a GB-targeted nanocarrier to deliver anti-angiogenic/cytotoxic therapy. The platform consisted of a poly-l-lysine dendrimer (DGL) capable to form a complex with the negatively-charged shVEGF-DOX plasmid. The cationic polymeric nanocarrier was conjugated to a pH-MMP2 dependent ACPP specific for GB tumor microenvironment. This enzyme-responsive nanoformulation demonstrated its ability to accumulate selectively and to trigger cell apoptosis in vitro, as well as to inhibit blood vessels proliferation and to increase the survival of U-87 MG GB bearing mice. Another study, by Zhu et al., demonstrated enhanced delivery and activation of cell penetrating peptides (CPPs) by MMP-stimuli using multifunctional polyethylene glycol (PEG)–lipid conjugates. However, MMP2 shows only 1–2-fold increase expression in glioma compared to healthy tissue. Thus, Zhang et al. proposed using urokinase plasminogen activator (uPA), another protease involved in fibrin degradation, which is overexpressed 4–10 folds in glioma neo-vascular cells and glioma cells compared to non-malignant brain tissue. The GB targeted enzyme-responsive nanocarrier they developed consisted of PEGylated PLA NPs loaded with PTX conjugated to uPA-sensitive ACPP. The in vitro experiments demonstrated a higher cellular uptake and NP penetration as well as increased apoptosis and growth inhibition of C6 cells and C6 3D spheroids, respectively. In vivo experiments in mice bearing C6 glioma revealed that the ACPP conjugated NPs show higher accumulation levels in the tumor. The ACPP modified NPs were found in glioma microenvironment parenchyma cells and stroma cells, both neovascular cells and in tumor-associated macrophages. Furthermore, the ACPP modified NPs were shown to have higher anticancer effect than the controls by extending the survival of the C6 glioma-bearing mice. To that end, enzyme-responsive nanocarriers seem to be promising candidates for controlled and programmed drug delivery to the brain exploiting specific enzyme overexpression in GB.

1.2.2. Redox-Responsive Nanocarriers

Brain tumors present altered microenvironment compared to physiological conditions in terms of pH and redox potential that can be exploited by designing stimuli-responsive nanocarriers capable of releasing the cargo exclusively under tumor-specific conditions. As in other solid tumors, GB microenvironment presents high levels of reactive oxygen species (ROS) that has long been known to be implicated in cancer development and progression. To prevent the ROS related damage, antioxidant systems are upregulated by cancer cells as an adaptive response to restore redox homeostasis. The main redox buffer system is based on glutathione (GSH). GSH participates in many metabolic processes such as the detoxification of xenobiotics as well as in the balance maintenance of the thiol status of proteins which is crucial for enzyme catalyzing activity, signal transduction, and gene expression. The elevated GSH levels in GB and their ability to reduce disulfide bonds to sulfhydryl groups has been exploited for developing nanocarriers capable of releasing their cargo under these pathological conditions. Several types of nanocarriers have been designed as redox-responsive drug delivery systems to overcome the BBB and treat GB, taking advantage of the disulfide chemistry.

For example, Ruan et al. synthesized PTX loaded, human serum albumin (HSA) nanoparticles by desolvation method, and stabilized by intramolecular disulfide bonds as a chemotherapeutic alternative for GB treatment. The NPs were functionalized via PEG linker to a Substance P (SP) peptide which targets neurokinin-1 (NK-1) receptors, overexpressed in GB, in order to target and cross the BBB. SP-HSA-PTX NPs exhibited a redox-dependent PTX release under GSH presence. SP-HSA-PTX NPs were successfully up taken into endothelial brain capillary cells and U87 GB cells due to the SP peptide. Moreover, treatment with SP functionalized NPs showed prolonged survival of U87 GB-bearing mice. Jiang et al. reported the synthesis of redox-responsive and angiopep-2 functionalized, virus-mimicking polymerosomes (PS) for selective and efficient delivery of saporin (SAP), a highly potent natural toxin capable of inactivating ribosomal activity. Angiopep-2 functionalization conferred the nanocarrier the capability to target and bind to LRP-1, thus increasing the cellular uptake. The nanocarriers showed an 80% release of the total payload under GSH-reductive conditions whereas under physiological conditions the total payload release was 15%. In vivo studies using an orthotopic U-87 MG-Luc mouse model, confirmed the potent anti-tumor efficacy of the ANG-PS-SAP nanomedicine. Mice treated with ANG-PS-SAP showed reduced systemic cytotoxicity, higher tumor accumulation, reduction in tumor volume and prolonged survival.

In order to improve O4-benzylguanine (BG) tumor accumulation and efficacy, Stephen et al. established superparamagnetic iron oxide NPs (SPIONs) for targeted convection-enhanced delivery (CED) for GB treatment. The NPs consisted of a superparamagnetic core coated with a redox-responsive, cross-linked, biocompatible chitosan-PEG copolymer and covalently attached to the surface coating (NPCP). NPCP were covalently modified with chlorotoxin, a tumor targeting peptide that binds with high affinity to MMP2 and Annexin A2, both known to be overexpressed in GB. The nanocarriers were loaded with BG, an inhibitor of DNA repair protein MGMT in order to overcome TMZ resistance. In vitro studies confirmed the redox-responsive behavior of the NPCP-BG-CTX nanocarriers, BG was released in a reductive environment (GSH 100 x 10⁻³ m) and GB cells were more sensitive to TMZ. In vivo studies showed reduced systemic toxicity compared to the free BG. The superparamagnetic core of NPCP-BG-CTX allowed real-time monitoring of the BG delivery through MRI. Furthermore, the combined treatment of NPCP-BG-CTX and TMZ increased the overall survival of GBM6-luc GB-bearing mice compared to the untreated group.

1.2.3. Magnetic NP

Magnetic NPs (MNPs) technology has an important role in cancer drug delivery. These MNPs are formulated from magnetic
nanomaterials, that provide a magnetic-responsive NPs. This feature is used to induce apoptosis in cancer cells, manipulate drug delivery, and increase the treatment specificity. MNPs can induce apoptotic cell death by a procedure of elevating the temperature at the tumor site following magnetic field sessions, called hyperthermia.\textsuperscript{[116]} MNPs were evaluated in vitro, in vivo and in clinical trials for GB treatment.

One of the most common magnetic nanomaterial is iron oxide. These NPs enable a combined treatment of hyperthermia and radiation.\textsuperscript{[117]} In a phase II clinical study, synthetic iron oxide NPs were directly injected into the tumor followed by heating using an alternating magnetic field. This study showed overall survival of 23.4 months following primary tumor diagnosis for the treated group compared to 14.6 months for the reference group that was treated with a combination of radiotherapy and chemotherapy (TMZ).\textsuperscript{[118]} Additional iron oxide MNPs are the magnetosomes that are biologically synthesized and obtained from magnetotactic bacteria lipid bilayer membrane. These MNPs are biocompatible and have a great potential once endotoxins and organic residues are removed. A study showed such MNPs made from MSR-1 bacteria containing iron oxide and Poly-l-Lysine coating (M-PLL). These MNPs were compared to chemically synthesized iron oxide MNPs. Briefly, they performed an in vivo experiment in order to examine and compare M-PLL and iron oxide MNPs hyperthermia efficacy. The results showed 100% of mild hyperthermia (43°C-46°C) caused by the M-PLL in the tumor while only 50% was achieved for the chemically synthesized iron oxide MNPs, suggesting that M-PLL have a higher heating capacity. M-PLL treated mice showed prolonged survival and tumor growth inhibition when applying an alternating magnetic field. Thus, M-PLL showed a better anticancer activity and efficacy.\textsuperscript{[119]} In another study, chain magnetosomes (CM) were made of AMB-1 1 magnetotactic bacteria that naturally form a chain structure. This unique organization prevents aggregation, results in preferred biodistribution in the tumor and homogenous heating. Similarly, CM activity was compared to chemically synthesized iron oxide NP. Here, they did not remove endotoxin, and demonstrated its controlled release by alternating the magnetic field. They showed that CM induces immune response better than iron oxide MNPs due to a better heat release and higher endotoxin content. In addition, the results show that early apoptosis is induced rather than late apoptosis for iron oxide MNPs. Finally, they performed an in vivo experiment, that showed a complete remission of the tumor for the groups that were treated with CM and 15 magnetic sessions, suggesting that these MNPs are very effective and can be used to eliminate GB.\textsuperscript{[120]}

Another very common MNPs are the gold NPs, that are known for their radio-sensitization activity. Gold NPs radio-sensitization activity is produced due to its high mass energy coefficient in soft tissues, results in increased energy at the tumor site when radiated. For instance, small gold NPs were designed in two different sizes (1.9 and 15 nm). These MNPs were photo-activated by low energy radiation, resulting in radio sensitization at the tumor site. Interestingly, the smaller size NPs showed stronger anticancer activity, perhaps due to the higher number of particles compared to the larger MNPs.\textsuperscript{[121]} Gold NPs were intensely investigated and developed in the recent few years. Gold NPs can be combined with chemotherapy agents or other drugs to get a synergistic treatment. Another study showed a combination of gold MNPs with cisplatin. Gold NPs were conjugated to cisplatin with an uptake peptide (GNP-UP-Cis) and were evaluated in vitro and in vivo for anticancer activity and specificity. MR-guided Focused Ultrasound (MRgFUS) method was used to increase drug delivery through the BBB. GNP-UP-Cis showed enhanced cancer cells proliferation, tumor growth inhibition and increased internalization compared to Cis alone and GNP-Cis. In addition, increased DNA damage and cell death were observed. Combination of the treatment with radiation showed synergistic effect of cell proliferation inhibition due to gold NPs radio-sensitization activity.\textsuperscript{[28]} As described, MNPs have a great potential for GB treatment as they have the ability to induce tumor hyperthermia and radio-sensitization and can be easily combined with chemotherapeutic agents.

1.3. Receptor-Targeting Nanoparticles

Since the BBB restricts the passage of most therapeutic agents from the blood to the brain, receptor-mediated transcytosis can offer a non-invasive trafficking system to deliver targeted carriers into the brain parenchyma (Figure 2).\textsuperscript{[122]} In addition, this approach allows selective targeting of tumor cells within the brain tissue, thus reducing toxicity in other tissues and non-tumor cells in the brain. Table 3 summarizes the receptor-mediated approaches discussed in the following sections.

1.3.1. Manipulating the Apolipoprotein Receptors for Crossing the BBB

Several studies aimed to target the apolipoprotein receptors for drug delivery to the brain. This strategy manipulates the endogenous role of the apolipoprotein receptors at the central nervous system (CNS), such as lipids transportation and homeostasis, for enhanced brain uptake of NPs. The apolipoprotein receptors, in particular the low density lipoprotein (LDL) family of receptors (LDL-R) and the LDL-R related proteins (LRP), are highly expressed on the brain endothelium that comprises the BBB, and on the cellular membranes of various cells within the CNS. Apolipoproteins are naturally assembled to lipid-based particles, and mediate their endocytosis by attachment to the LDL-R and LRP receptors. Furthermore, the LDL-R and LRP are the receptors for various ligands with endogenous brain functions and implications in CNS pathologies such as the β-amyloid precursor protein (APP), aprotinin, and transferrin. The upregulation of LDL-R and LRP on the membranes of various GB cell lines, provided the rational to target the apolipoprotein receptors to largely distribute anticancer therapies into the brain.\textsuperscript{[123-127]} Apolipoprotein E (APOE) is the most studied apolipoprotein used for targeting NP to the CNS. It is the main apolipoprotein in the brain, and is largely prevalent in the cerebral-spinal fluid (CSF) and the plasma. Two main approaches utilizing the APOE transporters were previously examined (Figure 3). The first approach relying on the high avidity of APOE to lipoprotein NP. Mimicking these naturally assembled lipid vesicles, coated NPs with certain amphiphilic macromolecules (i.e., polysorbate 80 and poloxamer 188) were shown to mediate the recruitment of APOE in the bloodstream. This further translated to the target-
Figure 2. Targets for ligand-directed delivery of nanoparticles across the BBB and into GB tumors.

...ing of these NPs to the corresponding receptors on the brain endothelium and the subsequent transcytosis into the brain parenchyma.\(^\text{[128]}\) The second approach involves the direct binding of APOE or its derivatives to the surface of the NPs, to further exploit the same transcytosis mechanism via the LDL-R and LRP receptors. A large body of work was performed by J. Kreuter and his group to define the mechanisms by which polysorbate 80-coating facilitates brain accumulation. In an early study, they demonstrated that DOX-loaded poly(butyl cyanoacrylate) NP coated with polysorbate 80 largely distributed to the brains of rats. The concentrations of DOX in the brain tissue were above 6 µg g\(^{-1}\) 2-4 h following IV injection, in contrast to non-coated NP that led to non-detectable concentrations of DOX in the brain (concentration of below 0.1 µg g\(^{-1}\)).\(^\text{[129]}\) In-vitro studies performed on NPs incubated in plasma showed the ability of the polysorbate 80 coated NP to adsorb apolipoproteins.\(^\text{[130]}\) The coated NPs were further evaluated for their anticancer efficacy using intracranial 101/8 GB in Wistar rats. In this study, animals treated with the polysorbate 80-coated DOX NPs demonstrated significantly increased survival rates and above 20% long-term remission, compared with untreated animals or animals treated with non-coated DOX NP or free DOX.\(^\text{[131]}\) This without causing any short-term general or neurotoxicity which is associated with free DOX treatment, suggesting that this method is efficient for delivering chemotherapy into the brain. Nevertheless, concerns were raised regarding a possible disruption of the BBB as the amphiphilic character of the surfactant may lead to solubilization of lipids at the membranes of endothelial cells. Furthermore, modulation of the capillaries tight junctions was observed 45 min following IV injection to the mice.\(^\text{[132]}\) Cytotoxic immune response was observed in the brains of rats treated with the polysorbate 80 formulations.\(^\text{[131]}\) Then, the requirement for conjugation of the entire polysorbate 80 was doubted, with the discovery that certain NPs coated with poly(ethylene glycol) (PEG) chains (the hydrophilic component of the polysorbate 80 molecule) were able to adsorb APOE on their surfaces when incubated in rat plasma. In accordance, PEG coated poly(hexadecylcyanoacrylate) (PEG-PHDA) NP penetrated into rat brain endothelial cells (RBEC) in vitro, whereas blocking the LDL-R inhibited cellular internalization of these NPs.\(^\text{[133]}\) Furthermore, PEG-PHDA NP penetrated into the brains of mice and rats following IV injection, and were distributed in the brain tissue to a larger extent compared with non-PEGylated or the polysorbate 80 coated NP, without causing disruption of the BBB.\(^\text{[134]}\) It is well established that other PEGylated NP such as liposomes can mediate delivery of drugs through the BBB and accumulate in GB.\(^\text{[135]}\) Nonetheless, more comprehensive investigation of the mechanisms governing this BBB permeation is required. The latter is mostly attributed to the prolonged circulation and escape from uptake by macrophages, formerly termed the “stealth” properties of PEGylated liposomes, allowing increased extravasation via impaired vasculature (i.e., the EPR effect) and damaged the BBB in the tumor site. To bypass the need for attachment of apolipoproteins in the blood, the approach of directly binding APOE to the surface of NP was taken. Huang et al. recently demonstrated that APOE3 targeting of lipoprotein-like NP achieved much higher accumulation in GB and penetrated deeper into the core of the tumor compared with PEGylated NPs.\(^\text{[127]}\) In this study, calcium phosphate (CaP) was assembled with siRNA to form a solid core further coated with two layers of lipids, and decorated with...
Table 3. Receptor targeting NPs for GB therapy.

| Target                      | Delivery system                                      | Therapeutic agent       | Study type                              | n                  |
|-----------------------------|------------------------------------------------------|-------------------------|-----------------------------------------|--------------------|
| Apolipoproteins receptors   | Poly(butyl cyanoacrylate) NPs coated with polysorbate 80 | DOX                     | Preclinical, orthotopic 101/8 GB rat model | [129–132]          |
|                            | PEG-PHDC                                             | N/A                     | Preclinical, orthotopic mice and rats GB models | [133, 134]        |
|                            | CaP - siRNA complex, coated with two layers of lipids, decorated with APOE3 | ATP5 siRNA              | Preclinical, orthotopic GB mouse model   | [137]              |
|                            | Chimeric polymersomes (CP) NPs decorated with APOE peptide (receptor-binding domain) | Saporin                  | Preclinical, intracranial U87 GB mouse model | [94]               |
|                            | LRP-1 ligand Kunitz domain peptide covalently linked to PTX (ANG1005) | PTX                     | Preclinical, intracranial U87 GB mouse model and phase II clinical study | NCT01967810        |
| EGFR                        | [121]-labeled anti-EGFR 425 murine monoclonal antibody (121-mAb 425) | Radioactive             | Phase II clinical study                 | NCT00589706        |
|                            | EGFR(V) antibody conjugated to EnGeneIC delivery vehicle (EDV), loaded with DOX (EGFR(V)-EDV-Dox) | DOX                     | Phase I clinical trial                  | NCT02766699        |
|                            | Monomethyl auristatin F (MMAF) conjugated to the anti EGFRvIII antibody ABT-806 (ABT-414) | synthetic anti-microtubule agent monomethyl auristatin F (MMAF) | Phase II/III clinical studies | NCT02343406, NCT02573324 |
|                            | PEI25-PEG-EGF                                         |                         | Preclinical, orthotopic GB mouse model | [170]              |
| Transferrin receptors       | Tranferrin-conjugated magnetic silica PLGA            | DOX and PTX             | Preclinical, intracranial U87 GB mouse model | [184]              |
|                            | Polysorbate 80 coated PLGA NPs loaded with Tf-methotrexate conjugates | Methotrexate (MTX)      | Preclinical, orthotopic C6 glioma rat model | [185]              |
|                            | Cyclic 9 amino-acid peptide CRTiGPSVC (CRT)- PLGA conjugate | PTX                     | Preclinical, orthotopic C6 GB bearing mice | [186]              |
|                            | Diphtheria toxin-Transferrin conjugate (TF-CRM107)    | Diphtheria toxin        | Preclinical, orthotopic GB U251 MG mouse model, and phase III clinical trial (terminated) | [188–190]          |
|                            | TF-conjugated polymersomes                            | DOX                     | Preclinical, orthotopic C6 glioma rat model | [191]              |
|                            | Liposomal delivery system, surface modified with transferrin and a cell penetrating peptide | DOX                     | Preclinical, intracranial U87 GB mouse model | [192]              |
|                            | Cationic liposome coated with an anti-TIR single-chain antibody (SGT-53) | Plasmid DNA encoding normal human p53 | Preclinical, T98, U87, U251, and LN-18 GB mouse model, and phase I clinical trial | NCT00470613        |
|                            | siRNA-loaded lactoferrin nanoparticles (AKB–LfNPs)    | Aurora kinase B (AKB) siRNA | Preclinical, GL261 GB mouse model | [201]              |
| Insulin receptor            | Human serum albumin (HSA) NPs decorated with insulin or with anti-insulin receptor antibody (29B4), using PEG as a crosslinker | Loperamide              | Preclinical, ICR (CD-1) mice            | [204]              |
|                            | Amphiphilic diblock copolymer of poly (dimethylsiloxane) -block-poly (2-methyl-2-oxazoline), conjugated to anti-human insulin receptor antibody (clone 83-14) | N/A                     | human BBB in vitro model               | [205]              |
|                            | PEGylated immunoliposomes, conjugated to 83–14 murine monoclonal antibody | EGFR antisense plasmid DNA | Preclinical, intracranial U87 GB mouse model | [206, 207]        |

(Continued)
Table 3. Continued.

| Target          | Delivery system                                                                 | Therapeutic agent | Study type                        | n  |
|-----------------|----------------------------------------------------------------------------------|-------------------|-----------------------------------|----|
| αvβ3 Integrin   | Cyclic Arginine-Glycine-Aspartic acid-o-Tyrosine-Lysine                           | PTX               | Preclinical, intracranial U87 GB mice model | [213] |
| Poly(amidoamine) (PAMAM) dendrons with c(RGDyK) peptide on the surface of the NPs | methotrexate       | Preclinical, intracranial U87 GB mice model | [214] |
| c(RGDyK)-modified polyethylene glycol-polyethylenimine (PEG−PEI) | pORF-hTRAIL gene delivery | Preclinical, intracranial U87 GB mice model | [215] |
| Cyclic RGD peptide: poly(trimethylene carbonate) multifunctional liposomal delivery system (c(RGDyK)/pHA-LS) modified with cyclic RGD (c(RGDyK)) and p-hydroxybenzoic acid (pHA) | PTX               | Preclinical, intracranial U87 GB mice model | [216] |
| c(RGDyK)-decorated Pluronic micelles [RGD-PF-DP] | DOX                 | Preclinical, intracranial U87 GB mice model | [217] |
| Ultrasound fluorescent core-shell silica NPs, Cornell prime dots (C’ dots), functionalized with cRGD | Dasatinib          | Phase II clinical trials            | [219] |
| Self-assembling RGD-surface-functionalized Dox-loaded lipid-core nanocapsules (Dox-LNC) | DOX               | Preclinical, intracranial U87 GB mice model | [220] |
| cRGD-modified micellar NPs (cRGD-Epi/m) | Epirubicin          | Preclinical, intracranial U87 GB mice model | [221] |
| polyglutamic acid (PGA)-PTX-E-[c(RGDyK)₂] (bis-cyclic RGD) | PTX               | In vitro using U87 GB cells, and in vivo using 4T1 breast cancer model | [222] |
| α2β1 integrin   | Integrin α2 antibody-directed, DOX-encapsulating liposomes (ITG α2-Dox-LPs)     | DOX               | In vitro BBB model                 | [223] |
| Connexin        | Stable vector nanogels, conjugated with monoclonal antibodies to connexin 43     | Cisplatin         | Preclinical, C6 glioma model       | [224] |
| P-Selectin      | Dendritic polyglycerol sulfate (dPGS)                                           | PTX               | Preclinical, GL261 and U87 GB mouse models | [57] |

APOE3. These NPs, mimicy of the natural high-density lipoproteins (HDLs), aimed to address the extremely challenging task of delivering siRNA into GB cells in the brain. Activating transcription factor-5 (ATF5) siRNA was entrapped in the CaP NP core, to target the corresponding anti-apoptotic transcription factor over-expressed in GB. The NPs were in size range of 20–40 nm, ideal to retain prolonged circulation and extravasation via the leaky endothelium and were able to protect the siRNA from degradation in physiological fluids for more than 8 h. Furthermore, the APOE3-decorated NPs demonstrated much higher accumulation in intracranial GB 4–24 h following IV injection, compared with the non-decorated NPs. Finally, prolonged survival of tumor bearing mice treated with APOE3-decorated ATF5 siRNA carrying NPs was observed compared to mice treated with non-decorated NP or negative-control siRNA NPs. Nevertheless, the use of a full-size protein as a targeting moiety presents various hurdles, including alteration in the size of the NPs, limited loading capacity, low conjugation ratio and challenging purification. Hence, the approach of minimizing the size of the targeting moiety to a short binding sequence was further developed. Such attempts were carried by Jiang et al, demonstrated the development of chimeric polymersomes (CP) NPs decorated with APOE peptide- a tandem repeat over a short sequence (9 AA) of the receptor-binding domain of APOE. In accordance, assembly of this APOE peptide to the naked CPs did not affect the size and zeta potential of the NPs. These NPs were used for the entrapment of saporin, a protein which is known to upregulate various apoptotic-related genes. The delivery of these NPs to intracranial U87 GB tumors following IV injection was validated using BALB/c nude mice. Furthermore, the efficiency of the saporin therapy was shown by a remarkable reduction in tumor growth. Strikingly, due to multi-receptor interactions, the APOE peptide decorated NPs accumulated in brains of mice twice than NP decorated with angiopoep-2 peptide. The latter is a peptide sequence derived from the Kunitz domain that is common to several LRP-1 ligands, including the β-amyloid precursor protein (APP) and aprotinin. Targeting moiety was covalently linked to the chemotherapeutic drug PTX (each to 3 drug molecules), to form a peptide-drug conjugate aimed at brain tumors known as ANG1005. Preclinical evaluation was performed on a U87 intracerebral mouse model, and demonstrated 15% prolonged survival compared with vehicle treated mice. Furthermore, it was found safe at therapeutic concentrations in a phase I clinical trial (NCT00539344), and maximum tolerated dose (MTD) was determined to be 650 mg m$^{-2}$.[139] Eight out of 27 patients treated with 420 mg m$^{-2}$ of the above NPs had a stable disease with a median of 51 days. Following, ANG1005 was in a fast track at the FDA, and a phase II clinical trial for efficacy evaluation on patients with high-grade glioma was launched (NCT01967810). In addition, ANG1005 demonstrated clinical benefit in a phase II clinical trial for the treatment of recurrent brain metastasis from breast cancer, and is considered as a leading approach amongst brain-targeting peptides. To conclude, large efforts were dedicated to targeting the apolipoprotein receptors as brain delivery strategy since 1995 up to date. Trends have shifted from using large, toxic surfactant-based side moieties relying on passive adsorption of APOE in the bloodstream, towards direct binding of APOE itself and then reducing sizes of the targeting moiety to a minimum comprising receptor-binding sequences. Such sequences led to high accumulation of drugs in brain tumors.
and increased anti-cancer activity, hence they are considered as a promising approach for the challenging task of harnessing nanotechnology for the delivery of therapeutics to the brain.

1.3.2. Targeting the Epidermal Growth Factor Receptor

In the last few years, major scientific and technological developments have led to a better understanding of the molecular characterization of GB. Many studies have shown that one of the most common genetic aberrations associated with primary GB is in the Epidermal Growth Factor Receptor (EGFR) gene. EGFR is a transmembrane receptor tyrosine kinase (RTK), a member of the ErbB family of receptors. EGFR monomers can form either homodimers or heterodimers with other RTK family members. This process can activate several signaling pathways such as PI3K/AKT, STAT3, Ras/Raf/Mek/ERK, and PLC. Activation of these pathways enhance cellular processes including cellular differentiation, proliferation, invasion, and survival. Amplification of the EGFR gene was found in more than 40% of primary GB patients. Moreover, ~50% of those patients also harbor a deletion in this gene referred to as EGFRvIII or delta-EGFR. The deletion of exons 2–7 of the EGFR gene results in a constitutively active variant of EGFR that can potentially promote tumor growth and confer a more aggressive tumor phenotype. Furthermore, it has been shown that changes that lead to EGFRvIII are late episodes following the amplification of EGFR. Therefore, EGFR and/or EGFRvIII have been considered as a potential therapeutic target in GB, recently evaluated in many preclinical and clinical studies. To that end, two main approaches have been used: a) inhibition of EGFR pathway activity, and b) usage of EGFR or EGFRvIII expression as a target for drug delivery. The first approach is based on small molecule inhibitors of EGFR/EGFRvIII, tyrosine kinase inhibitors (TKIs), that interfere with the signal transduction of this receptor. Preclinical studies of the 1st and 2nd generation of TKIs showed promising results such as prolonged survival and a decrease in tumor growth. Clinical trials have failed to demonstrate their benefit in patients, either alone or in combination. Therefore, although several TKIs such as Gefitinib (ZD1839; Iressa) and Erlotinib (OSI-774; Tarceva) are FDA approved for a broad spectrum of diseases, none of them was approved for GB. However, the third generation of EGFR TKIs such as AZD9291 demonstrated an effective crossing of the BBB and are currently tested in GB preclinical studies and in a phase II clinical trial (NCT03732352).

The second approach which exploits the EGFR/EGFRvIII expression as a target, includes EGFR/EGFRvIII antibodies,
vaccines, chimeric antigen receptors (CARs), and RNA-based therapies. The EGFR/EGFRvIII antibody therapies have a range of different mechanisms of action, such as different targets or different conjugated toxins, radioactive isotopes or even bispecific T-cell engaging antibodies (BiTE). Antibodies that target the L2 domain of EGFR should prevent ligand binding and dimerization of this receptor. Moreover, EGFRvIII is not responsive to this type of antibodies due to the deletion in the ligand-binding domain. To that end, mAb806 (ABT-806) which targets EGFRvIII and overexpressed-EGFR was engineered. A preclinical study showed promising results of tumor growth inhibition, additionally, it was found safe in GB patients in a phase I clinical trial (NCT01255657).

As mentioned before, antibodies can be conjugated to a toxin or radioactive isotopes in order to enhance tumor cell killing activity. A phase II study of a labeled anti-EGFR 425 murine monoclonal antibody significantly prolonged survival in newly diagnosed GB patients. Furthermore, there is an ongoing phase I clinical trial to evaluate the safety and immunological effect of EGFR-targeting EnGeneIC delivery vehicles harboring DOX (EGFR(V)-EDV-Dox) in recurrent GB patients (NCT02766699). Another approach is antibody-drug conjugate (ADC), such as Deputaxizumab Mafodotin (ABT-414). ABT-414 is composed of the anti-microtubule agent monomethyl auristatin F (MMAF), linked to ABT-806. A phase II study demonstrated improvement in overall survival when combined with TMZ in recurrent EGFR amplified GB patients (NCT02343406).

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RNA-based therapies targeting EGFR/EGFRvIII are a potential innovative treatment for GB. RNA interference (RNAi), antisense (AS) oligonucleotides and, double-stranded RNAs are different strategies to selectively activate apoptosis in GB cancer cells. One of the strategies described in a preclinical study is via synthetic dsRNA (polyinosine-polycytosine [poly I:C]), which strongly activates apoptosis. This study reported that EGFR-targeted [poly I:C] was very effective treating EGFR overexpressing GB. It induced rapid apoptosis in the targeted cells, tumor regression with no observed adverse effect in healthy tissues, without recurrence one year post treatment. Another strategy is by activation of Protein kinase RNA-activated (PKR) via AS RNA complementary to the unique exon 1 to 8 junction, which expressed in the truncated form of EGFRvIII, Δ (2–7) EGFR. Activation of PKR causes cell death by apoptosis and inhibition of protein synthesis. In a mouse model, the complementary 39-nucleotide AS RNA triggered death of cells expressing the truncated form of EGFR and significantly inhibited tumor growth.

However, there are still obstacles that need to be overcome such as efficient penetration of the BBB and efficient tumor-targeted delivery. Therefore, these treatments should be further evaluated in preclinical models.

1.3.3. Transferrin Receptor Targeting

The transferrin receptors (TRs) are attractive targets for drug delivery to the brain. TIR1 and TIR2 are transmembrane glycoproteins with high homology in their extracellular domain. They are highly expressed on the luminal membrane of brain endothelium and are known to undergo receptor mediated endocytosis. Their natural ligand is transferrin (Tf), which is an iron binding protein that controls the extracellular iron levels. In addition to expression on normal tissues, TRs are overexpressed on cancer cells. This could represent a strategy of the tumor for optimal import of iron, which is crucial for cell proliferation. In brain tumors, brain microvascular endothelial cells and GB cells express high levels of both TRs, making them attractive for targeted therapies. Therefore, Tf can be conjugated to NPs and serve as a targeting moiety for the delivery of diagnostic or therapeutic agents to the brain, and into tumor cells. Indeed, Tf and anti-TR antibodies have been used for active targeting of GB cells in preclinical models.

Tf-conjugated magnetic silica poly-lactic-co-glycolic acid (PLGA) NPs were loaded with DOX and PTX. When tested in U-87 MG tumor bearing mice, these NPs were the most effective in tumor growth inhibition, compared to the same NPs loaded with only PTX or DOX. In contrast, the non-targeted combination NPs exhibited the least antitumor activity, emphasizing Tf importance in targeting the drugs to the tumor site. Another example is polysorbate 80 coated PLGA NPs that were loaded with Tf-methotrexate conjugates. These NPs were tested in a C6 glioma rat model and exhibited decreased toxicity, improved penetration and antitumor activity, compared with the non-targeted NPs. Another approach is the use of CRT peptide, which is a cyclic 9 amino-acid peptide CRTIGPSVC, which targets the Tf-TfR complex. PLGA NPs were conjugated to CPR and loaded with PTX. These NPs showed favored pharmacokinetics, better accumulation in the tumor site, and prolonged survival of C6 tumor bearing mice, compared to the non-targeted NPs, NPs targeted with Tf, and Taxol.

Immunotoxins (ITs) are antibody-toxin conjugates. One such IT that utilized Tf as a targeting moiety is Tf-CRM107. This is a diphtheria toxin with CRM107 point mutation conjugated to human Tf by a thioester bond. Tf-CRM107 demonstrated a dose-dependent inhibition of tumor growth in U251 MG tumor bearing mice, and its cytotoxicity was superior to the non-targeted toxin. This IT has also progressed to clinical trials. A phase I study following local administration (intra-tumoral convection-enhanced delivery) revealed no severe toxicities. 35% of the patients showed tumor response and improved survival in phase II study. These trials were conducted in patients with recurrent high-grade brain tumors, including patients with GB refractory to conventional therapies. Unfortunately, in an early phase III clinical trial, Tf-CRM107 failed to show superiority over the
standard of care, and CNS toxicity was observed, therefore this trial was terminated.\cite{190}

Another type of NPs targeted to the TFR, are liposomes and polymersomes. For example, Tf-conjugated polymersomes loaded with DOX exhibited improved intracellular delivery against C6 glioma cells in rats, compared with the non-targeted version of the polymersomes and the free drug. This also resulted in tumor volume decrease and better survival.\cite{191} In a recent study, dual functionalized liposomes were prepared, by surface modifications with Tf for targeting, and penetrating peptide for cell uptake augmentation.\cite{192} These liposomes were loaded with DOX and erlotinib and were tested in vivo in U87 MG tumor bearing mice. High concentrations of the drugs were found in the mice brain, and the treatment demonstrated effective antitumor activity resulting in reduced tumor volume and improved survival, compared to the combination of the free drugs, non-modified or Tf/penetratin only liposomes loaded with this combination.\cite{192} Another example is the SGT-53, which is a cationic liposome that encapsulates a plasmid DNA encoding normal human p53. The surface of this liposome was decorated with an anti-TFR single-chain antibody fragment, and it was shown to cross the blood-brain barrier in several GB mouse models. This liposome has been shown to limit the development of chemoresistance to TMZ, and to overcome resistance to TMZ and anti PD-1 immunotherapy, leading to improvement in survival.\cite{193,194,195} This nanocomplex has already gone through Phase I clinical studies in patients with advanced solid tumors, and was well tolerated in systemic administration.\cite{196,197}

Lactoferrin (Lf) is a cationic Tf family protein which binds nucleic acids.\cite{198} Its biodegradability, hydrophilicity, and ability to target Lf-receptors, overexpressed on BBB and glioma cells,\cite{200} make it an attractive candidate to deliver ONTs to GB. Kumari et al. developed Aurora kinase B (AKB) siRNA-loaded Lf nanoparticles (AKB–LfNPs) and achieved effective AKB silencing and cell cycle arrest on GL261 cells. Tumor growth inhibition and increased survival was further shown in an orthotopic mouse model of GB, following IV treatment with AKB–LfNPs, either alone or in combination with TMZ.\cite{201}

As presented in this section, many kinds of TIR targeted NPs have been investigated in preclinical studies, and some even progressed to clinical trials. These were shown to accumulate within the brain, and inhibit tumor growth compared to their non-targeted forms, as displayed in the few examples given above.

1.3.4. Insulin Receptors Targeting

Insulin receptors (IR) are members of a family of receptor tyrosine kinases (RTKs) that also comprises the insulin-like growth factor 1 receptor (IGF-1R). Upon interaction with their ligands, insulin, and insulin-like growth factors 1 and 2 (IGF-1 and IGF-2), multiple downstream signaling pathways are activated, such as the mitogen-activated protein kinase (MAPK) and the phosphatidylinositol 3-kinase (PI3-K)/AKT pathways, which are involved in cancer development and progression.\cite{202} The IR mediates the transport of blood-borne insulin into the brain, however, the use of insulin as a targeting moiety in vivo is limited, due to its short serum half-life (10 min) and the hypoglycemia caused by exogenous administration of insulin.\cite{203} Therefore, other ligands have been developed to actively target nanocarriers to the brain. Human serum albumin (HSA) NPs were decorated with insulin or with a specific anti-insulin receptor antibody (29B4), using polyethylene glycol (PEG) as a crosslinker. Loperamide passage across the BBB was enabled by the insulin bound NPs and 29B4 bound NPs. In contrast, NPs bound to the control antibody IgG2a, were not able to cross the BBB. Moreover, loperamide crossing of the BBB was completely blocked when the 29B4 antibody was injected 30 minutes before the insulin-bound NPs. These results demonstrated that the transport mechanism is mediated by the IR.\cite{204} Another example is the design of polymersomes, composed from amphiphilic diblock copolymer of poly (dimethylsiloxane)-block-poly (2-methyl-2-oxazoline), conjugated to anti-human IR antibody (clone 83-14). These NPs demonstrated specific binding and uptake in a human BBB in vitro model comprised of the brain microvascular endothelial cell line hCMEC/D3 expressing the human IR.\cite{205} In addition, PEGylated immunoliposomes, conjugated to 83–14 murine mAb, were used to deliver human EGFR antisense plasmid DNA to EGFR-dependent glioma. Indeed, U87 glioma cell line growth was inhibited in about 70–80% following treatment with the liposomes.\cite{206} These PEGylated immunoliposomes were further conjugated to both mouse transferrin receptors with a monoclonal antibody (8D3), to enable transport of the liposomes across the mouse BBB and to human insulin receptor with 83-14 monoclonal antibody, to facilitate internalization of the liposomes into tumor cells. The liposomes were loaded with plasmid DNA, encoding a short hairpin RNA (shRNA) directed against human EGFR. Weekly IV administration of the shRNA-based therapy resulted in diminished EGFR tumor expression, and in major improvement in the survival (88% increase) of mice bearing intra-cranial brain cancer.\cite{207} Recently, an aptamer that binds the human IR was generated and demonstrated inhibition of IR dependent signaling and reduction of cell viability in IR expressing glioma cell lines. Furthermore, upon binding to the IR, the aptamer rapidly internalized into the cancer cells. These results suggest the aptamer as RNA-based molecule that can be further developed as inhibitory drug candidate or as a tool for IR targeted delivery.\cite{208}

1.3.5. Adhesion Molecules Targeting

Tumors, including GB, are highly heterogeneous, with tumor cells displaying stem-like properties, supporting cells and blood vessel forming endothelial cells. Tumor cells, and especially cancer stem cells (CSCs) interact with themselves, the extracellular matrix (ECM) and the tumor microenvironment via cell adhesion molecules (CAMs). In addition to their role in cell adhesion, CAMs also regulate tumor development and progression, cell growth, cell motility, signal transduction as well as inflammation. There are five main classes of CAMs: integrins, the immunoglobulin (Ig) superfAMILY, selectins, cadherins, and cluster of differentiation (CD) molecules.\cite{209}

Targeting GB via CAMs hold great therapeutic potential. Different pattern of CAMs expression between normal brain tissue, GB and moreover GB CSCs may highlight this potential. In this section we will review the different approaches targeting these molecules.
Integrin Targeted Nanoparticles: Attachment of cells to the ECM is mediated by Integrins. Those heterodimeric cell surface receptors can also interact with cell surface and soluble ligands. Diverse subunits (18 different α and 8 β), can combine to form 24 different arrangements. Integrins are tightly linked to cancer due to their dual involvement in disease progression. On one hand, these adhesion molecules mediate tumor cell proliferation, migration, invasion and survival. On the other hand, they play a key role by activating different cellular pathways in the formation of new capillary blood vessels. Due to their involvement in GB, integrins hold great potential in GB treatment, either for diagnostics, or targets for drug delivery.\textsuperscript{[210,211]}

Integrin αvβ3 is highly expressed in malignant glioma but not in normal brain cells. Ligands for αvβ3, including arginine-glycine-aspartic acid (RGD) have been extensively explored for glioma-targeted therapeutics in the past two decades.\textsuperscript{[212]} Its role in cancer was evaluated in numerous studies, that assessed its potential role as a therapeutic or as a targeting agent for GB treatment. Zhan et al. were among the first to design a PTX-loaded cyclic arginine-glycine-aspartic acid-d-tyrosine-lysine (c(RGDyK))-poly(ethylene glycol)-block-poly(actic acid) micelle (c(RGDyK)-PEG-PLA-PTX). These micelles showed enhanced in vitro and in vivo anti-tumor activity compared to PEG-PLA-PTX micelles.\textsuperscript{[213]} Similar approaches using different drugs or polymers were used with numerous nanoparticles. In order to generate a platform for multivalent binding and cellular targetting, McNerney et al. developed Poly(amidoamine) (PAMAM) dendrons with c(RGDyK) peptide on the surface. Binary dendron-RGD conjugates were synthesized with methotrexate drug molecule or other functionalized agent.\textsuperscript{[214]} Polyethylene glycol-polyethyleneimine (PEG-PEI) nanoparticles were modified with c(RGDyK) to improve their binding affinity to GB cells and facilitate specific gene delivery to intracranial GB in vivo.\textsuperscript{[215]} Jiang et al. generated a cyclic RGD peptide-poly(trimethylene carbonate)-based nanoparticles encapsulating PTX (c(RGDyK)-NP/PTX). This nanoparticle formulation showed increased targetting to glioma tumors in vivo.\textsuperscript{[216]} A multifunctional liposomal delivery system modified with cyclic RGD (c(RGDyK)) and p-hydroxybenzoic acid (pHA) was developed for dual targettation to integrin αvβ3 and to the dopamine receptors on the BBB (by pHA), c(RGDyK)/pHA-LS augmented the cytotoxicity of liposomal DOX and showed a strong anti-gloma effect both in vitro and in vivo.\textsuperscript{[217]}

Integrin αvβ3 was also used as a target for delivery of drugs combinations. For example, c(RGDyK)-coated pluronic micelles carrying DOX and PTX [RGD-PF-DP] showed significantly better performance than non-coated micelles in its ability to cross the BBB, internalize into GB cells and cause their apoptosis. RGD-PF-DP further exhibited specific tumor accumulation in a mouse model of intracranial GB following in vivo fluorescence.\textsuperscript{[218]} Other RGD peptide mimetics were also used for NPs targetting. Ultrasound fluorescent core-shell silica nanoparticles, Cornell prime dots (C dots), were functionalized with cRGD and were further attached to dasatinib, creating nanoparticle-drug conjugates (Das-NDCs).\textsuperscript{[219]} Antonow et al. developed self-assembling RGD-surface-functionalized Dox-loaded lipid-core nanocapsules (Dox-LNC) to target αvβ3 integrin over-expressing cancer cells. RGD-functionalized Dox-LNC had an improved uptake capacity by U87MG cells.\textsuperscript{[220]} Epirubicin, a potent chemotherapeutic agent, was loaded into a cRGD-coated micelle via a pH-sensitive hydrazone bond (cRGD-Epi/m) by Quader et al. The uptake and penetration of cRGD-Epi/m was evaluated in U87MG 3D-spheroids and showed a major increase versus the non-coated micelles. Furthermore, intracranial GB growth was inhibited and high concentrations of epirubicin were measured in the tumors, following mice treatment with cRGD-Epi/m.\textsuperscript{[221]} Our group had previously shown that polyglutamic acid (PGA)-PTX-E-[c(RGDfK)]₇ (bis-cyclic RGD) nanoscaled conjugate can also successfully target GB tumor cells.\textsuperscript{[222]}

Other integrins were used as targets for nanoparticle drug delivery for GB as well. Integrin α2β1 is a heterodimeric transmembrane glycoprotein that mediates adhesion of cells to the ECM. Integrin α2 antibodies were conjugated to liposomes encapsulating DOX (ITG α2-Dox-LPs), enabling both targetting and blockage of the integrin signaling. Consequently, ITG α2-Dox-LPs showed selective binding and potent inhibition of GB cells migration.\textsuperscript{[223]}

Connexin Targeted Nanoparticles: Connexins are major constituents of gap junction channels, formed by six connexin subunits that assemble at the interface between adjacent cells allowing direct cell−cell communication. The C-tail of connexin 43 (Cx43) interacts with tight and adherence junction molecules and E-cadherin and Cx30 interacts with actin microfilaments and microtubules.\textsuperscript{[209]} Nukolova et al. created a nanocarrier coated with monoclonal antibodies to Cx43, which expression was shown in a C6 glioma model, in both tumor cells and astrocytes around the tumor. High loading of cisplatin was achieved into this vector nanogels, enabling effective tumor growth inhibition with reduced toxicity and prolonged survival of tumor bearing mice.\textsuperscript{[224]} Balkusheva et al. from the same group enhanced the formulation to include another targetting antibody to brain-specific anion transporter (BSAT1) and achieved additional reduction in tumor growth and increased survival of tumor bearing rats treated with the double-targetted nanogels.\textsuperscript{[225]}

Selectins Targeted Nanoparticles: Selectins are transmembrane proteins and include E, L, and P selectins. Although E-selectin expression was seen in high grade glioma blood vessels, its role in GB needs to be further investigated.\textsuperscript{[209,226,227]} Our group, together with Calderon’s and Haag’s groups, synthesized a polyglycerol sulfate (dPGS) nanocarrier, that binds to P/L selectins and successfully crosses the BBB. dPGS targets P-selectin expressed on both GB cells and on tumor endothelium, therefore accumulating in intracranial tumors. PTX was conjugated to dPGS and delivered in combination with thrombospondin-1 peptidomimetic (TSP-1 PM). The combination of a potent chemotherapy with an anti-angiogenic peptide rendered a major synergistic anticancer effect that was shown in both murine and human orthotopic GB mouse models. None of the side effects observed with free PTX were monitored following treatment with dPGS-PTX.\textsuperscript{[57]}

1.4. Targeting the Brain Tumor Microenvironment and Immune-Mediated Approaches

The immune system plays a crucial role in tumor development, as it is one of the key components in the tumor microenvironment (TME). The interactions between the immune cells and
the tumor cells have been shown to facilitate tumor prolifera-
tion, invasion and angiogenesis in the TME.[228] Thus, immune
system targeted therapy may be a promising approach to mod-
ify the course of the disease. Immunotherapy is a therapeutic
approach which goal is to activate the immune system against
the tumor, help it recognize the tumor cells and subsequently
kill them.[229] To that end, a deep understanding of the processes
and interactions of the immune cells and the tumor is required.
As the immune system is composed of many cell types work-
ing together and communicating in various axes, there are sev-
eral treatment tactics targeting different interactions (Figure 4).
GB poses a great challenge for immunotherapy, due to its non-
immunogenic nature, which is manifested in the inhibition of
dendritic cells (DC) maturation, induction of T regulatory cells
(Tregs), tumor-associated macrophages (TAMs), and myeloid-
derived suppressor cells (MDSCs).[228] Much efforts have been
dedicated to reduce the brain immunosuppressive TME. How-
ever, no immunotherapy have showed efficacy in clinical trials
for GB until now.[230] Combined therapy approaches of anti-GB
treatment and modulation of the tumor TME from immunosup-
pressive to anti-cancer activity have been suggested.[229] Because
many GB targeted therapies affect pathways that are also im-
portant for the immune system, novel strategies combining im-
munotherapy with anticancer nanotherapies are being seriously
evaluated.

1.4.1. Immune-Checkpoint Modulators

Modulation of immune checkpoints using antibodies has the po-
tential to modulate the immunosuppressive brain TME. How-
ever, current immunotherapies have not yet been demonstrated
to improve survival for GB patients in a clinically significant
manner partially due to the restricted incapability of antibod-
ies to cross the BBB[231] and the low immunogenicity of GB,
referred to as a “cold tumor.” The most notable checkpoint in-

Figure 4. Therapeutic targets in GB microenvironment. NPs can be incorporated with therapeutic agents targeting receptors, cytokines or tran-
scription factors overexpressed by stromal cells in GB microenvironment (i.e., astrocytes, tumor-associated macrophages/microglia, endothelial
cells, and immune cells). Alternatively, DC-targeted NPs can be incorporated with GB antigens to elicit antigen-specific T cell antitumor immune
response.
hibitor antibodies for the treatment of GB are anti-cytotoxic T lymphocyte antigen 4 (CTLA-4) and anti-programmed cell death-1 (PD-1).[230,231] To overcome this drug delivery challenge, the immune checkpoint inhibitors can be conjugated to a nanocarrier that can cross the BBB. One study demonstrated this approach by conjugating immune checkpoint antibodies to poly(β-l-malic acid) (PMLA), a natural biopolymer that can cross the BBB by using TIR-mediated transcytosis. Another delivery method evaluated in the same study included conjugation of PMLA to Angiopep-2 (AP-2), a ligand for the low-density lipoprotein receptor-LRP-1.[232,233] This delivery system showed promising results such as the activation of the local immune system and prolonged survival in an intracranial GL261 GB-bearing mouse model.[234]

1.4.2. Targeting Tumor-Associated Microglia/Macrophages

An additional component of the immune system, which is well known to correlate with poor prognosis of GB patients are TAMs. These cells usually derive from monocytes precursors in the central nervous system (CNS) and present an M2-like, anti-inflammatory and immunosuppressive phenotype, thus promoting tumor progression and resistance to chemotherapy.[235,236] However, macrophages with a M1-like, pro-inflammatory phenotype, are known to have an anti-tumor activity.[228] Therefore, shifting the macrophage phenotype from M2 to M1-like could improve patient prognosis and prolong survival. To that end, macrophages targeted PbAE-477 mannosylated NPs, encapsulating mRNA of M1-related factors were developed. The mannose moiety was added to the NP surface using polyglutamic acid, to target the mannose receptor on the TAMs. To imprint TAMs with a potent pro-inflammatory and cytotoxic M1-like phenotype, co-delivery of Regulatory Factor 5 (IRF5) and IKKα (phosphorylates IRF5 and activates it) mRNA was used. The NPs were injected IV, in combination with radiotherapy, into a transgenic mouse model of PDGFβ-derivered glioma. The combined therapy showed decreased tumor growth and increased survival. However, as a monotherapy, only a mild effect on tumor progression was detected.[237] GB cells promote M2 TAMs activation state, via the secretion of different cytokines, such as GM-CSF, TGF-β, and IL-10. These cytokines activate STAT3 transcription factor which induces transcription of anti-inflammatory M2-related factors and thus changes the behavioral profile of microglia.[229] Therefore, the STAT3 pathway is a promising therapeutic target. Inhibition of STAT3 was tested using various small molecules inhibitors,[239,240] such as Napabucasin (BBI608), a STAT3 inhibitor that is now in Phase I/II clinical trials for GB (NCT02315534).[241] Another potent anti-inflammatory cytokine that enhances the immunosuppressive microenvironment is Transforming Growth Factor-β (TGF-β), secreted mainly by TAMs and enhancing the invasive capability of GB cells.[242] TGF-β is strongly associated with tumor cells proliferation, migration, invasion and angiogenesis.[243] In vitro studies showed that silencing TGF-β type II receptor (TβRII) using shRNA in the tumor cells decrease TGF-β signal transduction and the invasion of these tumor cells.[244] The above mentioned studies, along with others, led to clinical trials that are now being conducted using TGF-β antibodies, TGF-β antisense oligomers and the TFG-β2 inhibitor trabedersen.[245] CCL2 (also named MCP-1), is another highly investigated chemokine, known to be extremely important in the recruitment of the immune system cells in GB.[246] especially infiltration of monocytes and Treg cells, and was linked to angiogenesis, invasion and aggressiveness of GB.[247,248] CCR2, CCL2 receptor, is overexpressed in human GB tissues.[249] Preclinical studies showed decrease in the accumulation of TAMs and MDSCs in the tumors of mice bearing GB following systemic treatment with anti-CCL2 monoclonal antibody. Prolonged survival was achieved when anti-CCL2 antibody was given in combination with TMZ.[250,251] Colony-Stimulating Factor-1 (CSF-1) is another crucial cytokine for M2-like TAMs. It has been shown to stimulate GB invasion and TAMs infiltration, and the microglial epidermal growth factor (EGF) expression, which enhance epithelial cells migration.[252,253] Preclinical studies showed that inhibition of the CSF-1-EGF axis, using CSF1R inhibitor, can reduce M2-macrophage polarization in GB, and block the malignant progression.[254] Moreover, a phase II clinical study assessing pexidartinib, a CSF1R inhibiting small molecule, in GB patients is currently being held (NCT01790503).

1.4.3. Targeting Tumor Associated Reactive Astrocytes

Astrocytes are the most abundant glial cells in the brain and have been shown to be involved in physiological homeostasis and in a variety of brain pathologies as well as brain malignancies.[255] Several studies have shown that astrocytes and their interactions with tumor cells play a key role in promoting GB progression.[221] Tumor-associated reactive astrocytes enhance GB malignancy through ECM and cytoskeleton modulation, pro-inflammatory and anti-angiogenic factors and chemokines secretion (i.e., production of MMP2, VEGF, IL-10, IL-6, TGF-β, CXCL4). In addition, GB cells secrete factors that increase astrocytes growth, differentiation and maturation, such as metabolic, neurogenic and morphogenic factors. Hence, targeting the interactions between astrocytes and GB cells might have a therapeutic value in overcoming GB drug resistance and decreasing its malignancy.[256]

Several therapeutic targets aiming to disrupt the crosstalk between astrocytes and GB cells are under preclinical studies. One group of compounds disrupting these interactions, which showed prominent results, is CXCR4 antagonists. Astrocyte-secreted CXCL12, binds to CXCR4, which enhances the metabolic activity and cell proliferation of GB cells.[257] CXCR4 inhibition by Peptide R which acts as a specific CXCR4-antagonist, showed impairment in U87 GB cell growth in vitro and in vivo.[238] Another potential target is GDF-15, which is overexpressed by tumor-associated astrocytes and have been shown to facilitate GB cell proliferation and immune-escape. Depletion of GDF-15 by siRNA significantly reduced GB tumor growth in mice and resulted in enhanced T cell infiltration.[259,260] In addition, glial-derived neurotrophic factor (GDNF), secreted by astrocytes in a paracrine manner, facilitates GB cell invasion through the activation of its receptor RET and its downstream protein AKT. Inhibition of RET by pyrazolopyrimidine-1 (PP1), reduced GB cell invasion and tumor growth in GB mice model.[261]
1.4.4. GB Vaccines

GB vaccines include a vast range of therapies such as direct exposure to GB antigens coupled with immune-stimulating molecules as well as stimulated patient-derived DCs, which are antigen presenting cells that regulate both innate and adaptive immune response. DC-based vaccines have emerged as a promising immunotherapy for GB. DC purified population from patient peripheral blood mononuclear cells (PBMC), can be activated by tumor associated antigens (TAA), which are most commonly derived from constitutively active mutations. Then, the autologously stimulated DCs are reinfused into the patient and act by activating the anti-cancer T lymphocytes. Moreover, DC vaccines can be further personalized by analyzing the tumor presented antigens of a specific patient following tumor resection and using these antigens to activate DCs isolated from the same patient. Several clinical studies have demonstrated some encouraging results. A Phase 2 study demonstrated the efficacy of ICT-107 (activated autologous DCs with immunogenic epitopes of GB TAA) in newly diagnosed GB patients following resection and chemotherapy (TMZ). The progression-free survival (PFS) was higher in the ICT-107 group than the control (Autologous dendritic cells that have not been activated with antigens), while overall survival is still being assessed (NCT01280552). Trivax is another DC vaccine that uses autologous interleukin-12 secreting dendritic cells activated with autologous tumor lysate. Trivax is currently being tested in clinical trials in combination of TMZ and radiotherapy (NCT01213407). EGFRvIII expression in GB was also used as a specific target for vaccine development. The mutant protein sequence of EGFRvIII creates a unique antigenic epitope. Rindopepimut (CDX-110) is an injectable peptide vaccine that consists of the unique amino-acid sequence of EGFRvIII conjugated to keyhole limpet hemocyanin. A recent tree multicenter Phase II studies (ACT II, ACT III, and ACTIVATE) showed that Rindopepimut combined with TMZ induced robust EGFRvIII-specific immune responses, both humoral and cellular, and promising progression-free and overall survival in patients after chemoradiation, resected and EGFRvIII-expressing (EGFRvIII+) GB. However, Rindopepimut failed to show increased survival in the Phase III clinical trial (ACT IV, NCT01480479). Despite the negative results in the ACT IV trial, Rindopepimut is still being evaluated for recurrent GB patients (ReACT, NCT01498328). Gliovac (ERC1671) is another type of vaccine for advanced GB patients, composed of autologous and allogeneic cell fragments and whole tumor cells generated from glioma tumor tissues, which potentially stimulate the immune system response against the TAAs on the patient tumor. A small study, on 9 recurrent GB patients treated with Gliovac, reported increased survival and low toxicity following treatment. A Phase II clinical trial in recurrent GB patients has recently been initiated and will evaluate the safety and effectiveness of Gliovac in combination with Avastin, Leukine, and Cyclophosphamide (NCT01903330).

Another personalized therapeutic approach is the use of neoantigens as an anti-GB vaccine to activate T cells. Neoantigens are TAA derived from a tumor mutated protein which can generate robust immune response. Several clinical studies have tested this approach by detecting GB-specific neoantigens from newly diagnosed GB patients based on the analysis of whole-exome sequencing (WES) and RNA-seq. A mixture of the identified neoantigens was selected and injected into the patients with an adjuvant (poly-ICLC), in addition to radiotherapy following surgical resection. The treatment generated neoantigen-specific CD4+ and CD8+ T cells response and an increase in tumor infiltrating T cells and T memory cells. The goal of many cancer vaccines is to produce tumor-specific CD8+ T cells, which will then infiltrate into the tumor. Their potency in GB was shown in a few clinical trials, as it was correlated with prolonged survival. One approach of generating an anti-tumor immune response is by delivery of a picornavirus vector encoding for a tumor specific antigen. The high immunogenicity and the small genome size of picornavirus, that can be modified to encode tumor antigens, highlights its potential for cancer immunotherapy. Picornaviruses with the ovalbumin antigen coding vector, was tested on mice bearing orthotropic GL261-Quad tumor, a murine GB cell line that highly expresses ovalbumin. Upon vaccination with the engineered picornavirus, administered either to the non-tumor brain hemisphere or intraperitoneally, a significant increase in the levels of tumor antigen specific CD8+ T cell infiltration to the CNS was observed, as well as inhibition of tumor growth. Another immunology-based strategy for GB treatment is by chimeric antigen receptor (CAR) T cells. CARs were engineered to recognize the EGFRvIII via single-chain antibody variable fragment (ScFv), which was coupled to intracellular and transmembrane activation domains of immune cells receptors, such as those on T cells. Cells that express those CARs can recognize and kill GB cells that exhibit the antibody target molecule, EGFRvIII, on their surface. A preclinical study in xenogeneic models of human EGFRvIII(+) GB treated with EGFRvIII CARs, showed a specific effect against EGFRvIII expressing cells, reduction in tumor growth, and enhanced survival in GB bearing mice. Due to the promising results that were reported in the in-vivo models, the safety and effectiveness of EGFRvIII CARs as a treatment for GB are currently evaluated in phase I clinical trials (NCT02331693, NCT02844062, NCT02209376, NCT01454596, NCT02664363).

Although immunotherapy, especially immune system targeted nanotherapy, has evolved greatly over the past few decades, further research is needed in order to refine this therapeutic approach for GB.

1.4.5. Targeting Angiogenesis

GBs are highly vascularized tumors, expressing high levels of pro-angiogenic factors which facilitate microvascular formation and endothelial cell proliferation and migration. Hence, several anti-angiogenic agents have been evaluated in preclinical and in clinical studies for GB treatment. As GB growth is dependent on oxygen and nutrients supplied from the blood, reducing and normalizing the tumor blood vessels and preventing vascular regrowth, angiogenesis inhibitors represent a promising approach. In addition, angiogenesis inhibitors can interfere with VEGF-mediated recruitment of monocytes and reduce VEGF-mediated immune suppression, thus improving the efficacy of immunotherapies. However, clinical trials showed only a transient response to bevacizumab (humanized anti-VEGF monoclonal antibody), while the majority of the patients eventually
experienced relapse due to different resistant mechanisms.\[280\] Furthermore, different strategies to block angiogenic pathways did not show an advantage in treating GB. For instance, treatment with cediranib, a tyrosine kinase Inhibitor which blocks VEGF-receptors, showed a transient vessel normalization, as well as a reduction in cerebral vasogenic edema, but did not show any improvement in overall survival nor progression-free survival in Phase III trials. However, Phase II clinical trials using Afiblercept (VEGF decoy receptor) showed benefit in combination with radiation and TMZ in recently diagnosed patients.\[281,282\] Another strategy, proposed by Fujita et al., was to target Laminin-8, a vascular basement membrane component overexpressed by glioma cells. They conjugated anti-laminin-8 α4 and β1 chains oligonucleotides to a Polycefin bioconjugate. When treated U87 GB bearing mice, they observed a significant reduction in vascular density and prolonged mice survival.\[283\] Their results suggested that laminin-8 is an important mediator of GB angiogenesis and a potential target for anti-angiogenesis therapy. Considering the molecular profile of different GB subtypes, GB patients might respond differently to various antiangiogenic therapies. Specific molecular biomarkers, such as MGMT promoter methylation status, EGFR, PDGFR-α, and c-KIT expression, are now under examination as predictive bio-markers for personalized antiangiogenic treatments.\[278\] Molecules such as E7080 (Inhibitor of VEGFR2/3, FGFR1, PDGFR-β), sunitinib (inhibitor of VEGFR-2, PDGFR, c-kit), cabozantinib (inhibitor of VEGFR-2, c-met, Tie-2, and c-kit) and others are now under clinical trials.\[282\]

To conclude, angiogenesis is a critical hallmark of GB, thus seems a promising target for therapy. However, the lack of beneficial antiangiogenic treatments shows the complexity of angiogenesis mechanisms in GB. Studying the alternative mechanisms of resistance and combining several anti-angiogenic agents may improve patient outcome.

1.4.6. Oncolytic Viruses

Oncolytic viruses are naturally occurring or genetically engineered viruses that infect and kill only cancer cells. Unlike gene therapy where a viral vector is used as a carrier, the virus itself is used as an active drug reagent.\[286\] Most viruses can better replicate in cancer cells than in normal cells, since protection mechanisms against viral infection are impaired in most cancer cells.\[285\] However, blocking viral replication in normal cells, while retaining it in cancer cells is still a challenge. In order to achieve cancer cell-specific replication, several attempts to engineer the virus genome have been undertaken. The most advanced and promising approaches aimed to target GB are reviewed herein.

1.4.7. Herpes Virus (HSV-1)-Based

G47Δ is a third-generation oncolytic herpes simplex virus-1 (HSV-1) developed on the basis of the previous generation G207\[286\] with improved antitumor activity. The Escherichia coli (E. coli) LacZ gene was inserted, inactivating an essential subunit of ribonucleotide reductase (RR).\[287,288\] G47Δ showed cytotoxic activity on GB cancer stem cells and antitumor activity in animal models of several solid tumors including glioma.\[289,290\] Following the Phase I–IIa study, a Phase II study on patients with recurrent GB started in 2015 in Japan (UMIN000015959). C134 is a chimeric HSV-1 vector\[291\] lacking the γ134.5 gene and therefore with deficient protein synthesis. Due to its incompetent replication, γ134.5 HSV has been developed as a therapeutic vector for CNS-based malignancies.\[292,293\] The chimeric HSV reduced tumor growth and increased survival in two murine brain tumor models.\[293\] C134 is currently on a Phase I clinical trial, for patients with recurrent GB (NCT03657576). M032 combines the oncolytic activity of G207\[286\] with the recruitment of a targeted inflammatory response through the production of interleukin-12 (IL-12) to kill tumor cells.\[294\] In preclinical models, M002, a similar HSV construct that expresses murine IL-12 instead of human, was more effective than G207.\[295\] M032 is currently on a Phase I clinical trial for patients with recurrent glioma (NCT02062827). rQNestin 34.5 is an HSV-1-based oncolytic viral vector in which a nestin promoter drives expression of γ1 34.5, providing increased tumor specificity by replicating only in glioma cells and not in astrocytes.\[296,297\] rQNestin34.5v2 will infect a glioma cell, and then spread to all glioma cells in the surroundings to kill them too. rQnestin34.5v2 is currently on a Phase I clinical trial to evaluate its safety and effectiveness to treat malignant glioma (NCT03152318).

1.4.8. Adenovirus-Based

DNX-2401 (tasadenoturev or Delta-24-RGD) is an adenovirus-based vector selective for glioma due to two modifications: i) modified E1A protein that cannot bind to Rb and inactivate it; ii) RGD motif.\[289\] Rb which is dysregulated in nearly all gliomas.\[299,300\] An RGD peptide was further incorporated into DNX-2401 to target integrins αvβ3 and αvβ5, which are expressed on many cancer cells including glioma, and therefore facilitate the virus entry into tumor cells.\[301,302\] In preclinical studies DNX-2401 showed an oncolytic effect on tumor cells and a remarkable antitumor immunologic response.\[303,304\] In a Phase I dose-escalation study DNX-2401 was evaluated as single treatment.\[305\] In combination with TMZ (NCT01956734), or with IFN-γ (NCT02197169). Necrosis, inflammation and infiltration of immune cells including T-BET+ and CD8+ T cells to the tumor sites was shown following treatment.\[306\] A Phase I trial is currently evaluating the response of patients with recurrent GB to DNX-2401 armed with OX40L (NCT03714334). An ongoing Phase II study is assessing the response of recurrent GB to DNX-2401, followed by pembrolizumab (NCT02798406). CRad-S-pk7 is a conditionally replicating adenovirus, which E1A gene is regulated by a surviving promoter, and the viral fiber is attached to a polylysine. Prolonged survival following treatment was achieved in mice bearing intracranial glioma xenografts.\[307\] CRad-S-pk7 delivery to Neural Stem Cells (NSC) improved survival in several murine preclinical models of human GB.\[308-310\] A Phase I study of CRad-S-pk7 combined with radiation and chemotherapy for patients with newly diagnosed GB is currently ongoing (NCT03072134).
1.4.9. Others

Polio–rhinovirus chimera (PVSRIPO) is a live attenuated poliovirus type 1 which ribosome entry site was substituted with that of human rhinovirus type 2. This replacement ablates its ability to take advantage of the host ribosomes, translate its genome and spread in neurons. PVSRIPO binds with high affinity to the T-cell receptor, via CD155, which is broadly up-regulated on malignant cells and highly expressed in antigen-presenting cells. Convection-enhanced delivery of PVSRIPO to 61 patients with recurrent GB showed promising results, with higher survival rate than controls and no neuroviral potential (NCT01491893).

H-1 Parvovirus (H-1PV), is single-stranded DNA, small virus with no envelope, which oncosuppressive activity was demonstrated in numerous preclinical studies in several tumor models including GB. The first Phase I/IIa clinical trial of H-1PV in patients with malignant brain tumors showed that treatment was safe and well tolerated. H-1PV crossed the BBB, showed favorable pharmacokinetics, induced antibody formation and T cell responses. Infected tumors exhibited high infiltration of cytotoxic T cells and activation of microglia/macrophages. Survival was clearly improved compared to controls, therefore ParvOryx01 trial data confirmed H-1PV safety and tolerability.

Reovirus (REOLYSIN) is a double-stranded RNA virus that uses the activated ras signaling pathways of host cells to enhance viral disassembly and preferentially infect and lyse tumor cells. Several studies have shown reovirus efficacy in animal models of brain tumors. Phase I studies that treated patients with different advanced malignancies with locally administered reovirus with or without radiation have been completed without significant toxicities.

Toca 511 + Toca FC: Toca 511 is a retroviral vector which encodes cytosine deaminase that catalyzes the conversion of cytosine into uracil. When used in combination with 5-FU (Toca FC), it kills cancer cells and depletes immune-suppressive myeloid cells, resulting in a permissive tumor microenvironment that enables a durable systemic antitumor immunity, leading to selective elimination of the tumor. In a Phase I trial (NCT01470794), recurrent GB patients were treated in the resection cavity with Toca 511, followed by Toca FC that was administered orally. Long-lasting responses have been reported and the treatment will be further evaluated.

Measles Virus (MV) derivatives were genetically engineered to express the human carcinoembryonic antigen (CEA), a biologically inert marker peptide with limited immunogenicity. MV enters cells via the CD46 receptor which is overexpressed in tumor cells, and have an exceptional safety profile as minimal toxicities. Several studies have shown MV-CEA efficacy in preclinical studies on several tumors including GB. A Phase I clinical trial of MV-CEA for recurrent GB is currently in progress (NCT00390299).

TG6002 is a vaccinia virus with targeted deletions of the J2R gene and the I4L gene, which encode the large subunit of the ribonucleotide reductase, combined with the suicidal system FCU1/5-fluorocytosine. Significant antitumor effects were observed in preclinical models of GB following systemic injection of TG6002 and 5-fluorocytosine. TG6002/ Flucytosine is currently on a Phase I/II clinical trial (NCT03294486) for recurrent GB. As shown, oncolytic viruses possess great potential for GB therapy and are largely investigated using various platforms and approaches. Table 4 summarizes the ongoing clinical trials testing the use of different oncolytic viruses for GB treatment.

1.5. Targeting Glioma Cancer Stem Cells

GB tumors are not a homogenous mass of cancer cells, rather they are a small ecosystem with heterogenic glioma cells and recruited stromal cells. As such, a specific population of stem cells was identified in tumors in 1993, with two groundbreaking articles describing stem cells in leukemia, which led to their discovery in many other cancer types. Nevertheless, from its beginning, the field of cancer stem cells suffered from two major problems. First, proper identification of the stem cells population within the tumors is challenging. Secondly, once identified, the therapeutic benefits of this approach were unclear. Stem cells, both in healthy tissue and in tumors, are defined as multipotent cells with the ability to self-renew, meaning that stem cells are progenitors of many different cells in the tissue, due to their division and differentiation potential. CD133, a transmembrane protein which was initially shown to be a hematopoietic stem cell marker, was also found to be a biomarker for glioma stem cells (GSCs). A few studies have shown that CD133+ cells from the tumor are the small and exclusive population capable of proliferation and renewal of the tumor, suggesting a lineage hierarchy in the tumor. Besides CD133, several transcription factors were also found to be related to stem cells, several of which are known as embryonic/developmental transcription factors (e.g., NESTIN, GFAP, SOX2, OX4, and CD44). As it comes to therapeutic advantages, the GSC hypothesis predicts that even when a certain therapy is efficient, as long as the cancer stem cells are spread and remain in the brain, the patient will eventually experience disease relapse. As such, GSCs have been correlated with prognosis and must be considered and treated. To that end, some treatments targeting GSCs were studied.

Barbarisi et al. aimed to enhance the efficacy of the drug quercetin with a nanocarrier. Hyaluronic acid (HA) is a bioavailable polymer, very abundant in the brain ECM, and HA receptors (e.g., CD44, RHAMM) are highly expressed in GB cells. Therefore, it was not surprising that an HA nanohydrogel remarkably improved the cellular uptake of quercetin to human GB cells and decreased cells viability. The anticancer effect was potentiated when quercetin was given in combination with TMZ.

Another GSC target, Dynamin2 is a GTPase large enzyme, mainly related to endocytosis, shown to regulate tumor progression and poor prognosis in prostate cancer. Inhibition of dynamin2 using Dyn34-2 and CyDyn4-36 reduced cell growth of a TMZ resistant GB cell line and GSCs, GSCs migration and neurospheres formation in vitro, and GB tumor size in vivo. Although targeting GB with NPs has been widely studied, NPs targeting GSCs are less abundant. Kim et al. have developed a delivery platform called scL, which is a liposomal complex conjugated with anti-TIR single-chain variable fragment (TRscFv) as a targeting ligand. In this study, these scL nanoparticles were loaded with a wild type p53 gene fragment and were able to target the GSCs.
in vitro and increase the WT p53 gene expression, followed by a decrease in both GSCs and non-GSCs. An in vivo experiment showed that the scL NPs accumulated in the tumor and resulted in its growth inhibition.\textsuperscript{[348]} Yang et al. delivered microRNA-145 using cationic polyurethane with a branched chain of polyethylene imine. In this study, they showed that miR145 is related to high-grade glioma and GSCs, and effective tumor accumulation of the particles was achieved in a mouse model. The NPs delivered successfully the miR145 and inhibited the GSCs radiotherapy and chemotherapy resistance of the GSCs, resulting in prolonged survival.\textsuperscript{[349]} Ozra et al. used a different approach with gold NPs, known for their ability to cross the BBB. In this study, the gold NPs were coupled with TNZ, and bound to the GSC targeting moiety l-aspartate. These gold NPs induced apoptosis in 82\% of the GSCs, in comparison to TMZ alone which achieved only 42\% apoptosis.\textsuperscript{[350]}

### 1.6. Local Drug Administration

The simplest strategy to overcome the BBB is to bypass it by local delivery to the tumor.\textsuperscript{[351]} Thus, various therapeutic agents and different ways of administration are being investigated for safe and improved local delivery. Several methods including intra-tumor (IT) injection, intra-arterial injection (IA), intrathecal Cerebral Infusion (SIACI), and convection-enhanced delivery (CED) will be reviewed in this section.

#### 1.6.1. Gliadel (PCPP-SA with BCNU) and Controlled Release

Sustained controlled-release of macromolecules were first developed by Folkman and Langer in 1976.\textsuperscript{[352]} They established the inert ethylene vinyl acetate copolymer (EVAc), that was applied for the treatment of diabetes, asthma and glaucoma. Subsequently, significant progress was accomplished by the synthesis of new biodegradable polymers like polyanhydride poly[bis(p-carboxyphenoxy)propane–sebacic acid] (PCPP–SA).\textsuperscript{[353]} Due to its hydrophobic nature, the degradation of PCPP–SA is limited to its surface, leading to a constant rate of drug delivery. The PCPP–SA polymer rate of breakdown can be controlled (between days to years), by changing the ratio of the CPP to SA.\textsuperscript{[354]} PCPP–SA can be manufactured in several different shapes for the delivery of different compounds. Other critical advances in polymer science include the development of the biodegradable fatty-acid dimer–sebacic acid (FAD–SA); poly(lactide-co-glycolide)
polymer, that deliver 5-FU to brain tumors; gelatin–chondroitin-sulphate-coated microspheres, that release cytokines in vivo, and polyethylene glycol-coated liposomes which encapsulate anthracyclines.\[354-357\]

Carmustine (BCNU) is an alkylating agent of low molecular weight which crosses the BBB and exerts a modest prolongation of survival on patients with brain tumors.\[358,359\] Its relatively short half-life and severe toxicity have limited its use and make it a likely candidate for modification taking advantage of the polymer technology.

Gliadel (PCPP-SA with BCNU) was the first preclinical studied BCNU–polymer which confirmed that stable controlled release of BCNU can be accomplished both in vitro and in vivo with a polymer system.\[360,361\] This polymer system led to a remarkable improvement in BCNU delivery, achieved via local administration. Survival of animals with glioma was notably prolonged,\[362\] BCNU–polymers were well tolerated in primates, either alone or in combination with radiotherapy,\[363\] setting the stage for clinical trials. In a Phase I/II study, BCNU loaded in PCPP–SA polymers was given in three doses to 21 patients, and it proved to be well tolerated and safe.\[364\] Consequently, BCNU polymers were proved to be effective and safe for the treatment of recurrent glioma in a Phase III prospective, placebo-controlled, randomized clinical trial. Consequently, in 1996, Gliadel was approved as the first polymer-based treatment against recurrent malignant brain tumors.\[365\] Following Westphal et al. results, in 2003 Gliadel was approved by the FDA for treatment of newly diagnosed malignant glioma.\[365\]

Since then, the use of Gliadel wafers has been controversial with questionable survival benefit.\[366-369\] Following review of 19 studies with 795 patients, Bregy et al. have concluded that treatment with Gliadel resulted in a mean overall survival (OS) of 16.2 months, while control survival following Stupp standard of care (surgery and adjuvant chemoradiotherapy)\[37\] is ≈14 months. Although they may slightly inhibit tumor invasiveness and increase survival in GB patients, Gliadel wafers are associated with a high rate of complications.\[366\]

1.6.2. Other Locally Delivered Therapeutics

TMZ, the water soluble alkylating agent, is used systemically for treating GB. Similar to other chemotherapies, TMZ suffers from poor bioavailability following systemic administration. Poly(lactic acid-glycolic acid) (PLGA) is a polymer that has been shown to be biodegradable and biocompatible in the brain.\[370,371\] PLGA wafers were prepared with 50% w/w TMZ using a pre-encapsulation process and showed stable release of a high dose of TMZ during 4 weeks. The drug was coated with the polymer that controlled the release rate of the drug. TMZ and BCNU were also co-loaded in wafers, that were evaluated in intracranial 9L gliosarcoma rat models, showing both safety and efficacy.\[372\] In addition, PLGA/PEG loaded with TMZ-etoposide exhibited enhanced stability of both drugs that retained cytotoxic capability upon release in vitro. In vivo studies revealed a significant overall survival benefit in postsurgery 9L orthotopic gliosarcomas, treated with intracavity delivered PLGA/PEG/TMZ/etoposide and enhanced with adjuvant radiotherapy.\[373\]

Oruno˘gluet al. evaluated the antitumor activity of local administration of PLGA-DSP-PEG hybrid nanoparticles loaded with curcumin using a rat glioma-2 (RG2) GB model, showing a remarkable tumor growth inhibition compared to untreated and IV administration.\[374\] Further, Taghizadehghalehjoughi et al. assessed the effects of injecting directly into the brain the Topoisomerase-I inhibitor irinotecan (IRI) and the Mtorc1 inhibitor metformin hydrochloride (MET) loaded PLGA NPs for the treatment of GB using an in vivo rat brain tumor model. They found that the tumor volume was about 12–38% in the treated group compared to untreated.\[375\]

Local administration of anti-angiogenic agents like bevacizumab (BV), Sunitinib (inhibitor of VEGFR, c-kit, and PDGFR), and cetuximab (EGFR inhibitor), have also been evaluated in GB. Wang et al. found that delivery of the proteasome inhibitor bortezomib (shown to effectively induce cancer cells to undergo apoptosis and previously considered ineffective in GB) via IA administration using an Alzet mini-osmotic pump increased the median survival in a mouse model of GB from 23 to 37 days (40% increase in survival) when compared to untreated or IV Bortezomib.\[376\] Liu et al. assessed the therapeutic effect of a low-dose BV treatment in glioma-bearing animals administered intratumorally. Tumor-bearing mice treated IT with BV had a median survival of 40 days compared to 17 days for the untreated group and 27 days for the IV treated group. In addition, there was a significant reduction in the density of the tumor blood vessels (≈50% reduction) and SOX2+ cells (cancer stem cell marker) in the IT treated tumors compared to the IV treated group. Furthermore, a preclinical study conducted by Gravina et al. showed that following addition of PRX-177561 (CXCR4 antagonist) to BV the median survival increased from 47 to 144 days when compared to the untreated group, while the addition of sunitinib increased median survival from 47 to 107 days.\[377\] Chakraborty et al. aim to determine a maximum tolerated dose of Cetuximab through SIAI following osmotic BBBI disruption with mannitol, and to evaluate the safety of the technique in 15 patients with recurrent GB. They found that a cetuximab dose of up to 250 mg m−2 was safe and well-tolerated. Adverse effects were similar to IV cetuximab administration and included a rash or thrombocytopenia, and rare intracranial hemorrhage.\[378\]

1.6.3. Convection Enhanced Delivery

Convection enhanced delivery (CED) is being used to deliver drugs to the brain while bypassing the BBB. Since the spreading of drugs in the brain is limited to simple Fick’s diffusion law which determined an opposite correlation between the diffusion coefficient and the particle diameter, the flux rates of large molecules are extremely low. For example, it can take up to 2 days to an antibody to move 1 mm in brain tissue.\[379\] In the process of CED, microcatheters are inserted through bur holes directly into the tumor allowing the drug flow by hydrostatic pressure gradient.\[380\] CED is most efficient to nanoformulated drugs due to their small diameter which allows higher diffusion rates that lead to higher and homogenous distribution and longer half-time than the free drug.\[381\] The BBB limits DOX use in the treatment of GB. Finbloom et al. showed antitumor efficacy
of nanocarrier-DOX using CED in glioma-bearing mice using a lower dose in orders of magnitude than IV administration. In another study, CED-TMZ affected GL261 and KR158 mice models by improving the therapeutic index compared to non-CED intratumor injection and increased the influx of T cells into the tumor site. These recent findings suggest that the use of CED may improve the efficacy of chemotherapeutic drugs for GB therapy. There are two clinical trials recruiting for a Phase 1 study. The first seeks the dosage, the safety and the toxicity of poliovirus immunotherapy with PVSRIPO administered by CED for children with recurrent GB (NCT02869243). The second seeks to elucidate the differences in the delivery method of hrBMP4 (a recombinant protein of TGF-β superfamily) between intra-tumor and interstitial CED in a continuous infusion manner via intracranial catheters (NCT02973789). Two clinical trials have reached to Phase III; one studied the safety and efficacy of AP 12 009 (a specific inhibitor of TGF-β2 pathway) compared to standard chemotherapy in patients with secondary GB. This study has terminated due to lack of patient recruitment (NCT00761280). The second Phase III clinical trial compared the Glade wafers to CED administration of Cintredekin Besudotox (a human IL13 protein linked to bacterial toxin) that targets the IL13 which is highly expressed in GB. The trial showed only a poor survival benefit compared to the CED administration which was attributed to poor drug distribution (NCT00076986). The current challenges of CED can be divided into two aspects. First, the pre-treatment challenges including catheter design and placement, number of catheters, infusion rate and duration, drug nature and encapsulation. The failure of the only published Phase III study can be attributed to the second essential aspect, the post-treatment challenge regarding the method of drug distribution evaluation. The majority of studies reviewed by Halle et al. in GB treatment via CED, did not perform proper evaluation of the drug distribution as 30% of reviewed studies used histology which is not relevant in clinical use and only a few used CT or MRI. Therefore, there is a need to develop trackable drugs with some contrast agent or conjugated drugs with minimal effects in the drug properties and affinity. CED is a quiet new technology, expanding the drug administration options in neuro-oncology by overcoming the BBB challenge that could allow new breakthroughs in the future of GB treatment.

1.6.4. Tumor Treating Fields

Novocure Ltd. is the exclusive manufacturer of the FDA approved (PMA Number P100034/S013, since 2015), Optune system based on tumor treating fields (TTF) for anti-cancer therapy. It is used for the treatment of patients with recurrent and newly diagnosed GB with over 14,000 patients treated globally. TTF is based on introducing alternating electric fields of intermediate frequency in a personalized manner to the supratentorial area of the brain. The transducer array is designed according to the tumor morphology using MRI scans to maximize the electric field intensity at the tumor site. The alternating fields cause misalignment of tubulin, thus interfering and disrupting the formation of mitotic spindle inhibiting cancer cell division and resulting in apoptosis. TTF therapy has minimal toxicity with comparable efficacy to chemotherapy. The transducer array is a wearable, portable device composed of ceramic insulators, adhesive hydrogels, and medical tape placed on the patient’s shaved head. It has to be replaced every few days and the patient’s head must be reshaven to achieve optimal results. Patients are required to take care of the device basic maintenance by replacing the transducer arrays and recharging the batteries. Although they can carry on with their daily routine during the treatment; patients must constantly carry the TTF generator and the batteries in a designated backpack. Despite the technical and social limitations, high treatment compliance may prolong patient survival. The 5-years overall survival rate of GB patient treated with Optune combined with TMZ treatment was 13% compared to 5% for patients treated with TMZ alone. Novocure has also FDA approval (through the HDE pathway) for mesothelioma treatment with TTF (the NovoTTF-100L delivery system) and has ongoing phase II clinical trials in brain metastases (NCT02831159), non-small cell lung cancer (NCT02973789), pancreatic cancer (NCT0377491), ovarian cancer (NCT03940196), and a Phase I clinical trial in liver cancer (NCT03606590). One challenge of the Optune system is that it cannot be used together with electronic medical devices (defibrillators, pacemakers, etc.). This can limit the number of patients that could use this technology. Recent preclinical studies suggest that the TTF have some effect on the BBB which perhaps allow the crossing of drugs that were unable to penetrate otherwise into the brain. These findings may lead to combined treatments utilizing TTF also for temporal opening of the BBB.

1.7. Glioblastoma Tumor Models

In order to evaluate the therapeutic potential of novel targets and drug delivery systems, an experimental model which properly recapitulates the clinical settings of the disease is required. As GB is a highly heterogeneous tumor which may originate from different cell types, and composed of various microenvironment components as well as the BBB, many in vitro and in vivo models have been developed over the years. Hence, we set to review some of these models and discuss the advantages and disadvantages of each approach.

1.7.1. Human GB Models

Several human and murine GB cell lines have been generated and broadly used for GB research and drug development. Human GB cell lines such as U-87 MG, U-251 MG, U-373 MG, and LN-229 were generated from GB patients and are commercially available. These human cell lines develop GB-like tumors when intracranially injected into mice, in contrast to T98G and A-172 which are not tumorigenic. LN-229 undergoes apoptotic cell death when stimulated with Fas-ligand and is a suitable model to study apoptosis. U-251 MG and U-87 MG are the most studied GB cell lines in the past few decades. In contrast to U-251 MG, U-87 MG exhibits less infiltrative phenotype with a disrupted BBB. Thus, testing drug delivery systems designed to cross the BBB using U-87 MG model may be misleading. Orthotopic U251 mice xenografts show high similarity to the human disease. However, these cell lines
are considered to be sub-cloned due to a genetic drift, therefore, do not represent their origin. Indeed, U-251 MG and U-373 MG were found to have the same origin but present different drug-sensitivity. Nonetheless, these cell lines were successfully used for drug screening and development. For instance, U-373 MG model was used to evaluate the efficacy of bevacizumab while U251 was used to evaluate BCNU and rapamycin treatments. Furthermore, genetic manipulations can be applied on these cell lines and others to generate GB specific molecular alterations such as EGFRvIII mutation which represents 50% of GB. It was recently recognized that growing cells in the presence of 10% serum, results in cell differentiation and leads to genetic alterations. In contrast, culturing patient-derived tumor spheroids in serum free medium, supplemented with several growth factors, enriches the culture for glioma stem cells and maintains their genetic characteristics. This method may be used to identify GB-specific molecular targets and prognosis markers.

### 1.7.2. Murine GB Models

In the attempt to mimic the clinical setting by using patient-derived GB cells, a major drawback is the lack of infiltrating immune cells that lack the adaptive immune system so that they will not be rejected. Thus, human xenograft models are not suitable for testing immunotherapies in vivo and may not be representative for various treatments as the immune system may affect drug efficacy and resistance. In contrast, murine GB cell lines may represent an appropriate syngeneic model such as GL261, CT-2A, and SMA-560, which are inoculated into immunocompetent mice. GL261 and CT-2A were generated by injection of the alkylating agents 3-methylcholantrene and 20-methylcholanthrene, respectively, into C57/BL6 mice. SMA-560 was obtained from a rare case of spontaneous GB tumor in the VM mouse strain. While GB are considered to be poorly-immunogenic tumors, GL261 are partially immunogenic. However, they carry KRAS and TP53 mutations which resemble both GB cells and macrophages. SMA-560 represents anaplastic astrocytoma and expresses class I MHC and TGF-β. Hence, SMA-560 is commonly used to test immune modulators, GB vaccines and T cell therapy. The CT-2A model represents several GB features such as intra-tumoral heterogeneity, invasiveness and chemoradiation resistance. An additional, widely used, rodent GB model is the C6 rat GB cell line. C6 tumors closely resemble the human disease regarding their morphological characterization, invasion and microenvironment activation, and can be grown in both rats and in mice. In order to investigate GB molecular subtype, several methods have been developed to transform normal murine CNS cells into GB initiating cells. These methods include lentiviral vectors and genetically-modified mouse models (GEMMs) to induce specific mutations in a specific cell type. These models are suitable for cell-origin studies and to investigate tumor-initiating oncogenic processes. One advantage of GEMMs is the development of de novo tumors, which may offer more reliable models for tumor-host interaction studies.

### 1.7.3. 3D GB In Vitro Models

3D models provide a platform for the investigation of the tumor microenvironment in vitro and can be used for rapid drug screening in a setting which better recapitulates the in vivo scenario. For instance, Musah-Eroje et al. have developed a basement membrane extract (BME) – based 3D model to study TMZ resistance mechanisms. Using this model, they have revealed hypoxia-induced TMZ-resistance mechanism of human GB cells. Gomez-Roman et al. have established a 3D system based on a polystyrene scaffold (Alvetex). They found differences in GB cell response to TMZ, bevacizumab and erlotinib between 2D culture and their 3D model. In addition, 3D models are suitable for multicellular cultures. Our group have demonstrated the use of a 3D model of GB using a modified hanging drop method, which is comprised of GB cells, astrocytes and brain endothelial cells, for the evaluation of a P-Selectin-targeted drug delivery system. In order to investigate GB-TAMs interactions, Heinrich et al. have reported a 3D bio-printed “mini brain” which incorporate both GB cells and macrophages. They found that when incorporated into their bio-printed model, naïve macrophages have acquired TAM-specific phenotype. Moreover, Ozturk et al. have developed a 3D bio-printed model combining perfused vascular channel for drug delivery studies. Their 3D imaging modality allows for long-term imaging and drug-response assessment. Moreover, many efforts are dedicated to the development of 3D multicellular BBB models for the evaluation of drug delivery methods to the CNS. These advanced BBB models show more similarities to the in vivo BBB properties than the traditional, TransWell based models. Bergmann et al., have developed a BBB organoid model which consists of brain endothelial cells, pericytes and astrocytes. Their models presented in vivo BBB characteristics such as the expression of tight junction proteins, various molecular transporters and drug efflux pumps that can be used for high-throughput drug-screening.

### 2. Current Clinical Studies and Future Outlook

GB therapy encounters many obstacles, arising from intrinsic tumor properties such as high tumor heterogeneity, lack of population-shared and targetable mutations, drug resistance, and tumor invasiveness. In addition, therapeutic agents are not effective unless properly designed to cross the BBB and accumulate in the tumor site. As discussed here, nanoparticles comprise a promising strategy to overcome these limitations. Combining strategies and different targets, both in the intrinsic tumor properties and in the tumor microenvironment, may improve current therapies and prolong patient survival. Designing NPs which target both BBB-expressing ligands such as APOE or integrins, and shared tumor cell-expressing molecules such as EGFR, may serve as suitable delivery systems. Selectins were found to be expressed on both brain endothelial cells and GB cells which make them promising candidates for this dual targeting approach. Loading such NPs with potent chemotherapy and biological agents such as TKIs, may improve treatment outcome and prevent acquired tumor cell resistance. In addition, ONTs provide a platform which can target shared oncogenic mechanisms. Delivering p53 mRNA or PTEN siRNA may overcome the tumor heterogeneity limi-
tation. However, there is still a need for successful ONT delivery systems and nanotechnology platforms have the potential to come to the rescue in these cases.

As our understanding of the brain microenvironment greatly advanced in the last decade, combining targeting approaches to GB cells together with targeting of TAM, with CSFR1 or STAT3 inhibitors for example, may not only reduce the oncogenic potential of the tumor cells, but can also harness the immune system to fight against the tumor. Although many attempts have failed to benefit patient outcome over the past years, several clinical trials are testing the use of novel nanomedicine approaches to treat GB. These trials are focused on advanced drug delivery methods such as targeted liposomes, polymeric nanoparticles, gold nanoparticles and local administration for the use of potent cytotoxic drugs or biological agents (Table 5) which may pave the way for much needed new therapeutics for GB.

Taken together, there has been an incredible progress in our understanding of the mechanisms that regulate GB initiation and progression in the last 3 decades. However, several open questions remain which their answer may dictate our ability to develop new therapeutics for this devastating disease. There are still many questions that researchers in academia, pharmaceutical companies and clinicians are working to answer. The big questions for the next decade on, and hopefully less, include why do GB patients respond so differently to the disease in terms of severity, time to recurrence and overall survival? Do these differences arise from the genetic background of the patients or perhaps environmental attributes that affect the patient immune response? Is the immune system capable of acquiring full or partial immunity to disease recurrence? How do the tumor cells evolve during the disease course, escape from the immune system, and acquire mutation-based drug resistance? What is the cell of origin of GB? Is it a common universal target? And is there an overexpressed marker that can be used for better targeting? Answering these questions can be translated to more personalized, cell type-specific, effective, and safe treatments for GB in the future.

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### Conflict of Interest

The authors declare no conflict of interest.

### Keywords

blood brain barrier, drug delivery, glioblastoma, nanoparticles, targeted therapies

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### Table 5. Current clinical trials evaluating the safety and efficacy of GB-targeted nanomedicines.

| ClinicalTrials.gov Identifier | Research name | Nanocarrier | Drug | Phase | Related articles |
|------------------------------|--------------|------------|------|-------|-----------------|
| NCT02766699                  | Safety, and Immunogenicity of EGFR(V)-EDV-DOX in patients with recurrent GB (Cerebral EDV) | EGFR(V)-EDV | DOX | Phase 1 | [420] |
| NCT00944801                  | PEGylated liposomal DOX with TMZ and radiotherapy in newly diagnosed GB | polyethylene glycol | DOX | Phase 1 and 2 | [421] |
| NCT01663012                  | NKTR-102 in bevacizumab-resistant high grade glioma | Irinotecan (Camptothecin) | Early Phase 1 | N/A |
| NCT03020017                  | NU-0129 for patients with recurrent GB or gliosarcoma undergoing surgery | small spherical gold nanoparticle | Targeting the anti-apoptotic protein Bcl2L12 | Phase 2 | [422] |
| NCT03463265                  | ABI-009 (Nab-Rapamycin) in newly diagnosed GB and recurrent high grade glioma | nanoparticle albumin-bound (Nab) | Rapamycin Avastin Radiation | Phase 2 | [423] |
| NCT02340156                  | Combined TMZ and SGT-53 for recurrent GB | DOTAP:DOPE cationic liposome | p53 cDNA (Plasmid) and Temozolomide | Phase 2 | [424] |
| NCT02858895                  | CED of MDNA55 for adults with recurrent or progressive GB | Fusion protein targeting IL-4 receptor | Pseudomonas aeruginosa exotoxin A | Phase 2 | N/A |
| NCT00104091                  | Safety and efficacy study for recurrent Grade 4 malignant brain tumors | EGFR-targeted, convection enhanced delivery | Pseudomonas exotoxin bound | Phase 2 | [425] |
| NCT00734682                  | Nanoliposomal CPT-11 for recurrent high-grade gliomas | Liposome nanoparticle | Irinotecan | Phase 1 | N/A |
| NCT03119064                  | BrUOG 329 GBM Onyvide With TMZ | Nanoliposome | Irinotecan | Phase 1 and 2 | N/A |
| NCT02022644                  | Image-assisted CED of liposomal-irinotecan In recurrent high grade glioma | | | | |
| NCT03603379                  | DOX-loaded anti-EGFR-immunoliposomes in high-grade gliomas | Anti-EGFR-immunoliposomes | DOX | Phase 1 | [426] |
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