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REVIEW

Overcoming the Blood–Brain Barrier: Successes and Challenges in Developing Nanoparticle-Mediated Drug Delivery Systems for the Treatment of Brain Tumours

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Abstract: High-grade gliomas are still characterized by a poor prognosis, despite recent advances in surgical treatment. Chemotherapy is currently practiced after surgery, but its efficacy is limited by aspecific toxicity on healthy cells, tumour cell chemoresistance, poor selectivity, and especially by the blood–brain barrier (BBB). Thus, despite the large number of potential drug candidates, the choice of effective chemotherapeutics is still limited to few compounds. Malignant gliomas are characterized by high infiltration and neovascularization, and leaky BBB (the so-called blood–brain tumour barrier); surgical resection is often incomplete, leaving residual cells that are able to migrate and proliferate. Nanocarriers can favour delivery of chemotherapeutics to brain tumours owing to different strategies, including chemical stabilization of the drug in the bloodstream; passive targeting (because of the leaky vascularization at the tumour site); inhibition of drug efflux mechanisms in endothelial and cancer cells; and active targeting by exploiting carriers and receptors overexpressed at the blood–brain tumour barrier. Within this concern, a suitable nanomedicine-based therapy for gliomas should not be limited to cytotoxic agents, but also target the most important pathogenetic mechanisms, including cell differentiation pathways and angiogenesis. Moreover, the combinatorial approach of cell therapy plus nanomedicine strategies can open new therapeutical opportunities. The major part of attempted preclinical approaches on animal models involves active targeting with protein ligands, but, despite encouraging results, a few number of nanomedicines reached clinical trials, and most of them include drug-loaded nanocarriers free of targeting ligands, also because of safety and scalability concerns.

Keywords: glioma, blood–brain barrier, blood–brain tumour barrier, nanoparticles, targeting

Classification of Brain Tumours

The most frequent brain tumours (gliomas) originate from glial cells, and range from low infiltrating to highly aggressive. In the 2007 World Health Organization (WHO) classified gliomas within four grades, basing on histopathologic features, such as mitotic index, anaplasia, cytological atypia, microvascular proliferation, and necrosis: grade I (ie pilocytic astrocytoma), grade II (ie astrocytomas and oligodendrogliomas), grade III (ie anaplastic astrocytomas and oligodendrogliomas), and grade IV (ie glioblastoma multiforme). In 2016 WHO included in the classification also molecular diagnostic criteria for infiltrating gliomas, including mutation of

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isocitrate dehydrogenase, deletion of 1p/19q chromosome, and histone mutations.[1] However, malignant or high grade (III and IV) gliomas are characterized by very poor prognosis. Furthermore, 8–10% of the adult patients with cancer develop brain metastases, with considerably variable incidence among different primary cancers. Lung, breast, colon, kidney cancer or melanoma can lead to brain metastases, 70% of which originating from lung and breast cancer.[2]

Current Therapy of Gliomas

Surgery is the first-line treatment both in low and high-grade gliomas[3] and the extent of resection has demonstrated a positive prognostic effect.[4] Several techniques have been designed to refine tumour resection: neuronavigation, use of 5-aminolevulinic acid,[5] and intra-operative magnetic resonance imaging (MRI). There is evidence that the combined use of these techniques improves the rate of gross total resection. The choice and the timeframe of subsequent adjuvant chemotherapy and radiation therapy (alone or as combined treatments) is still considered controversial. A survey within the European Low-Grade Glioma Network showed a relevant heterogeneity in the usage of chemotherapy. Generally, oral temozolomide (TMZ) is the first-line treatment after surgery for high-risk low-grade gliomas, or at progression, although, according to the Radiation Therapy Oncology Group, combination of radiotherapy with procarbazine, lomustine and vincristine regimen has been indicated as the gold-standard treatment.[6] While investigations are currently underway to evaluate the potential role of chemotherapy in low-grade gliomas, combined chemotherapy/radiotherapy approaches are currently practiced after surgery in high-grade gliomas. Radiotherapy is related to important side effects, such as post-radiation leuko-encephalopathy, nerve damage, hair loss, vomiting, infertility, and skin rash. Moreover, the effectiveness of chemotherapy is limited by toxic effects on healthy cells, tumour cell chemoresistance, and poor selectivity of anticancer drugs. Finally, the blood–brain barrier (BBB) is the major limit for the delivery of chemotherapeutic agents.[7] Thus, the chemotherapeutics currently employed for high-grade gliomas are still limited to few chemical compounds. Currently, owing to the Food and Drug Administration (FDA), oral TMZ is the standard chemotherapy for glioblastoma and anaplastic astrocytoma. Bevacizumab (Avastin®) is a monoclonal antibody that specifically binds vascular endothelial growth factor (VEGF). Despite FDA accelerated approval for bevacizumab for brain tumours, basing on its efficacy towards recurrent glioblastoma, its use has been involved with many controversies. Indeed, this anti-angiogenic therapy failed to improve patient overall survival, despite showing efficacy in shrinking or halting tumour growth.[8] In 1996, FDA approved biodegradable polyanhydride wafers loaded with carmustine (Gliadel®) for chemotherapy of recurrent high-grade gliomas. Patients with recurrent tumours benefit of an 8 weeks survival increase, when wafers were placed at the second surgery. Instead, the survival increase was 2.3 months in patients with early diagnosed tumours, undergoing primary resection followed by wafer placement.[9]

Experimental Drugs for Gliomas

Apart from currently approved chemotherapy, several drugs belonging to various therapeutic categories are currently under investigation for high-grade glioma treatment: the main mechanisms underlying their activity towards glioma are summarized in Figure 1. Advantages and disadvantages of such therapeutic drugs are listed in Table 1. In the following sections, the most important attempts and findings at preclinical and clinical level concerning such drugs are briefly described.

Cytotoxic Agents

Different marketed cytotoxic drugs have been tested off-label in preclinical glioma models and clinical trials, including nitrosoureas (alkylating agents), platinum salts, inhibitors of topoisomerase I (etoposide) and II (camptothecin, irinotecan, topotecan), mitotic inhibitors that is taxanes derivatives (paclitaxel, docetaxel), anthracyclines such as doxorubicin (DNA intercalation and topoisomerase II inhibition), and paclitaxel–cisplatin–vincristine (PCV) combination.[10]

Prodrugs of Cytotoxic Agents

Lipophilic prodrugs with molecular weight (MW) lower than 500 Da, and capable of forming less than 8 hydrogen bonds, should be able to overcome the BBB:[11] the cytotoxic drug chlorambucil was modified accordingly, with improved brain delivery.[12] However, recent interest has been growing concerning higher MW compounds, such as fatty acid – paclitaxel (PTX) prodrugs. In particular *cis*-linoleic acid conjugate with PTX (CLA-PTX) resulted in much higher plasmatic half-life and brain accumulation than free PTX, with encouraging therapeutic effect on brain tumour bearing rats.[13]

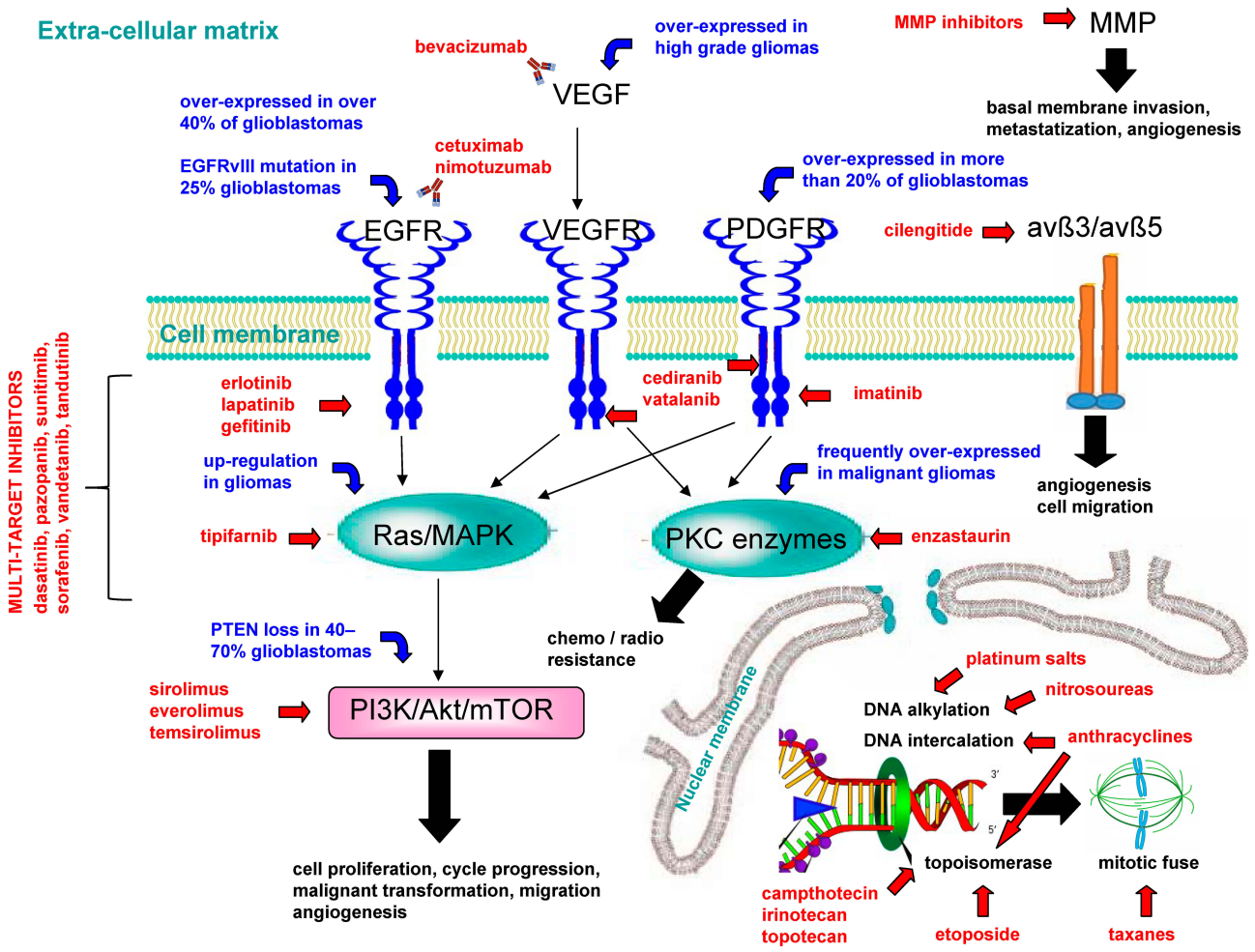

Figure 1 Main mechanisms of experimental drugs used against high-grade gliomas.
Abbreviations: avß3/avß5, avß3/avß5 heterodimers; EGFR, epidermal growth factor receptor; EGFRvIII, mutant EGFR; MMP, matrix metalloproteinase; PDGFR, platelet-derived growth factor receptor; PI3K/Akt/mTOR, phosphoinositide 3-kinase/protein kinase B/mammalian target of rapamycin; PKC, protein kinase C; PTEN, phosphatase and tensin homolog; Ras/MAPK, Ras mitogen-activated protein kinase; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor.

Angiogenesis Inhibitors

The expression level of VEGF directly correlates with tumour grade, with a nearly 10-fold gap between high and low-grade gliomas.[14] Thus, endothelial cells are a suitable target for high-grade glioma treatment. Apart from monoclonal antibody bevacizumab, that selectively binds VEGF, potential therapeutic agents include thalidomide and VEGF receptor (VEGFR) inhibitors, belonging to receptor tyrosine kinase (RTK) inhibitors category.[15] Cediranib and vatalanib are orally bioavailable VEGFR inhibitors, with simultaneous inhibitory activity on tyrosine-protein kinase KIT (c-Kit) and platelet-derived growth factor receptor (PDGFR), that, currently, are undergoing clinical trials for high-grade gliomas.[16]

Epidermal Growth Factor Receptor (EGFR) Inhibitors

EGFR, together with downstream signalling pathways (such as phosphoinositide 3-kinase/protein kinase B/mammalian target of rapamycin - PI3K/Akt/mTOR and Ras mitogen-activated protein kinase - Ras/MAPK), regulates cell survival, proliferation, angiogenesis and migration. EGFR is overexpressed in over 40% of glioblastomas.[17] Furthermore, 25% of glioblastomas are characterized by the expression of an EGFR mutant (EGFRvIII), which is defective of the extracellular ligand-binding domain.[18] Erlotinib, lapatinib, and gefitinib, three RTK inhibitors acting on EGFR, have been tested at preclinical and clinical stage for malignant gliomas treatment, with limited efficacy.[15] Also, cetuximab and nimotuzumab, two mouse

Table 1 Advantages and Disadvantages of Experimental Drugs Against High-Grade Gliomas

	Advantages	Disadvantages
Cytotoxic agents	• Complete oral bioavailability of TMZ[149] • Capacity of nitrosoureas and temozolomide to cross the BBB • Clinical efficacy of nitrosoureas chemotherapy combined with radiotherapy[150] • Clinical efficacy of the combination of etoposide and platinum salts[149] • Availability of newer platinum derivatives (eg oxaliplatin) with activity against resistant tumors[10]	• Important side effects: nausea, myelosuppression, hepatotoxicity, DOX cardiotoxicity, cisplatin nephrotoxicity, etc • Generally i.v. administered
VEGFR inhibitors	• Orally administered • Potential availability of biomarkers indicative of tumour responsiveness to VEGF inhibition[151] • Vascular normalization induced may reduce interstitial fluid pressure and allow better drug penetration[16] • Reduced tumour hypoxia[16] • Well tolerated	• Continued tumour growth in monotherapy[152]
Anti-VEGF antibodies	• Size reduction in xenograft models[153] • Good clinical efficacy also in monotherapy[154]	• Severe toxicities, especially in combination with irinotecan[71] • i.v. administration
EGFR inhibitors	• Potential identification of EGFR inhibition sensitive phenotypes[155]	• Limited efficacy both as mono and combination therapy, or radiotherapy[9] • Severe toxicities[9]
Anti-EGFR antibodies	• Orally administered • Binding to both wild-type and EGFRvIII receptors[18] • Increased survival in combination with radiotherapy in mouse models[157] • Responsiveness as monotherapy in EGFR overexpressing patients refractory to standard chemotherapy[158] • Well tolerated	• No difference in survival between normal and EGFR over-expressing patients[156] • i.v. administration
PDGFR inhibitors	• Orally administered • Promising direct antitumor activity in preclinical models[159] • Sensitize glioma cells to radiation[159] • Through vascular normalization, it facilitates the tumor penetration of cytotoxic drugs[159] • Well tolerated	• Poor clinical efficacy as monotherapy[9]
Ras/MAPK inhibitors	• Orally administered • Safety and efficacy of tipifarnib in combination therapy[9]	• No clinical efficacy of tipifarnib monotherapy[160]
PKC inhibitors	• Orally administered • Well tolerated and promising efficacy in highly pretreated patients[161]	• High doses are poorly tolerated with cardiovascular side effects[162]
PI3K/Akt/mTOR inhibitors	• Everolimus and sirolimus orally administered • Tumor growth was retarded in some patients[34] • Well tolerated	• Significant activation of Akt, potentially resulting in a reduced time to progression[34] • No clinical efficacy as monotherapy[163]
Multi target inhibitors	• Multiple target inhibition with a single agent • Pazopanib, sorafenib, sunitinib, tandutinib, vandetanib orally administered • Sorafenib overcomes BBB[164]	• No clinical benefit as monotherapy[9]

(*Continued*)

Table 1 (Continued).

	Advantages	Disadvantages
MMP inhibitors	• MMP overexpression in gliomas	• Negligible clinical efficacy[165] • Severe toxicities[165]
Integrin inhibitors	• Minimal toxicity also at high doses[166]	• Limited clinical efficacy as monotherapy[166]

Abbreviations: Akt, protein kinase B; BBB, blood–brain barrier; DOX, doxorubicin; EGFR, epidermal growth factor receptor; EGFRvIII, mutant EGFR; MMP, matrix metalloproteinase; PDGFR, platelet-derived growth factor receptor; PI3K/Akt/mTOR, phosphoinositide 3-kinase/protein kinase B/mammalian target of rapamycin; PKC, protein kinase C; Ras/MAPK, Ras mitogen-activated protein kinase; TMZ, temozolomide; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor.

anti-human EGFR antibodies, were employed in clinical trials for recurrent high-grade glioma patients.[9,19]

PDGFR Inhibitors

PDGFR is an RTK, which is overexpressed in more than 20% of glioblastomas,[20] and, like EGFR, it is upstream of Ras/MAPK and PI3K/Akt/mTOR signalling pathways.[21] Imatinib, an inhibitor of PDGFR, c-Kit, Abelson murine leukemia viral oncogene (Abl), and arginase (ARG), was tested in several clinical studies involving high-grade glioma patients.[15,22]

Ras/MAPK Inhibitors

The Ras/MAPK signalling pathway is downstream to several RTK inhibitors, such as fibroblast growth factor receptor (FGFR), insulin-like growth factor (IGFR), and the aforementioned EGFR, VEGFR, PDGFR. Therefore, it is responsible for cell proliferation and migration processes, as well as for cell cycle progression, and malignant transformation. Ras/MAPK is also upstream to PI3K/Akt/mTOR signalling pathway.[15,21,23] Furthermore, malignant gliomas are characterized by Ras/MAPK up-regulation.[23] Oral tipifarnib, a farnesyl transferase inhibitor with demonstrated radio-sensitizing effect is currently undergoing several clinical trials for high-grade gliomas.[24]

Protein Kinase C (PKC) Inhibitors

PKC enzymes belong to a serine/threonine kinases family, and it is downstream to different RTKs, including VEGFR and PDGFR.[25] PKC is involved in chemoresistance and radioresistance in malignant gliomas .[26] Furthermore, PKC is frequently overexpressed in malignant gliomas.[26–28] Enzastaurin, a lipophilic, orally administered PKC inhibitor, underwent different clinical trials as mono or combination therapy, but with limited clinical benefit.[9]

PI3K/Akt/mTOR Pathway Inhibitors

PI3K/Akt/mTOR is down-regulated by phosphatase and tensin homolog (PTEN), which, in turn, is altered (deleted, inactivated, or mutated) in nearly 40–70% glioblastomas.[29,30] Sirolimus, an orally bioavailable peptide macrolide, which is able to overcome the BBB and acts as mammalian target of rapamycin (mTOR) inhibitor, is currently employed in clinical trials for malignant gliomas.[31] Also, everolimus and temsirolimus, two analogs of sirolimus, respectively, orally and i.v administered, have been tested in clinical trials.[32,33] However, mTOR inhibitors induce significant activation of protein kinase B (Akt) in 50% of the patients, potentially causing a reduced time to progression: simultaneous inhibition of mTOR and Akt could overcome this limitation.[34]

Multi-Target Inhibitors

The simultaneous inhibition of different aberrant signalling pathways should result in great clinical benefit for targeted therapies. Apart from the combination of different drug therapies, multiple-target inhibition can be achieved through employment of small molecule inhibitors of newer-generation.[15] Dasatinib, pazopanib, sunitinib, sorafenib, vandetanib, tandutinib are currently in evaluation in clinical trials for glioblastoma, alone or in combination therapy.[9]

Matrix Metalloproteinase (MMP) Inhibitors

MMPs are proteolytic enzymes active also in physiological conditions. In tumours, they promote basal membrane invasion, metastatization and angiogenesis, being directly secreted by tumour cells or by the surrounding stroma, under tumoural stimulus. Marimastat, the most studied MMP inhibitor, showed promising in vitro inhibition of

malignant glioma, but no advantage in clinical trials with glioblastoma patients.[35,36]

Integrin Inhibitors

The expression of avß3 and avß5 heterodimers is increased in malignant gliomas, and it has been hypothesized that they contribute to regulate angiogenesis and migration.[37] Cilengitide is a potent antagonist of both avß3 and avß5. Several clinical trials testing cilengitide in combination therapy regimens are currently ongoing.[9]

Rationale for Employment of Nanomedicines in Glioma Therapy

The main mechanisms underlying the rationale of employing nanomedicines for glioma treatment are summarized below (Figure 2).

Passive Targeting

The permeability of adjacent brain vasculature changes during the growth of brain tumours. At their early stage, the growth of tumour cells depends only on normal brain vessels and the BBB is intact. With tumour progression, glioma cells invade the surrounding healthy tissues. When a large enough volume (>0.2 mm^3) is reached by the tumour cell cluster, a structural damage will affect the BBB, and blood–brain tumour barrier (BBTB) will be formed.[38] Within this concern, claudin-1, a protein specifically expressed in the tight junctions of endothelial cells, is down-regulated in vessels surrounding high-grade gliomas, thus increasing the permeability of the BBB.[39,40] This "leaky" BBTB is a common feature of high-grade gliomas, because of their increased metabolic requirements. Furthermore, it has been demonstrated that VEGF, associated with the high angiogenic nature of high-grade

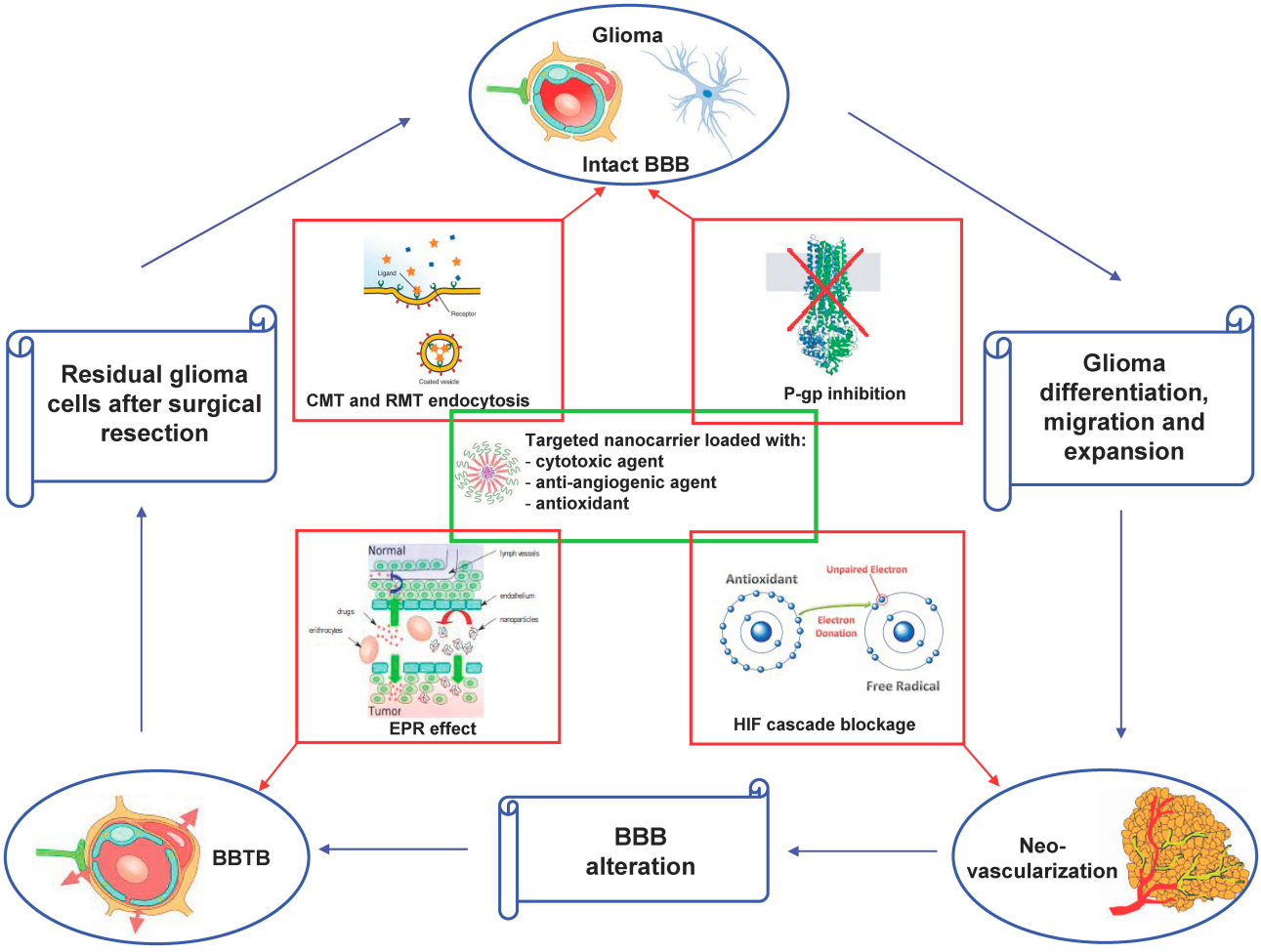

Figure 2 Rationale of employing nanomedicines for glioma treatment.
Abbreviations: BBB, blood–brain barrier; BBTB, blood–brain tumour barrier; CMT, carrier-mediated transport; EPR, enhanced permeation and retention; HIF, hypoxia-induced factors; P-gp, P-glycoprotein; RMT, receptor-mediated transport.

gliomas, increases the BBB permeability, by stimulating angiogenesis in response to hypoxia.[41,42] The "leaky" BBTB can be targeted for drug delivery purposes, by exploiting the so-called enhanced permeability and retention (EPR) effect. Since the pores cut-off size at the BBTB is highly variable, the size of nanocarriers largely influences the extent and efficacy of drug delivery. In fact, the vascular leakage is significantly reduced in the tumours within the brain microenvironment compared with other districts, and it is affected by the tumour stage and/or to the tumour model employed.[43] For instance, the critical cut-off size of the BBTB for intracranial U87MG xenografts ranges between 7 and 100 nm.[44] Despite the reduced size of vessel fenestrations may limit the potential of passive targeting strategy to address brain cancer, drug-loaded nanocarriers free of targeting ligands showed promising efficacy in orthotopic brain glioma models, thus allowing a translation to clinical therapy.[45]

Active Targeting

However, high-grade gliomas rapidly infiltrate the surrounding healthy tissue, where the BBB is not altered and the EPR effect cannot be achieved.[46] Indeed, in human patients, contrarily to animal tumour models, chemotherapy is only adjuvant, and it should mainly be addressed towards the eradication of residual tumour cells after surgery or radiotherapy. This cell population includes cells migrating from the tumour into healthy tissue or that feed the tumour from distant sites still active. Thus, the most relevant area for chemotherapy is the one surrounding the glioma, the so-called BAT (Brain Adjacent to Tumour), including cells in the invasion phase, that do not still affect the integrity of the BBB. Since BAT should be the main target of chemotherapy, the development of formulations easily crossing the intact BBB is essential.[47] In this case, suitable active targeting of nanocarriers is needed to reach the target tissue, by exploiting carrier and/or receptors overexpressed at the BBB. Carrier-mediated transporters (CMTs) are entailed in the transport of essential small molecules into the brain. Receptor-mediated transporters (RMTs) are abundantly expressed at the BBB, being exploited by large endogenous biomolecules.[2,48] Specific peptide receptors are included in RMT, eg low-density lipoprotein (LDL) receptor, transferrin (Tf) receptor, lactoferrin (Lf) receptor, insulin receptor, and receptors for insulin-like growth factors (IGF-1 and IGF-2).[49] CMT and RMT ligands have been frequently exploited for drug delivery as molecular "Trojan horses" for nanocarriers.[50–55]

Overcoming Extrusion Mechanisms at the BBB

Additionally, in the BBB endothelial cells (on the luminal side), as well as in glioma cells, the P-glycoprotein (P-gp) is present. P-gp is a 170-kDa glycoprotein associated to the cell membrane, belonging to the superfamily of ATP binding cassette (ABC) transporters, that actively extrudes from cells chemically different substrates. P-gp can hamper BBB overcoming by chemotherapeutic drugs, and it is responsible for chemoresistance in glioma cells.[56,57] Thus, P-gp inhibition can be considered as a potential dual strategy, suitable both to enhance drug penetration in the brain, and to reduce glioma chemoresistance. Thus, specific inhibitors of ABC subfamily member 1, such as elacridar and tariquidar, and anti-estrogen tamoxifen, were investigated for glioma treatment, owing to their anti-extrusion effect at the BBB.[58,59] Apart from chemical modulators, also nanoparticulate systems can inhibit the P-gp drug efflux mechanism: indeed they can deliver the drug within the cells by endocytosis, therefore making it less susceptible to the membrane-bound drug efflux mechanisms, since it is in the form of drug-matrix aggregates. Also, some ingredients employed in the formulation of nanocarriers, such as surfactants, can contribute to the P-gp inhibition.[60] Furthermore, co-delivery in the same nanocarriers of cytotoxic drugs and P-gp inhibitors (such as ketoconazole) has also been attempted in preclinical models.[61]

Inhibition of Tumour Differentiation, Migration and Neo-Vascularization

Lastly, from a histological point of view, in malignant gliomas, areas of necrosis, hypoxia and microvascular hyperplasia are present, with pseudo-palisades of cells migrating from the original necrosis area.[62,63] Accordingly, it has been recently shown that cancer stem cells (CSC) are responsible for the initiation step of human glioblastoma and medulloblastoma in intracranial mouse xenografts.[64] Unfortunately, the CSC location has yet to be precisely disclosed, but it has been demonstrated that hypoxia-induced factors (HIF) are responsible for CSC phenotype and can even shift normal cells to CSC.[65,66] Since HIF are involved both in angiogenesis and in maintaining CSC, a suitable treatment for high-grade gliomas

should be able to down-regulate their expression. This can occur by scavenging of reactive oxygen species (ROS). Indeed, during hypoxia, rising intracellular ROS concentration overcomes glutathione (GSH) guard levels. ROS can stabilize HIF, allowing VEGF transcription and consequent angiogenesis.[67–70] This mechanism underlies the rationale for employing anti-oxidant and anti-VEGF agents in glioma therapy. Indeed, the combination of anti-angiogenic bevacizumab and cytotoxic irinotecan is approved for recurrent glioblastoma.[71,72] Furthermore, in mice bearing intracerebral glioblastoma, pre-treatment with Tempol, a ROS scavenger, followed by TMZ chemotherapy, suppressed tumour growth and increased survival rate.[73] Within this concern, nanocarriers should be able to achieve co-delivery of anti-VEGF drugs and anti-oxidants, together to cytotoxic agents within the tumour tissue, owing to the aforementioned mechanisms. A recent experimental study showed an increase in bevacizumab activity and permeation through the BBB, when loaded within solid lipid nanoparticles (SLN).[74] Also treatment cerium oxide nanoparticles (nanoceria), with ROS scavenging properties, caused decreased expression levels of VEGF in a human astrocytoma cell line, associated with reduced motility and capacity of endothelial cells to form new capillaries.[75] Recently, camptothecin (CPT), a topoisomerase inhibitor, was employed in a co-delivery nanoparticulate system, that could stop the stabilization of HIF in brain tumours. For this, a suitable CPT prodrug was synthesized, by linkage with a tetraethylene glycol (TEG) spacer and α-lipoic acid (ALA). The obtained CPT-TEG-ALA prodrug can be cleaved by oxidation, thus acting as a ROS scavenger, and release CPT in its active form within glioblastoma cells. CPT-TEG-ALA was loaded in nanoemulsion along with α-tocopherol, an additional ROS scavenger, thus preventing HIF production.[76]

Preclinical Nanomedicines

Several approaches involve the alteration of the BBB, in order to increase brain penetration of nanocarriers loaded with chemotherapeutic drugs against glioma tumours. They include both invasive methods,[77] such as convection-enhanced delivery[78,79] and post-surgical implantation,[80–82] and noninvasive BBB opening through focused ultrasound (FUS).[83] Indeed, ultrasound with a frequency below 1 MHz can induce reversible and temporary BBB opening with the aid of microbubbles (Figure 3). This technique can be employed to deliver theranostic agents for the detection and treatment of various brain diseases, and it

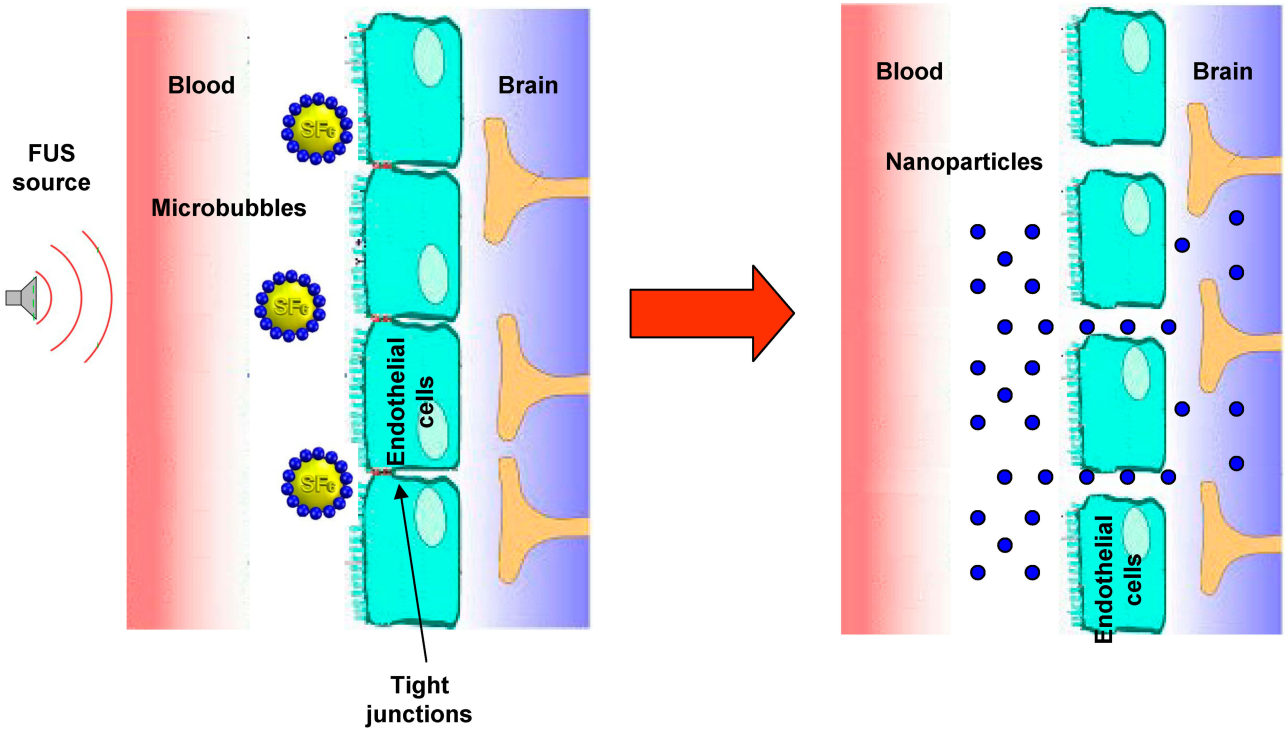

Figure 3 Scheme of brain delivery of nanoparticles with focused ultrasound (FUS) technique.
Abbreviation: SF_6, sulfur hexafluoride.

has been already subject of clinical trials.[84] In particular, Cu-Se ultrasmall nanoparticles and rare earth nanoparticles, labelled with near-infrared (NIR) dyes, have been employed, at preclinical level, to monitor FUS-induced temporary opening of the BBB and following recovery,[85] as well as to detect glioma in orthotopic animal models.[86] In further studies, the aforementioned Cu-Se ultrasmall nanoparticles, loaded with doxorubicin (DOX) and labelled with NIR dyes, were used concurrently with FUS and photodynamic therapy, and demonstrated good efficacy against orthotopic tumour models.[87]

Anyway, the nanocarriers discussed here below include only those used to overcome the BBB/BBTB without disruption, aiming to deliver anti-cancer drugs to the brain tumours. They can be made of different matrixes, including metals (ie calcium phosphate, iron oxide), lipids (SLN), phospholipids (liposomes), proteins (bovine serum albumin – BSA), synthetic polymers (ie poly-lactide – PLA; poly-lactide-glycolide – PLGA; PEG: polyethyleneglycol), natural polymers (ie chitosan) and polymer-lipid hybrid nanoparticles (PLN).[88]

Requested features of such nanomedicines are the following:[46]

- They should stabilize the drug from physicochemical or biological standpoints;
- They should avoid opsonization and, therefore, clearance by the reticuloendothelial system (RES), prolonging plasma circulation time and allowing EPR effect;
- They should be endowed with selective targeting strategies to the brain;
- They should not stimulate immune response.

However, the fact that almost 98% of drugs are unable to overcome the BBB is the main reason for employment of nanocarriers in high-grade glioma treatment. Two approaches have been documented in literature: the first employs plain nanocarriers, the second exploits active targeting, in order to enhance uptake of the nanocarriers by the endothelial cells at the BBB/BBTB.[89]

Within this concern, a huge number of nanocarriers aiming to glioma treatment have been engineered in the last decades. However, the following discussion will be focused only on the ones that underwent preclinical in vivo testing. Indeed, despite the presence of several in vitro investigation methods,[90] only preclinical animal models can be predictive of the real potential of administered nanomedicines, because of the simultaneous presence of biological barriers and metabolism/distribution. Pharmacokinetic and biodistribution experiments are relevant in order to foresee the metabolic fate of nanocarriers and loaded drugs, while glioma models allow to predict in vivo efficacy. Different mouse and rat glioma models are described in literature:[91] ethyl-nitrosourea induced orthotopic models in allogeneic or syngeneic healthy animals, orthotopic xenografts, or genetically engineered models. Within this concern, it should be pointed out that the glioma model employed can affect in a relevant manner the measured efficacy of the therapeutic drug delivery system under investigation, with the immune system playing a key role. In fact, today the so-called "immune privilege" of the brain is no more a retained concept.[92,93] Thus, if the absence of the immune system could cause an under-estimation of the therapeutic effect in xenografts, on the other side, the presence of the immunity in immuno-competent models can hamper the reproducibility of the glioma model itself. Indeed, the superior reproducibility of syngeneic models over allogeneic has been documented, owing to a lower graft rejection.[94] Furthermore, despite surgical resection is the primary approach for high-grade gliomas, the majority of preclinical models focus solely on drug treatment of solid intracranial tumours. Within this concern, recently a resection and recurrence orthotopic model has been developed, with potential for the investigation of tumour ablation combined with local and systemic chemotherapy.[95]

Plain Nanocarriers

Plain nanocarriers can improve drug delivery to gliomas by stabilizing the drug in the bloodstream, such as in the case of TMZ, which suffer from pH-dependent nonenzymatic chemo-degradation at the neutral pH, despite being the most employed drug for the treatment of brain cancers. Published reports demonstrated the therapeutic advantage of loading TMZ inside nanostructured lipid carriers (NLC), which is an optimized form of SLN with higher drug loading capacity.[96,97] Naked nucleic acid delivery is also associated with fast degradation, aspecific biodistribution and poor cell internalization. In fact, small interfering RNA (siRNA) and microRNA (miRNA) are promising tools to treat various diseases, but, due to their instability and poor delivery within target tissues, naked RNA is not robustly used. Thus, entrapment in positively charged nanocarriers can prevent from degradation/metabolism,

and favour cell internalization, both at the BBTB and in glioma cells.[98]

Nanocarriers can also increase drug half-life (for EPR effect), and/or favour endocytosis across BBTB endothelial cells, also owing to P-gp inhibition. In particular, this mechanism has been well documented for lipid nanocarriers, such as liposomes and SLN.[99] The physiological nature of the lipid matrix employed should improve natural brain uptake, and the employment of surfactants, such as Brij 78 or Polysorbate 80, to coat SLN can improve the nanoparticle brain uptake and drug activity, being the two molecules associated to P-gp inhibition.[100,101] However, SLN mechanism of action excludes any significant toxic effect on the BBB, as assessed by the absence of alterations to cell integrity and permeability, vessel blood flow, and choline active transport. Indeed, Western blot analyses of occludin and claudin-1 in the BBB cells, run following SLN administration, showed no modifications in protein expression.[102] Interestingly, in the case of the insoluble prototype anticancer drug named edelfosine (EDF) and of ion-paired idarubicin, also oral administration of drug-loaded SLN was effective against glioma subcutaneous models[101] and in enhancing drug concentration in the brain,[103] respectively.

Finally, plain nanocarriers can also be employed for stimuli-responsive formulations. Indeed, the combination of silver nanoparticles (AgNPs) with magnetic nanoparticle hyperthermia (MNPH) was used as a treatment in the glioma model.[104]

The most important plain nanocarriers, employed in in vivo preclinical studies for glioma non-invasive treatment, are shown in Table 2.

Functionalized Nanocarriers

Nanocarriers can be functionalized on their surface with suitable ligands, in order to exploit RMT and CMT at the BBB. As previously mentioned, several specific receptors, in particular for endogenous proteins and peptides, were employed as molecular "Trojan horse" for nanocarrier systems.[49] LDL receptor is overexpressed both at the BBB and in glioma cells and, therefore, it is a potential target to be exploited for the delivery of therapeutic agents. Apolipoproteins (Apo) are usually employed to target LDL receptors, but Apo being a high MW protein, usually shorter chimera peptides including the receptor-binding domain are employed.[50,105] Angiopep-2 (TFFYGGSRGKRNNFKTEEY, molecular weight 2.4 kDa), a peptide belonging to the Kunitz domain-derived family, is a potent ligand of LDL on the BBB employed for nanocarriers functionalization.[106] Within this concern, it should be pointed out that Polysorbate 80 is frequently employed as suspending agent for plain nanocarriers aiming to drug brain delivery. Indeed, it has been hypothesized that this nonionic surfactant can adsorb endogenous Apo E present in serum on the surface of nanoparticles.[107,108] Mammalian Lf is a cationic iron transporting glycoprotein (80 kDa), whose receptor is expressed on the endothelial side of the BBB. Studies with membrane preparations of mouse brain have shown that the Lf receptor at the BBB has two classes of binding sites: a high-affinity, with a dissociation constant K_d of 10.61 nM, and a low-affinity with a K_d of 2228 nM. Plasmatic concentration of endogenous Lf (~5 nM) is lower than the K_d of Lf receptors at the BBB. Thus, the competitive inhibition with endogenous Lf is avoided, allowing employment of Lf functionalized nanocarriers for drug delivery in glioma models.[109] Finally, apart from protein ligands, nanocarriers can be functionalized also with small molecules, recognized at the BBB from specific receptors/carriers, such as GSH receptor,[110] folate receptor and monocarboxylic acid transporter (MCT-1).[61,111]

However, different critical issues are associated with active targeting to the BBB. First of all, most of the RMT are not present exclusively at the BBB. For example, Tf receptor is expressed in monocytes, red blood cells, lungs, hepatocytes, and in the gut, along with the BBB. Within this concern, it is reported that active targeting of nanocarriers should benefit from employment of a "spacer" between the nanoparticles' surface and the grafted protein, and that grafting a low amount of protein should enhance selectivity for the BBB rather than nontarget tissues.[51] Moreover, nanocarriers grafted with physiological ligands (ie Tf) undergo binding competition with the corresponding endogenous protein, which can decrease targeting efficacy;[46] then, most of the available targeting proteins can cause immunogenic reactions; finally, some receptors on the BBB, such as insulin and Tf, control homeostasis of iron and glucose within the brain, and nanocarriers grafted with monoclonal antibodies against these receptors may down-regulate their activity and raise safety concerns.[112]

Functionalization of nanocarriers can be exploited also to target specific receptors overexpressed in glioma and/or at the BBTB. Previously mentioned overexpression of EGFR and EGFRvIII mutant at the BBTB allows a selective targeting.[113] Also, integrins play important

Table 2 Plain Nanocarriers Aiming to Glioma Therapy Employed in Preclinical Studies

Nanocarriers	Drug	Experimental in vivo Model	Achievements in vivo	References
AgNPs combined with MNPH	Ag^+	Efficacy in glioma rat model	Enhanced Bcl-2-associated X protein expression	[104]
Cationic SLN	PEGylated c-Met siRNA	Efficacy in U87 xenografts	Enhanced accumulation in brain tumour and down-regulation of c-Met levels	[93]
Liposomes	Oxaliplatin	Biodistribution and survival analysis in F98/Fischer glioma model	Increased brain oxaliplatin concentration and median survival time of glioma models	[167]
NLC	TMZ; GFP encoding pDNA	Efficacy in U87 xenografts	Gene transfection and enhanced antitumor activity	[96]
PLGA nanoparticles, SLN, NLC	TMZ	Efficacy in U87 xenografts	Best efficacy obtained with NLC formulation	[168]
Polymer nanogel	miRNA miR.34a	Efficacy in U87 xenografts	Significant tumor growth inhibition	[169]
Polysorbate 80 coated PBCA nanoparticles	DOX	Biodistribution in glioma models	Increased accumulation of DOX in brain tumour	[170]
Polysorbate 80 coated PBCA nanoparticles	TMZ	Biodistribution in healthy rats	Increased brain uptake of TMZ	[171]
Polysorbate 80 coated PBCA nanoparticles	Gemcitabine	Survival analysis in C6/Sprague Dawley rat glioma models	Prolonged survival of glioma models	[172]
Polysorbate 80 coated PLA nanoparticles	TMZ	Pharmacokinetic and biodistribution in rats	Enhancement in half-life of TMZ with higher deposition in the brain	[173]
Polysorbate 80 coated SLN	CPT	Pharmacokinetic and biodistribution in rats	Increased brain accumulation of CPT	[108]
Polysorbate 80 coated SPION	DOX	Biodistribution and efficacy in C6/ Sprague Dawley glioma model	Enhanced brain accumulation of SPION and increased anti- tumour efficacy under magnetic field	[174]
SLN	PTX	Rat brain perfusion experiment after intra-carotid administration	Enhanced PTX accumulation in brain; P-gp overcoming	[100]
SLN	EDF	Biodistribution and efficacy in subcutaneous mouse model	Good drug accumulation in brain after oral administration; P-gp overcoming	[101]
SLN	DOX	Pharmacokinetic and biodistribution in rats and rabbits	Enhanced DOX accumulation in brain	[175,176]
SLN	Idarubicin	Pharmacokinetic and biodistribution in rats	Enhanced idarubicin accumulation in brain after SLN oral administration	[103]
SLN, NLC	TMZ; vincristine	Efficacy in U87 xenografts	Improved glioma inhibition with NLC and drug co-delivery	[97]

Abbreviations: AgNPs, silver nanoparticles; Bcl-2, B-cell lymphoma 2; c-Met, tyrosine-protein kinase Met; C6, C6 cells; CPT, camptothecin; DOX, doxorubicin; EDF, edelfosine; GFP, green fluorescent protein; miRNA, microRNA; MNPH, magnetic nanoparticle hyperthermia; NLC, nanostructured lipid carriers, PBCA, poly(butyl cyanoacrylate); pDNA, plasmid DNA; PEG, polyethylenglycol; P-gp, P-glycoprotein; PLA, poly-lactide; PLGA, poly-lactide-glycolide; PTX, paclitaxel; siRNA, small interfering RNA; SLN, solid lipid nanoparticles; SPION, superparamagnetic iron oxide nanoparticles; U87, U87 cells; TMZ, temozolomide.

roles in tumour invasion and angiogenesis: in glioblastoma avß3 and avß5 integrin receptors are overexpressed on brain tumour cells and neo-vessels, favouring interaction with the extracellular matrix. Integrins can be specifically targeted by employing arginine-glycine-aspartic acid (RGD) peptides, such as cilengitide (cyclo [RGDfV]), a cyclic RGD peptide.[114] Nucleolin, instead, exists only in the nucleus of cells, thus offering an attractive target for cells characterized from a high internalization rate. F3 peptide, that specifically binds to nucleolin, was utilized to decorate nanocarriers, in order to realize glioma cell and neo-vasculature dual-cellular targeting.[115] Furthermore, as previously mentioned, MMPs are a group of zinc-dependent proteins acting as key modulators of tumour invasion and metastatization, due to their degrading capacity of the extracellular matrix. In addition, MMP-2/9 is also required for the tumoural angiogenic switch. Thus, MMP-2/9 conjugated low-molecular-weight protamine (ALMWP) was employed for glioma targeting of nanocarriers.[116] Also chlorotoxin (CTX), a small peptide (36-amino acid) derived from Leiurus quinquestriatus (scorpion) venom, can bind to MMP-2, and it was employed for nanocarriers functionalization.[117] Finally, IL-13Ra2 receptor is overexpressed in pilocytic astrocytomas and glioblastoma. Therefore, interleukin-13 (IL-13) can be used to target nanocarriers to glioblastoma tissue.

A summary of the most important functionalized nanocarriers employed in in vivo preclinical studies is shown in Table 3, and a scheme of the main active targeting strategies is displayed in Figure 4.

Nanocarrier-Mediated Cell Therapy

In addition to the selective BBB, glioma chemotherapy can be hampered from the fact that it is difficult to obtain a sufficiently high drug concentration in the tumour, in order to kill infiltrative malignant cells, without hampering healthy brain tissue. Thus, even if a relatively high drug accumulation within brain tissue is obtained, chemotherapeutics may undergo unwanted distribution in the extracellular matrix, or within intratumoral necrotic pockets, but without exerting the desired effects on target glioma cells. These limitations may be overcome by using stem cells as carriers of nanoparticulate delivery systems.[118] Indeed, cell-based therapies represent an innovative and valuable tool for cancer treatment, and recent interest is growing also for high-grade glioma therapy. A number of cells have been recently studied, either in preclinical or clinical trials for brain tumour targeting, and can be exploited also in combination with nanocarrier systems.

In particular, adult mesenchymal stem cells (MSC) show a tumour tropic ability, that can be exploited simultaneously to target tumour and deliver therapeutic agents.[119] With genetic modification, MSC are able to home cancer tissues and affect the tumor growth by the secretion of cytotoxic molecules. Moreover, MSC are able to trespass the BBB in physiological and pathological conditions, and preclinical studies showed that MSC engineered to express suicide gene enhance the antitumor response in glioblastoma animal models. Within this concern, the combinatorial approach of cell therapy, small-molecule chemotherapy and nanomedicine strategies can open new opportunities for glioma treatment (Figure 5). Internalization/binding of drug-loaded nanoparticles into MSC can be exploited to increase the antitumor efficacy by targeted delivery to the tumour microenvironment.[120] Indeed, PTX loaded PLGA nanoparticles showed an enhanced activity in brain tumor, which is ascribed to the sustained release of the drug.[121] In a recent work, a hybrid system consisting of MSC spheroids and methotrexate (MTX)-loaded nanoparticles was engineered, in order to increase retention at tumour site: this system improved tumour inhibition in a heterotopic glioblastoma murine model.[122] Even though drug-loaded MSC are a promising strategy, some major issues should be considered, such as the fact that the conjugation of nanodrugs to MSC surface could affect the tumour homing ability, as demonstrated by a lot of studies carried out on brain tumour xenografts.

Interestingly, also adipose-derived stem cells (ADSC) show tumour homing ability. This behaviour can be exploited to design drug delivery systems for brain tumours, such as cell-based carriers for nanoparticulate systems. Moreover, it is possible to endow such nanoparticles with stimuli-responsive properties for targeted drug delivery. Indeed, superparamagnetic iron oxide nanoparticles (SPION) were loaded with PTX and subsequently taken up by ADSC via endocytosis.[123] Drug release was then activated by high-frequency magnetic field in a glioblastoma murine model. The dual-modality therapeutic strategy including ADSC and smart nanoparticles may be further investigated in the near future for clinical translation.

Given that the "immune privilege" of the brain is no more a retained concept, also the recent advances in immunotherapy strategies offer new opportunities for a synergistic

Table 3 Functionalized Nanocarriers Aiming to Glioma Therapy Employed in Preclinical Studies

Nanocarriers	Drug	Functionalization	Experimental in vivo Model	Achievements in vivo	References
Polymeric nanoparticles	TMZ	Angiopep-2	Biodistribution of liposomes and efficacy in C6/ICR mouse glioma models	Enhanced brain distribution of DOX and promising efficacy in glioma models	[177]
Dendrimers	TRAIL DNA	Angiopep-2	Biodistribution of dendrimers and survival analysis in C6 mouse xenografts	Increased survival of xenografts	[106]
PCL nanoparticles	DOX	Angiopep-2	Pharmacokinetics, biodistribution and survival analysis in C6/Wistar rat glioma models	Enhanced brain uptake of DOX, prolonged survival of glioma models	[178]
PLGA nanoparticles	IP10	Angiopep-2; EGFRvIII scFv	Efficacy in U87-EGFRvIII cells xenografts	Reduced tumour growth, prolonged survival of glioma models	[179]
Liposomes	Daunomycin	Anti Tf Receptor antibody	Pharmacokinetic & biodistribution in rats	Increased daunomycin accumulation in brain	[180]
Calcium phosphate nanoparticles	ATF5 siRNA	Apo E	Glioma distribution, ATF5 expression and survival analysis in C6 mouse xenografts	Efficient tumour targeting and increased survival of xenografts	[181]
Polymersomes	Saporin	Apo E	Biodistribution and efficacy in U87 mouse xenografts	Specific brain accumulation of polymersomes, encouraging efficacy towards brain tumours	[182]
SLN	MTX prodrug	Apo E chimera peptide	Biodistribution and survival analysis in F98/Fischer rat glioma model	Increased brain accumulation of MTX; encouraging efficacy	[50]
Lipid nanoparticles	Porphyrin	Apo E3	Pharmacokinetics and biodistribution in mice, efficacy in U87 mouse xenografts	Selective drug accumulation in brain tumour compared to healthy parenchyma	[183]
Liposomes	–	Cetuximab	Biodistribution in U87 mouse xenografts	Increased brain accumulation of liposomes	[113]
Liposomes	DOX	Chlorotoxin	Biodistribution of liposomes and efficacy in U87 mouse xenografts	Brain accumulation of liposomes, reduced tumour growth	[117]
Polyionic micelles	Cilengitide	Cilengitide	Survival analysis in C6/Wistar rat glioma models	Prolonged survival of glioma models	[184]
Polymeric micelles	DACHPt	Cilengitide	Efficacy in U87 mouse xenografts	Reduced tumour growth	[185]
Liposomes	DOX	Cilengitide and Peptide 22 (LDL receptor)	Biodistribution and survival analysis in intracranial glioma-bearing mice	Prolonged survival time of glioma models	[114]
PEG–PLA micelles	PTX	EGFR/EGFRvIII targeting peptide	Pharmacokinetics in healthy rats, biodistribution and efficacy in U87 mouse xenografts	Specific micelles distribution to the brain, reduced tumour growth in glioma models	[186]

(*Continued*)

Table 3 (Continued).

Nanocarriers	Drug	Functionalization	Experimental in vivo Model	Achievements in vivo	References
PEG-PLA nanoparticles	PTX	F3 peptide (targeting nucleolin) and tLyp-1 peptide (targeting neuropilin)	Pharmacokinetics in rats; biodistribution and survival analysis in C6 mouse xenografts	Enhanced PTX accumulation and deep penetration at the tumour location; prolonged survival in xenografts	[115]
PEGilated liposomes	DOX	GSH	Pharmacokinetics, biodistribution and efficacy in U87 mouse xenografts	Enhanced brain retention of DOX; strong inhibition of brain tumour growth	[110]
Liposomes	DOX	IL-13	Efficacy in U251 mouse xenografts	Reduced tumour growth in glioma models	[187]
BSA nanoparticles	DOX	Lf	Pharmacokinetics in rats, biodistribution in C6/Wistar rat glioma model	Increased brain uptake of DOX	[188]
Cationic liposomes	DOX	Lf	Biodistribution and survival analysis in C6/Wistar rat glioma models	Increased accumulation of DOX in brain and prolonged survival time of glioma models	[109]
Liposomes	^{99m}Tc-BMEDA	Lf	Pharmacokinetic & biodistribution in mice	Increased brain accumulation	[189]
Olive oil nanoparticles	TMZ	Lf	Pharmacokinetic & biodistribution in healthy mice; biodistribution and efficacy in GL261/C57BL/6 mouse models	Enhanced brain distribution of TMZ and promising efficacy towards glioma	[190]
Olive oil nanoparticles	Aurora Kinase B siRNA	Lf	Gene silencing and survival analysis in GL261/C57BL/6 mouse models	Survival improvement of glioma models treated with nanoparticles and TMZ simultaneously	[191]
PEG-PCL polymersomes	DOX and tetrandrine	Lf	Pharmacokinetics, biodistribution and efficacy in C6/Wistar rat glioma model	Improved DOX distribution in brain, reduced tumour growth and increased survival in glioma models	[192]
PEG-PLA nanoparticles	PTX	Lf and tLyp-1 peptide (targeting neuropilin)	Pharmacokinetics in healthy rats, biodistribution and efficacy in C6 mouse xenografts	Enhanced tumour accumulation of PTX and increased survival of glioma models	[53]
NLC	TMZ and vincristine	Lf, RGD	Biodistribution and efficacy in U87 xenografts	Specific brain distribution of the drugs, promising efficacy in glioma models	[193]
Liposomes	DOX and vincristine	T7 and ^{D}A7R	Biodistribution of liposomes and efficacy in C6/ICR mouse glioma models	Enhanced brain distribution of liposomes and promising efficacy in glioma models	[194]
Liposomes	5-fluorouracil	Tf	Biodistribution of radiolabelled liposomes in healthy rats	Increased brain uptake of liposomes	[195]

(*Continued*)

Table 3 (Continued).

Nanocarriers	Drug	Functionalization	Experimental in vivo Model	Achievements in vivo	References
Liposomes	TMZ and bromodomain inhibitor	Tf	Biodistribution in mice and efficacy in U87 mouse xenografts and C57BL/6 mouse models	Improved liposomes distribution to the brain; promising efficacy in glioma models	[196]
SLN	MTX prodrug	Tf, Insulin	Biodistribution in healthy rats	Increased brain accumulation of MTX	[51]
Liposomes	DOX	Tf, Octaarginin	Biodistribution of liposomes and efficacy in U87 mouse xenografts	Prolonged survival of glioma models	[197]
SLN	Docetaxel	HBA	Pharmacokinetics and biodistribution in healthy rats	Enhanced drug brain uptake	[111]
SLN	Docetaxel ketoconazole	Folic acid	Pharmacokinetics and biodistribution in healthy rats	Enhanced drug brain uptake; P-gp overcoming	[61]
PEG-co-PCL nanoparticles	PTX	Activatable LMWP coupled to a MMP-2/9-cleavable peptide sequence	Pharmacokinetics in rats; biodistribution and efficacy in C6 mouse xenografts	Specific PTX accumulation in glioma; enhanced efficacy in xenografts	[116]

Abbreviations: ^{99m}Tc-labeled *N*,*N*-bis(2-mercaptoethyl)-*N'*,*N'*-diethylethylenediamine (^{99m}Tc-BMEDA); Apo, apolipoprotein; ATF5, activating transcription factor-5; C6, C6 cells; ^{D}A7R, ^{D}A^{D}T^{D}W^{D}L^{D}P^{D}P^{D}R sequence, which has a high affinity for VEGFR 2; DACHPt, (1,2-diaminocyclohexane)platinum(II); DOX, doxorubicin; EGFR, epidermal growth factor receptor; EGFRvIII, mutant EGFR; F98, F98 cells; GL261, GL261 cells; GSH, glutathione; HBA, β-hydroxybutyric acid; IP10, Interferon-γ-inducible protein; LDL, low density lipoprotein; Lf, lactoferrin; LMWP, low-molecular-weight protein; MMP, matrix metalloproteinase; MTX, methotrexate; NLC, nanostructured lipid carriers; PCL, poly(ε-caprolactone); PEG, polyethylenglycol; P-gp, P-glycoprotein; PLA, poly-lactide; PLGA, poly-lactide-glycolide; PTX, paclitaxel; RGD, arginine-glycine-aspartic acid; scFv, single-chain Fv fragments; siRNA, small interfering RNA; SLN, solid lipid nanoparticles; T7, HAIYPRH sequence, which can bind to Tf receptors; Tf, transferrin; TMZ, temozolomide; TRAIL, tumor necrosis factor (TNF) related apoptosis-inducing ligand; U251, U251 cells; U87, U87 cells.

combination of cell therapy with chemotherapeutic drug delivery systems, in order to target brain tumours.[124] For example, the combination of dendritic cell-targeted vaccines with nanoparticles loaded with anticancer drugs should lead to a superior anticancer activity.[125] Combination with nanocarrier loaded with checkpoint inhibitors could be another valuable therapeutic approach.[126] Furthermore, neutrophils (NE) have a native ability to traverse BBB/BBTB and penetrate the glioma site, where the tumour associated NE favour the continuous recruitment of circulating NE; in addition, local brain inflammation, following surgical tumour removal, activates NE migration towards to the inflamed brain. This amplification of inflammatory signals supports an enhanced brain tumour targeting of nanocarriers loaded in NE, such as PTX loaded liposomes[127] and DOX loaded magnetic mesoporous silica nanoparticles, which is evident also in resection and recurrence orthotopic models.[128] Finally, chimeric antigen receptor (CAR) T cells should be considered, too. They are T cells, generally autologous, ex vivo artificially engineered on their surfaces in order to recognize tumour-associated antigens. The CAR shows both an antigen-binding and a T cell activating function. These modified CAR T cells can produce the lysis of the cells presenting the associated tumor antigen when administered intravenously in patients. Currently, CAR-T cell therapy has been approved in B-cell lymphoma and leukemia, but clinical trials are ongoing for glioblastoma treatment.[129,130] Very recently CAR T cell administration showed to improve anti-glioma response.[131] Also in this case, CAR-T cells could be associated with nanocarriers in order to improve the therapeutic outcome, and CAR-T cell surface can also be modified to targeting purposes. Indeed, human epidermal growth factor receptor 2 (HER-2) functionalized CAR T cells demonstrated increased persistence over time.

Within this context, suitable attention should be devoted also to cell membrane-coated nanoparticulate systems. Wrapping nanoparticles with cell-derived membranes provide nanocarriers of a natural surface coating with complex biological entities, which are nearly impossible to synthetically replicate via ligand attachment. This allows to overcome some of the previously mentioned shortcomings of actively targeted nanocarriers, such as opsonization and incorrect ligand recognition. Cell

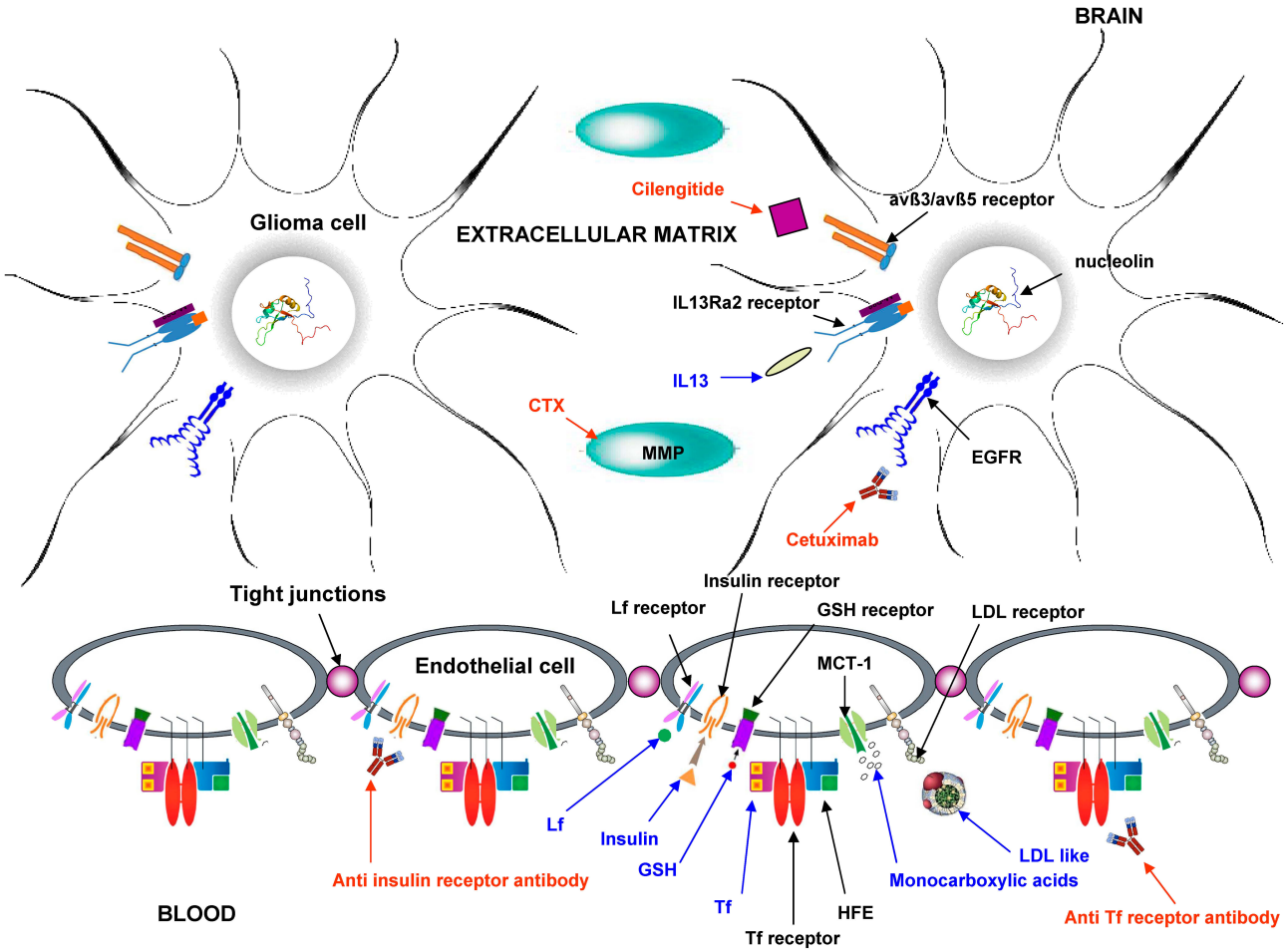

Figure 4 Scheme of the main mechanism used for active targeting of nanocarriers in glioma therapy. Blue: endogenous ligands; red: exogenous ligands.
Abbreviations: avß3/avß5, avß3/avß5 heterodimers; CTX, chlorotoxin; EGFR, epidermal growth factor receptor; GSH, glutathione; HFE, homeostatic iron regulator protein; IL 13, interleukin 13; LDL, low-density lipoprotein; Lf, lactoferrin; MCT-1, monocarboxylic acid transporter 1; MMP, matrix metalloproteinase; Tf, transferrin.

membrane coating technology was firstly introduced by employing red blood cell membranes, in order to provide "stealth" properties to synthetic nanoparticles. Currently, it has been applied to many cell types, including cancer cells.[132] Indeed, cancer cells possess the unique ability to self-target homologous cells (the so-called homotypic targeting), which can be translated to wrapped nanocarriers, showing also reduced immune clearance compared to uncoated ones (Figure 6). Recently, in a study involving PLGA nanoparticles coated with U87 glioma cell membrane fractions, an induction of cancer cell-specific immune response was demonstrated.[133]

Clinical Nanomedicines

Despite relevant evidences at the preclinical level, and several clinical studies addressing the employment of nanocarriers for brain tumours chemotherapy, particularly for glioblastoma, till now no new nano-drug has yet approved for brain tumour therapy. In general, the approval rate for novel nanomedicines is below 10%, mainly because of safety and efficacy profile failures during preclinical and clinical studies.[134] Indeed, regulatory agencies require manufacturers to perform accurate preauthorization studies to assess the quality, safety, and efficacy profiles of a new nanomedicine.[135,136] Furthermore, the difficulty to find out suitable preclinical models that truly represent what happens in the humans is a major flaw, that hampers clinical translation of nanomedicines.[95]

Within this concern, according to the European Commission Recommendation (2011/696/EU), 100 nm is the demarcating upper size limit where the properties of materials can change significantly from conventional equivalents, and the European Medicines Agency (EMA) also included an official definition of nanomedicine as being up to a size of 100 nm.[137] However, frequently nanomedicines have broader size range than the proposed

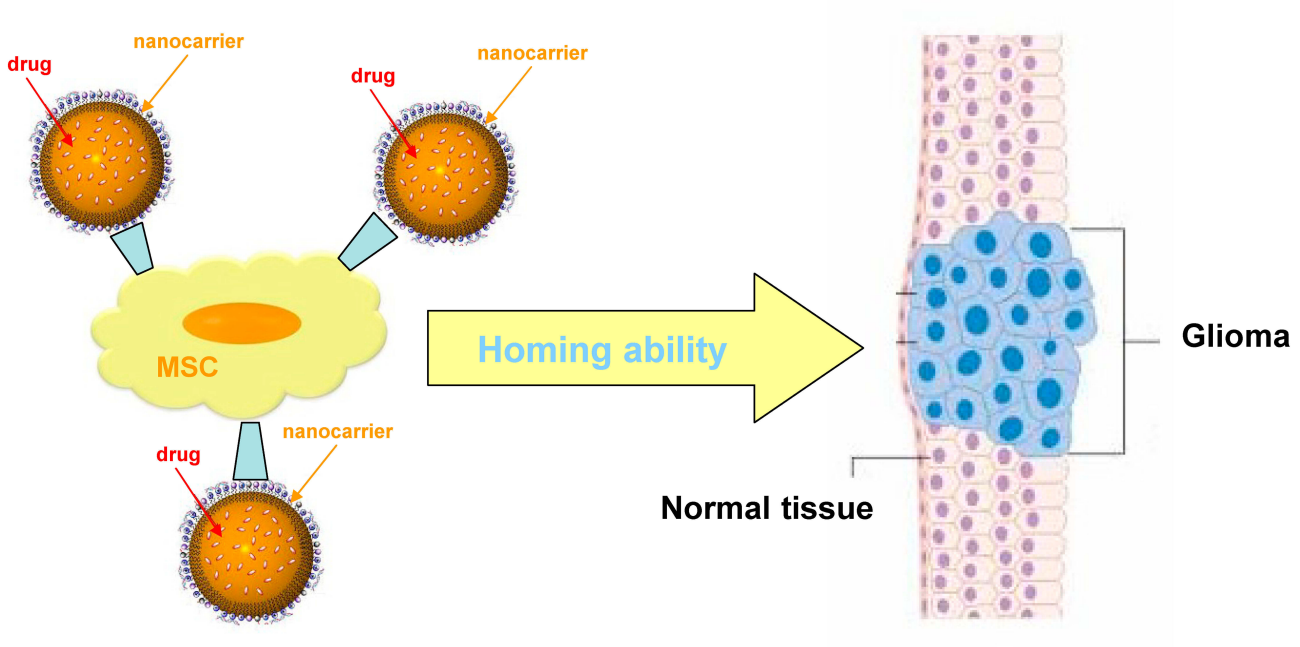

Figure 5 Scheme of nanocarrier mesenchymal stem cells (MSC) combined therapy of gliomas.

definition, inducing the EMA to include all "structures" with sizes of less than 1000 nm, that are designed to have specific properties,[138] can improve site-specific drug delivery and significantly alter toxicological profiles, thus allowing to perform a case-by-case evaluation.[139] Nonetheless, despite the numerous guidelines existing for the validation methods of chemical parameters in such matrixes, suitable implementation of this legislation should require validated analytical methods for nanoparticles' characterization, that, so far, do not exist.[140]

Some of the most important clinical trials with new emerging nanomedicines are here reported. Few of them involve active targeting mechanisms. In fact, despite the existence of good manufacturing practices for nanodrug delivery systems, that can be employed for translation to clinical trials,[141] actively targeted nanomedicines are associated with high costs and scale-up issues. A Phase I clinical study involving patients with recurrent glioma (grade 2–4) has been carried out by administering PTX-Angiopep-2 peptide–drug conjugate (GRN1005). Even if GRN1005 improved PTX permeation into tumour tissue, Phase II trial interim analysis did not show therapeutic response.[142,143] Tf conjugated diphtheria toxin (Tf-CRM107) showed in vitro toxicity towards glioma cells and it was effective in xenografts after local administration. Moreover, in phase I and II clinical trials, local administration of Tf-CRM107 resulted in low toxicity, encouraging response rate (35%) and promising overall survival (74 weeks) in patients with recurrent high-grade brain tumours. However, early Phase III clinical trials employing this approach were terminated due to disappointing preliminary results.[9,144] In another interesting phase I study, EnGeneIC delivery vehicle loaded with DOX was tested in patients with recurrent glioblastoma. The anti-EGFR monoclonal antibody Vectibix was used to target EGFR on cancer cells, thus leading to DOX release.[145]

However, the most important clinical evidences were obtained with off-label employment of already marketed liposomal DOX, acting mainly owing to the EPR effect. In a clinical study liposomal DOX administered in patients with high-grade gliomas improved overall survival.[146] Moreover, DOX loaded in pegylated liposomes (PEG-DOX) was efficacious and well tolerated in patients with recurrent high-grade glioma.[147] Indeed, encouraging results were obtained with PEG-DOX alone (mOS 26 weeks) or in combination with TMZ (median overall survival 32 weeks, 6-months progression-free survival 32%). Owing to the available studies, PEG-DOX, alone or in combination regimens, can actually be considered as a treatment option for recurrent high-grade gliomas, but only if no further chemotherapy is available; however, it should be further evaluated in larger clinical trials.[148]

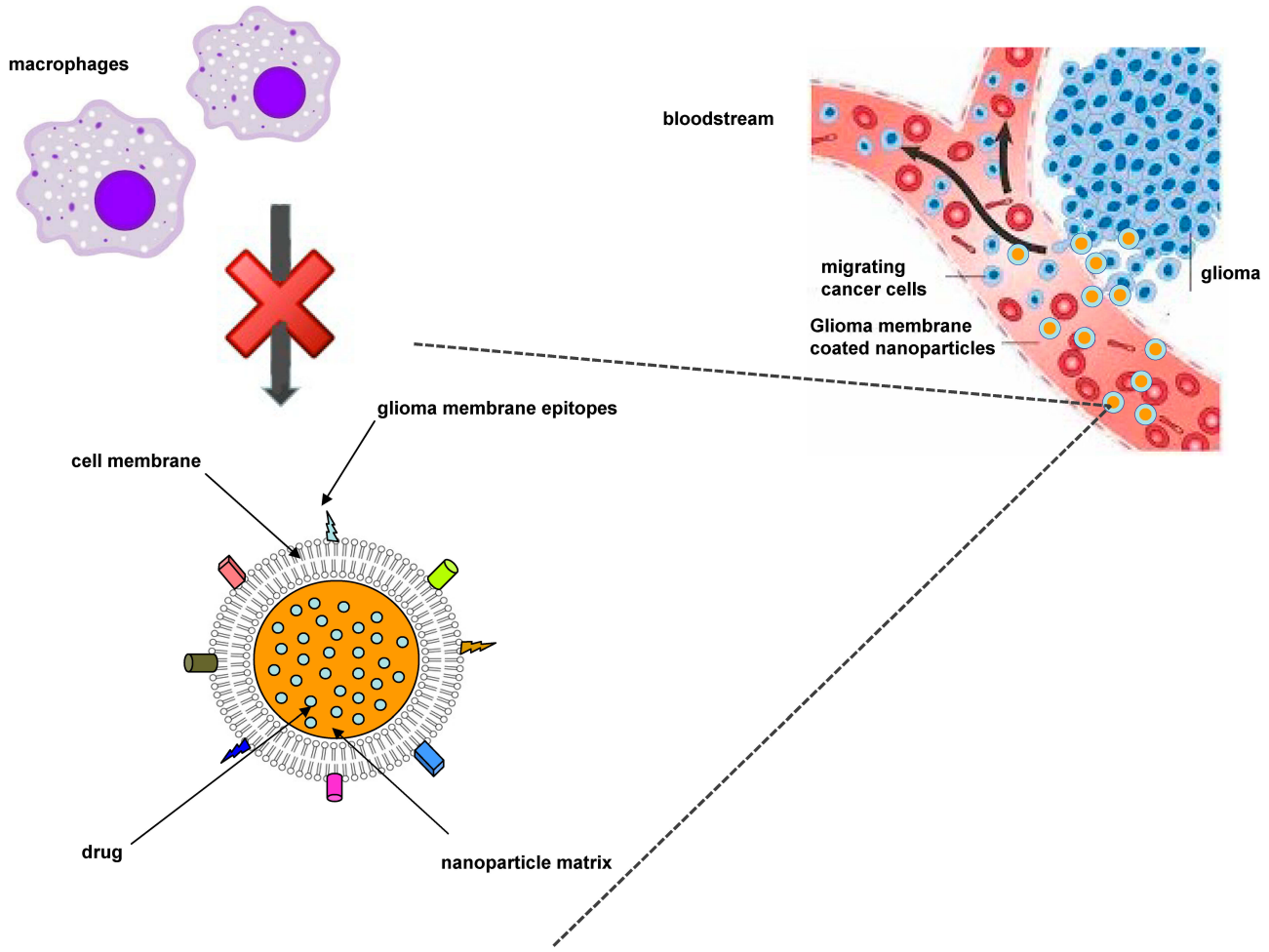

Figure 6 Scheme of homotypic targeting of glioma cell membrane-coated nanoparticles.

Conclusions

Malignant gliomas are still associated with a poor prognosis, despite recent advances in surgical treatment. Despite the large number of potential drug candidates, the efficacy of adjuvant chemotherapy remains unsatisfactory, primarily because of the BBB. Nanocarriers can favour delivery of chemotherapeutics to malignant gliomas owing to different mechanisms, including chemical stabilization of the drug in the bloodstream, EPR effect (because of the leaky BBTB), P-gp inhibition, active targeting through CMT and RMT, inhibition of cell differentiation and angiogenesis, cell-mediated targeting, or stimuli-responsive delivery. In particular, different and efficient active targeting approaches have been attempted in preclinical studies on animal models, mainly by employing protein targeting moieties. Nevertheless, a few number of nanomedicines reached the clinical trials, and most of them include drug-loaded nanocarriers free of targeting ligands, probably because of safety and scalability concerns.

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Disclosure

The authors report no conflicts of interest in this work.

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