Breaching Barriers in Glioblastoma Targeted Drug Delivery

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Abstract: Tailored nanocarriers have gained huge research focus for brain drug delivery, aimed at combating several neuro-oncological conditions, such as the glioblastoma. The progress of knowledge on the pathogenesis of glioblastoma has allowed identifying the major hurdles for efficient treatment, encompassing biological interfaces (blood-brain barrier and blood-brain tumor barrier), specificities of tumor microenvironment, as well as both bulk and glioma stem cell subpopulations. These findings provided new insights into the molecular basis of glioblastoma, being a strong driving force behind development of targeted nanomedicines in this area. Diversified nanoparticles have been designed to target glioblastoma surface markers, overexpressed receptors, aberrant genes and signaling pathways, in addition to contemplating barriers targeting strategies. Nanotechnologies claim important and unique features, including the versatility in promoting both passive and active drug targeting, making them excellent candidates for brain drug delivery and one of the most appealing to overcome the obstacles of the current glioblastoma treatment. In this short review, we will report the mechanisms of overcoming the blood-brain barrier as well as various studies relating to the applications of nanotechnologies as drug delivery carriers in glioblastoma treatment.

Keywords: Blood-Brain Barrier, Brain Tumors, Drug Delivery, Glioblastoma, Nanoparticles, Nanotechnologies, Target Therapy.

INTRODUCTION

The World Health Organization (WHO) divides gliomas in glioblastoma (GB) IDH-wildtype, astrocytoma IDH-mutant, and oligodendroglioma IDH-mutant, as well as 1p/19q co-deleted [1]. GB is the most common primary malignant tumor of the central nervous system (CNS), accounting for 12% to 15% of all intracranial tumors and 50% to 60% of gliomas [2]. The standard treatment of gliomas is a combination of surgical, radiotherapy and chemotherapy treatment. The extent of resection is a prognostic factor, but radical surgery is not always achievable. This is due to the widespread infiltration of the white substance and in order to preserve the functional areas. Multiple preoperative and intraoperative techniques have been developed to improve tumor detection and tumor resection, such as intra-operative magnetic resonance (MR), preoperative non-invasive mapping of the brain surface with identification of the motor and speech areas through the use of navigated transcranial magnetic stimulation (nTMS), electrophysiologic monitoring, neuronavigation, use of 5-aminolevulinic acid (5-ALA). The combined use of these techniques improves the rates of successful complete resection to 96% [3]. Radiotherapy and chemotherapy are burdened by important side effects, such as, respectively, post-radiation leukoencephalopathy and nerve damage, hair loss, vomiting, infertility insomnia and skin rash [4]. Moreover, the effectiveness of chemotherapy is limited by various factors such as toxic effects, tumor cell chemoresistance and poor selectivity of anticancer drugs. More, the blood-brain barrier (BBB) impairs the delivery of many chemotherapeutic agents.

Nanotechnology is considered an emerging field with potential application in cancer research and therapy. Nanoparticles (NPs) provide better penetration of therapeutic and diagnostic agents and a reduced risk in comparison to conventional treatments. By using NPs, it is possible to deliver the drug to the targeted tissue across the BBB, release the drug at the controlled rate, and avoid from multidrug resistance. NPs can be structured to carry therapeutic drugs and imaging agents, which are loaded on or within the nanocarriers by chemical conjugation or encapsulation. NPs can also be engineered to exploit many mechanisms for brain-targeted delivery, including receptor-mediated transcytosis, carrier-mediated transcytosis, and adsorptive mediated transcytosis. Reduction of toxicity to peripheral organs and biodegradability can also be achieved with these systems.

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ISSN: 1927-7210 / E-ISSN: 1927-7229/23

TARGETED DRUG DELIVERY USING NANOPARTICLES

Due to the poor prognosis of GB, NPs-based carriers have been intensively explored in an attempt to improve bioavailability of therapeutic molecules for brain uptake and their targeted delivery. Indeed, increasing the amount of the drug delivered to the target tumor cells is the greatest challenge for the development of novel cancer nanomedicines. After being systemically administered, NPs have to face several hurdles *in vivo* to reach their target site inside the cells. However, due to their ability of active or passive targeting, NPs have been considered one of the most appealing drug delivery systems to overcome the limitations of the current GB treatment [5].

The passive targeting drug delivery can occur through the enhanced permeability and retention (EPR) effect, which is linked to the anatomical differences between normal and tumor tissues. The passive method takes advantage of the unique tumor characteristics, including the high vascular density, the leaky vasculature and the inefficient lymphatic drainage. However, some NPs properties must be considered, including particle size, shape, and surface characteristics, as they influence the EPR effect [6]. However, despite passive targeting strategies usefulness in the treatment of tumors, they present some limitations. The EPR effect relies on the diffusion of drugs, but not all drugs diffuse efficiently through cells. Since brain tumors present a relatively weak EPR effect due to a dense brain matrix, the drugs concentrations is often insufficient at the tumor site. Furthermore, due to the inefficient lymphatic drainage in tumors, the interstitial fluid pressure increases, causing only the larger nanoparticles to accumulate. Thus, it is estimated that when administered intravenously, most of passively targeted nanoparticles (about 95%) do not reach the tumor since they accumulate non-specifically in other organs [7]. To overcome the limitations of passive targeting, the attachment of site-specific ligands on NPs surface can increase their uptake selectivity with the consequent cellular accumulation. Thus, active targeting takes advantage of the receptors generally overexpressed in tumor cells. Affinity ligands, such as antibodies, peptides, or aptamers, are capable of binding to antigens or receptors on the target cells, which lead to the internalization of NPs via receptor-mediated endocytosis and thereby enhancing the therapeutic effects. However, targeting moiety of NPs should be engineered without directly perturbing the receptor binding site's characteristics [8]. It has been described that NPs usually form a "corona" layer after systemic administration. This phenomenon occurs because proteins, peptides and other cellular apparatus circulating in the biological fluids tend to adsorb on surface of nanoparticles, generally modifying their initial physicochemical properties. It should be noted that protein corona may confer new biological identity to the NP, therefore interfering with their cellular uptake, circulation time and bioavailability [9].

Barriers to Targeted Drug Delivery

The BBB is a neuroprotective barrier, which presents different defense mechanisms to block the passage of noxious agents to the brain. To counteract these protective effects of BBB, alternative approaches have emerged, such as direct chemotherapy delivery to the brain as well as the passive targeting based on the EPR effect. However, the passive targeting strategy is not enough to reach the tumor invasive cells, once the EPR effect appears to be weak near the tumor area containing these infiltrating cells [10]. That is, not only the BBB, but also the blood-brain tumor barrier (BBTB), is known to prevent drugs from reaching the tumor bulk, contributing to chemotherapy resistance and recurrence of cancer. Therefore, new strategies for active targeting have been developed in order to circumvent effectively BBB/BBTB and enhance the efficacy of GB treatment [11].

The opening of tight junctions (TJs) in the cerebral endothelial cells (CECs) has been object of study in order to create reversible and transient disruptions between the TJs, resulting in an increased drug permeability. Different chemical (mannitol), biological (histamine and bradykinin) and physical (ultrasound and electromagnetic waves) stimuli have demonstrated ability to alter the integrity of TJ structure. The hyperosmolar agent mannitol has shown to contract the CECs by withdrawing water from them, thereby altering their shape with the consequent opening of TJs for a few hours. In turn, bradykinin acts at the level of B2 receptors of the CECs, leading to changes in the TJ integrity and an increased drug permeability. Some surfactants, such as polysorbate 80 and sodium dodecyl sulfate, have also shown ability to disrupt TJs [12]. Although this technique favors the entry of the drug into the brain, it is obvious that it has limited usefulness due to toxicity, since the neuroprotective function of the BBB becomes compromised. In addition, BBB disruption by itself is not enough to obtain a significant outcome in GB patients, bearing in

mind that drugs still need to overcome other physical barriers, such as brain parenchyma, to reach their target cells [13].

Efflux Transporter Inhibition

It is well known that the ATP-binding cassette (ABC) gene family is an active efflux transport system displaying a crucial role in protection of the BBB against the entry of substances into the brain. Indeed, drugs are usually substrates of the efflux transporters, such as P-glycoprotein (P-gp), multiple drug resistance protein 4 (MRP4) and breast cancer cell resistance protein (BCRP), which restrict their entry to the brain. Therefore, by knocking out or blocking efflux transporters, it is possible to increase the amount of drug that crosses into the brain, with the additional benefit of maintaining the integrity of the BBB [14]. In order to reverse the effect of drug efflux transporters expressed at the BBB level, several chemosensitizers and polymers have been successfully tested. P-gp modulators, including cyclosporin A, valspodar, verapamil, elacridar and tariquidar, have shown to inhibit P-gp activity in multiple preclinical studies, improving BBB crossing of drugs into the brain. Moreover, both natural (xanthan gum, gellant gum and alginates) and synthetic polymers (polyethylene glycol (PEG), Pluronics and Thiomers), as well as nonionic surfactants have gained attention due to their ability to inhibit the action of the P-gp efflux pump. Although, specific inhibitors of efflux transporters seem to bring advantage in brain tumor treatment, they have not revealed statistically significant results in clinical trials so far. This is not only because of their ineffective degree of inhibition, but also due to increased BBB permeability following the inhibition that may increase the entry of toxic elements into the brain tissue. Thus, before applying this method, some factors need to be evaluated, such as the required level of inhibition, the best drug-inhibitor combination according to the target tissue, as well as the overall safety of this therapeutic strategy [15].

Receptor-Mediated Transcytosis and/or Endo-cytosis

CECs display a set of receptors and/or transporter systems, such as glucose transporters (GLUT), low density lipoprotein receptors (LDLR), transferrin receptors (TfR), insulin receptors (IR) and nicotinic acetylcholine receptors (nAChR), which are substrate-selective. Since receptor-mediated transcytosis (RMT) is a selective pathway for trans-BBB transport, some targeting ligands mimicking receptor, binding fragments of the endogenous ligand have been developed in order to facilitate the entry of therapeutic systems into the brain with deeper tumor penetration [16]. Numerous studies have demonstrated an overexpression of TfRs on the surface of CECs. Several studies have reported the incorporation of an anti-TfR single chain antibody fragment (TfRscFv) in NPs to target TfRs. Its recombinant nature along with its small size (~28 kDa) make the TfRscFv a promising moiety in targeted DDS. Indeed, TfRscFv is preferable in human use to the transferrin molecule itself, not only because the previous characteristics, but also because it does not contain the fragment crystallizable portion of the monoclonal antibody (mAb), leading to low immunogenicity. It seems that high-affinity anti-TfR dosing significantly decrease brain TfR levels and impairs its efficient transcytosis across the BBB, so reducing anti-TfR affinity is expected to augment its brain exposure. LDLR is another receptor highly expressed in CECs, being also responsible for transporting several ligands conjugated to NPs across the BBB via RMT. Actually, LDLR acts as a ligand for both apolipoprotein B-100 (ApoB-100) and apolipoprotein E (ApoE), being the main protein constituents of lipoprotein particles. This knowledge has led to perform numerous *in vitro*/*in vivo* studies using NPs decorated with ApoB-100 or ApoE in order to facilitate passage through the BBB [17]. Angio-peps, derivatives from aprotinin with the Kunitz domain of human proteins, have attracted much attention because they exhibited high transcytosis capacity. ANG1005 is a product containing angiopep-2, and it has been tested in clinical trials for the transport of paclitaxel across the BBB via the lipoprotein receptor–related protein 1 (LRP-1) transport system. To date, ANG1005 already showed clinical benefit and prolonged survival in phase II trial, in addition to previous studies in which the ANG1005 was administered intravenously in murine models with subsequent observation of intracranial tumor regression. Since glucose transport to the brain involves GLUT, more specifically GLUT1 on the BBB, these transporters have gained more attention. In this context, it was developed a brain-specific drug liposomal carrier with a novel glucose-cholesterol derivative L, showing promising results in both *in vitro*/*in vivo* studies. Also, a mannose-vitamin E derivative conjugate demonstrated ability to cross the BBB efficiently via GLUTs. However, many other compounds have also been used to target GLUT1, including 7-chlorokynurenic acid, dopamine, glycosyl

derivatives of ibuprofen, and other glycoconjugates [18]. nAChRs are also found in the brain, so they have been exploited as a means to transport therapeutic systems across the BBB. Indeed, nAChR-mediated brain targeting appears to be a good strategy for the intracranial transport of DDSs. Some compounds, like the 29-amino acid peptide derived from rabies virus glycoprotein (RVG29) and the 16 aminoacid peptide CDX, have demonstrated high binding affinity to nAChRs, allowing successful delivery of therapeutic substances into the brain. CDX conjugated to paclitaxel-loaded micelles were found to decrease GB growth and to prolong survival in mice. In the case of RVG29, the conjugation of this peptide with micelles revealed great potential as carriers for small interfering RNA (siRNA) delivery to the brain. Furthermore, as nAChRs have affinity to positively charged quaternary ammonium groups or simple cations, nanoparticles coated with those groups or cations have shown ability to successfully cross *in vitro* BBB models of bovine, without changing the paracellular permeability [19].

In addition to the receptor-mediated endocytosis, there is a nonspecific process called adsorptive-mediated transcytosis (AMT), which does not involve receptors, and relies on the interaction of positively charged molecules with the negatively charged BBB membrane surface. Cationic proteins, such as albumin and immunoglobulin G, and cell-penetrating peptides (CPPs), constitute the major shuttles with ability to cross the BBB in a non-selective way. For instance, cationic bovine serum albumin (CBSA)-conjugated nanoparticles have been used for extended delivery of different therapeutic molecules into the brain. The CPPs, represented by short amphipathic and/or cationic peptides with different sequences, are derived from natural proteins and have been used for the transport of several therapeutic molecules into the brain. The most frequently used proteins in this context are the transcription-activating factor Tat, penetrating, and the Syn-B, demonstrating promising results in different studies with regards to enhanced brain drug uptake. However, it should be noted that AMT is an unspecific process, because there may be interaction with any negatively charged membrane, with no cell selectivity. This makes this strategy less attractive than other ones available for targeting in brain tumors. Moreover, cationic proteins and CPPs have been associated to some immunogenicity and toxicity, including enhanced peripheral and cerebrovascular permeability, also contributing to the low use of this technique. However, to overcome these limitations, there are some groups developing CPPs conjugated with a specific targeting ligand, thus attributing selectivity to this approach [4]

Tumor Microenvironment

The interactive loop between cancer cells and tumor microenvironment is quite interesting and has been subject of several studies, for its ability to interfere with these processes and subsequent responses. It is well known that cancer cells have a lower extracellular pH (pHe) than normal cells. Actually, the lowered pHe is primarily due to lactate secretion from anaerobic glycolysis in hypoxia and it can reach values around 5.7–7.2. An acidic pH stimulates both tumor growth and its metastases, being a strong negative prognostic indicator in patients with GB. Moreover, increased extracellular lactate levels have been associated high radio resistance [20]. For providing environmental targeting ability, innovative pH-sensitive shielding systems have been developed in recent decades. These systems are usually constituted by pH-sensitive molecules, such as peptides, polymers or lipids, which are unstable at acidic pH, disintegrate and promote the release of their contents, in this case anticancer drugs [21]. For instance, pH-responsive CH12K18PEG5k DNA NPs demonstrated to successfully silence a tumor-specific transgene in an intracranial mouse GB model. Even more complex, an activatable cell-penetrating peptide called dtACCP was used to decorate NPs intended to tumor-targeting. These NPs revealed to be dual-triggered by the acidic pH and the upregulated MMP-2 in the tumor microenvironment, having been successfully used to co-deliver doxorubicin (DOX) and plasmid expressing siRNA targeting the vascular endothelial growth factor (VEGF) [22]. Basically, these pH-triggered drug release systems promote drug accumulation at tumor site with less drug distribution and, consequently, decrease the damage to the healthy tissues. Angiogenesis is required for invasive glioma growth and metastasis, reason why growth factors involved in tumor angiogenesis, such as VEGF, as well as certain receptors, such as epidermal growth factor receptor (EGFR), integrins and platelet-derived growth factor receptor (PDGFR), have been intensively exploited as tumor-targeted strategies for delivering anti-angiogenic agents. Bevacizumab, a humanized anti-VEGF mAb used as anti-angiogenic agent, has been considered for cancer therapy, either due to its ability to inhibit the growth of new blood vessels, thus decreasing tumor perfusion, or its effects in reducing vasogenic edema caused by radiation necrosis. Bevacizumab exhibits a nonspecific mechanism of action, since it does not

target tumor-specific receptors or antigens. In turn, integrins are generally overexpressed primarily on tumor neovasculture and in several glioma-derived cell lines, promoting interaction between cells and the components of extracellular matrix (ECM). Especially, αvβ3 and αvβ5, appear to play an important role in regulating angiogenesis, not being expressed on healthy brain cells. This knowledge about integrins has enabled to study the effect of some integrin antagonists in GB: the use of cyclic arginine–glycine–aspartic acid (RGD) peptide and its analogs, for instance, showed to be a promising BBTB and glioma-targeted drug delivery strategy, mainly due to positive αvβ3 integrin–RGD interaction. RGD peptide has been successfully incorporated into different systems, such as micelles, liposomes and other NPs, to target GB. Cyclic RGD and peptide-22 dual-modified liposomes loaded with DOX showed to overcome BBB/BBTB barriers, inhibiting the growth of GB more effectively. Recent findings showed that pericytes, which are components of the neurovasculature critical to the maintenance of the BBB, also seem to contribute to tumor angiogenesis, growth, metastasis, and evasion of immune destruction. In this context, a new pathway that links glioma stem-like cells (GSCs) to the development of GB-specific endothelial cell-related pericytes (G-pericytes) has emerged, thus supporting the theory that GSCs are the potential progenitors of pericytes. When activated, pericytes trigger the release of important regulatory factors, such as VEGF-A, that act as signals for endothelial cells survival. Since this process contributes to the resistance of antiangiogenic therapies, selective elimination of G-pericytes may lead to the disruption of endothelial walls, thereby limiting GB tumor progression and, also enhancing the efficacy of anti-GB therapy. Indeed, therapeutic resistance was confirmed after tumor vessels with less pericytes reveal to be more susceptible to radio-chemotherapy. It should be point out that therapeutic targeting of GSC-derived pericytes is valuable, since it can attack multiple cancer hallmarks at once. On this subject, it was developed a pericyte-targeting DDS composed of TH10 peptide-conjugated nanoparticles loaded with DTX. This system aimed to target the neural/glial antigen 2 (NG2) proteoglycan, which is overexpressed in tumor pericytes. Positive results were achieved through the visualization of pericyte apoptosis and the decrease in micro vessel density of metastases.

A new channel pattern named vasculogenic mimicry (VM) has been introduced as a blood supply system independent of endothelial vessels in aggressive tumor cells, in which these cells are able to form extracellular matrix (ECM)-rich, vasculogenic-like networks to complement the endothelial-cell-dependent vasculature. These independent vascular channels do not present endothelial cells on the inner wall and are believed to be a key step for the sustained growth of tumors, as well as for the invasion and metastasis. Studies have revealed that VM exists in several malignant tumors, including gliomas, and it has been associated with a prognostic factor for poor clinical outcomes. Nevertheless, no significant difference in survival time between patients with VM-positive and VM-negative GBs has been observed [23]. Three factors with significant impact on VM are already identified: tumor stem cells, tumor microenvironment and hypoxia. It has been reported that aggressive VM-positive tumors exhibit matrix metalloproteinases (MMPs) (MMP-1, MMP-2, MMP-9), membrane type-1(MT1)-MMP, the basement membrane ECM component laminin 5γ2, vascular endothelial-cadherin (VE-cadherin), epithelial cell kinase (EphA2), phosphoinositide 3-kinase (PI3 K), focal adhesion kinase (FAK), transforming growth factor (TGF-β), cyclooxygenase 2 (COX-2), vascular endothelial growth factor receptor (VEGFR) and hypoxia-inducible factor (HIF) [23]. Multiple strategies have been employed in an effort to destroy brain glioma VM channels and therefore improve the GB prognostic. Anti-VM therapy is intended to downregulate the VM channel-forming indicators mentioned above, by using anti-sense oligonucleotides or mAb. For example, tetracycline and isoxanthohumol were shown to downregulate laminin5γ2, EphA2, VE-cadherin and MMPs; curcumin (CUR), a yellow pigment from Curcuma longa, appears to downregulate the EphA2/PI3K/MMP pathway. Thus, therapeutic strategies include the use of antisense oligonucleotides to the Ln-5 γ2 chain, antibodies to MMP-2 or MT1-MMP, as well as to down-regulate VE-cadherin and EphA2 genes, among other VM-associated genes and signaling pathways. Targeting VM in combination with anti-angiogenic therapy is expected to be synergistic, effectively blocking the supply of oxygen and nutrition to the tumor cells. Once VEGFRs are overexpressed on both VM and angiogenic cells, the development of a corresponding ligand-mediated DDS may be a promising strategy for the treatment of GB. Interestingly, the VEGFR-2 kinase inhibitors AZD2171 and SU1498 were tested *in vitro* and *in vivo* studies for GB, demonstrating ability to inhibit both VM channel formation and tumor growth. The vasculature of GB is atypical and leaky, resulting in fibrinogen extravasation

with subsequent deposition of insoluble fibrin, which promotes angiogenesis and tumor growth. Evidences suggest that fibrin deposition increases in a tumor grade-dependent manner, being characteristic to both primary and metastatic GB. Bearing in mind this malignancy, Cy7-labeled micelles were tailored with a fibrin-binding pentapeptide, CREKA (cysteine–arginine–glutamic acid–lysine–alanine), in order to improve the targeting in GL261 glioma mouse model by intravenous (IV) administration. These micelles not only demonstrated to passively accumulate at the glioma via the EPR effect, but also demonstrated the ability to increase retention by active targeting as early as 1 h after administration.

Targeting GSCs Surface Markers

CD133, the most widely recognized surface marker of cancer stem cells, has been used as a biomarker in GB. Several strategies have been explored to target CD133, including specific CD133 binding peptides, CD133 silencing, or CD133 mAbs. For example, as Sirtuin 1 (SirT1) gene was found to be exclusively expressed in CD133(+) radioresistant cancer stem cells, its silencing resulted in an enhanced effect of radiation-mediated apoptosis, in addition to the attenuation of GB growth in nude mice transplanted with GB-CD133(+). It was demonstrated that BMI-1 is highly enriched in CD133(+) cells when compared to CD133(−) cells, qualifying thus CD133(+) cells to acquire chemoresistance. This evidence supports the potential advantage of depleting CD133(+) cells by knockdown of BMI1 in GB [24]. There are therapeutic mAbs that can be used to target specific GSC surface moieties, thereby representing a good strategy to deliver anticancer drugs directly to the tumor. DDSs conjugated with CD133 mAbs have been developed to improve the efficacy of GB treatment. Antibodies can also be used to inhibit immunosuppressive niche development in GB, for example by targeting GSC-secreted factors, such as galectin-3 and TGFβ. ICT-107, a vaccine composed of autologous dendritic cells and six antigens highly expressed in GSCs, was tested along with radiation and TMZ in phase II trial for newly diagnosed GB, revealing positive outcomes with respect to progression-free survival [25]. Recently, it was found that the Survivin-Ran complex is responsible for the spindle formation in tumor cells, reason why GSCs depend on the interaction of these compounds for their survival. The disruption of the Survivin-Ran complex by a pharmacological inhibitor, such as LLP-3, has shown to hinder survival and growth of GSCs both *in vitro* and *in vivo*, leading to apoptotic death. Moreover, it was demonstrated that Survivin expression is higher in recurrent GB than in newly diagnosed GB, and that TMZ-resistant GB spheres have responded very positively to LLP-3 treatment. Another example is Nestin, which, similarly to GSC marker CD133, also exhibits different levels of expression in GB, serving as a promising marker for the isolation of GSCs in GB. An effective Nestin targeted peptide, termed AQYLNPS, was developed through *in vitro* phage display technology, demonstrating ability to specifically target Nestin-positive GSCs [26].

Bulk Tumor Cells

All efforts have been focused on the development of new therapeutic strategies for GB, considering different surface markers, as well as DNA repair and signaling pathways, in order to provide targeted therapies. Plasma-derived low-density lipoproteins (LDL) have been studied as DDS for tumors expressing LDLR, but the trouble of purifying them in large amounts, associated with their variability in size and composition, has limited their use. In order to solve these problems, synthetic forms of LDL have been explored and currently there are some synthetic LDL-conjugated nanocarriers being tested in GB cells with great success. Chlorotoxin (CTX), a 36 amino acid long peptide derived from scorpion venom, revealed the ability to specifically bind and inhibit both chloride channels and MMP-2 of GB cells. Two CTX derivatives, named CA4 and CTX-23, were shown to be even more promising in that they seem to have multiple effects on GB, both in tumor growth and angiogenesis [27]. DNA repair mechanisms allow GB cells to survive DNA damage caused by radio-chemotherapy, so the use of DNA-repair-inhibitors could be useful to enhance the effectiveness of GB treatment. There is good clinical evidence that patients with TMZ-resistant GB present an overexpression of unmethylated O6-methylguanine-DNA methyltrans-ferase (MGMT), being, therefore, a predictive biomarker of poor prognosis. Hence, targeting MGMT becomes a priority, representing one of the best ways for intervention in order to improve TMZ efficacy in GB [28]. For example, p53 appears to play an important role in down-regulating MGMT expression, thereby reversing TMZ resistance. While, on one hand, the wild-type tumor suppressor gene p53 has been incorporated in DDS, MGMT expression has been silenced through methylation of the MGMT promoter [29] on the other hand. Several clinical trials, such as GLARIUS and BELOB trials, have been conducted to

test alternative drug combinations beyond TMZ, in newly diagnosed unmethylated GB patients. The obtained results indicate that it may be reasonable to omit TMZ in treatment of GB and there seems to be more encouraging results on the cytotoxic effect of other alkylating agents, such as nitrosoureas on unmethylated. In addition to MGMT, the dual targeting of mismatch repair (MMR) and base excision repair (BER) may prove to be a promising cancer treatment, regardless of the impairment status of both DNA repair pathways [30]. So far as is known, MMR gene mutations lead to TMZ resistance in GB cells, reason why restoration of the MMR system is being investigated. Furthermore, personalized therapeutic strategies to target MMR-deficient tumor cells have been introduced, resulting in their death. By way of example, the synthetic lethal approach has gained popularity in this field, having already identified several synthetic lethal interactions with MMR-gene mutations so far; in turn, the potassium-sparing diuretic drug triamterene was found to be selective for MMR-deficient tumor cells, leading to DNA double-strand breaks of these cells [31]. In the BER system, poly(ADP-ribose) polymerase-1 (PARP-1) plays a crucial role, being overexpressed in the presence of DNA damage caused by both chemotherapeutic agents and ionizing radiation. Hence, PARP-1 inhibitors have been developed in the last years to limit BER activity and enhance the toxicity of radio-chemotherapy.

Nanoparticles Drugs Delivery in Glioblastoma Treatment

Nanotechnology has notably changed the classical modality in which diagnosis and treatment are achieved mainly due to recent advances in material engineering, drug availability, and advantage of targeting cancer cells. A wide range of NPs have been developed to deliver chemotherapeutic drugs, such as docetaxel [32,33], paclitaxel [34,35], doxorubicin or other small molecule chemotherapeutics [36,37]; or, to leverage antibodies [38,39], RNA [40,41], or peptides [42] in an attempt to enhance GB therapy. Despite these grand efforts, research conducted over the past decades has made only marginal advances with no real promise of a clinical path towards a viable curative treatment. In general, these nanocarriers share a number of common characteristics; they are made of synthetic materials, tend to accumulate and persist in liver and spleen causing severe side effects, and are incapable of passing the BBB. In contrast, natural evolution has resulted in proteins and viral particulates that can target to and transport through the BBB [43]. Inspired by the unique capabilities of biological NPs, a GB-targeting synthetic protein nanoparticle (SPNP) has been engineered comprising of polymerized human serum albumin (HSA) and oligo-(ethylene glycol) (OEG), loaded with the cell-penetrating peptide iRGD [44,45] as well as STAT3. The choice of HSA as the major matrix component was motivated by its rapid and well-understood clearance mechanisms, its demonstrated clinical relevance, and its exquisite biochemical compatibility with both, therapeutic agents and homing peptides. In addition, albumin-based nanocarriers have been shown to engage cell-surface receptors, such as SPARC [46] and gp60 [47], that are overexpressed on glioma cells and tumor vessel endothelium [48,49]. Encouraged by results showing the accumulation of SPNPs in GB tumors it sought to evaluate the therapeutic efficacy of STAT3i SPNPs in combination with focused radiotherapy. In the highly aggressive GB GL26 model, a significant increase in MS is observed in mice treated with the combined therapy with 87.5% of mice reaching the long-term survival timepoint. In these mice, it observed significantly reduced levels of STAT3, no apparent residual tumors, normal brain architecture, and a lack of inflammation in response to the treatment. It observed increases in both tumor-antigen specific CD8 T cells in the brain TME along with a decrease in immune suppressive M2 macrophages suggesting the activation of an anti-GB immune response. Finally, it observed minimal signs of toxicity in the liver and no significant differences in the cellular components of blood relating to kidney and liver function suggesting no overt off-target side effects occurred as a result of the treatment. To further explore the observed immune response, mice reaching the long-term survival time point were rechallenged with a second tumor in the contralateral hemisphere. Incredibly, in the absence of therapeutic intervention, all rechallenged mice survived to a second long-term survival timepoint. Rechallenged mice showed no overt signs of residual tumor, regions of necrosis, or disruption of the surrounding brain architecture. Together, these studies further suggest the activation of an adaptive immune response, potentially capable of eradicating secondary tumors resulting from the aggressive and infiltrative nature of GB. SPNPs combine the biological benefits of proteins with the precise engineering control of synthetic NPs to yield high efficacy (87.5% long-term survivors in a very aggressive intracranial tumor model), effective tumor delivery using systemically administered NPs, and possibilities towards long-term eradication of resistant cancer cells using immunomodulatory protein NPs.

Chimeric antigen receptor (CAR) modification has significantly enhanced anti-tumor activities of immune T or natural killer (NK) cells [50,51]. However, their efficacy in solid tumors is still limited due in part to their relatively low trafficking and tumor penetration ability. The presence of physiological BBB and BBTB further impedes the efficacy of these emerging therapeutics against GB in the brain. We speculated that the combination of CAR-engineering and highly motile neutrophils might sustain their anti-tumor N1 phenotype and yield excellent therapeutic efficacy in treating GB. Primary neutrophils are short-lived and resistant to genome editing [52], limiting their application in CAR-directed immunotherapy. Human pluripotent stem cells (hPSCs), which are more accessible to gene editing and capable of differentiating into neutrophils massively, could provide an unlimited source of high-quality CAR-neutrophils for targeted immunotherapy under chemically-defined, xeno-free conditions [53]. Neutrophils also preferentially phagocytose microbial pathogens with rough or long surfaces, such as S. aureus and E. coli, which should be considered for nanoparticle design in neutrophil-mediated drug delivery. Indeed, Safari *et al*. recently reported the preferred phagocytosis of intravenously administered elongated particles, without complicated surface modification, by circulating neutrophils [54]. Such an easy and bioinspired design in drug-loaded nanoparticles may maximize drug-loading in neutrophils and allow therapeutic levels of drug delivery at targeted sites. Chang *et al*. have design and screen four anti-GB chlorotoxin (CLTX)-CAR constructs with T or neutrophil-specific signaling domains by knocking them into the AAVS1 safe harbor locus of hPSCs via CRISPR/Cas9-mediated homologous recombination and identified an optimized CAR, composed of a 36-amino acid GB-targeting CLTX peptide, a CD4 transmembrane domain and a CD3ζ intracellular domain, for neutrophil-mediated tumor-killing [55,56]. The resulting stable CAR-expressing hPSCs are then differentiated into CAR-neutrophils, which sustain an anti-tumor N1 phenotype and exhibit enhanced antiGB activities under the hypoxic tumor microenvironment. A biodegradable mesoporous organic silica nanoparticle with a rough surface (R-SiO2) is synthesized and employed to load hypoxia-activated prodrug tirapazamine (TPZ) or clinical chemo-drug temozolomide (TMZ) and JNJ-64619187 (a potent PRMT5 inhibitor under clinical trial NCT03573310) into hPSC-derived CAR-neutrophils, which are unharmed by the nanoparticulated cargo and retain the inherent biophysiological properties of naïve neutrophils. CAR-neutrophils loaded with drug-containing SiO2 nanoparticles display superior anti-tumor activities against GB, possibly due to a combination of CAR-enhanced direct cytolysis and chemotherapeutic-mediated tumor-killing via cellular uptake and glutathione (GSH)-induced degradation of nanoparticles within the targeted tumor cells [56]. In an, *in situ,* GB xenograft model, hPSC-derived CAR-neutrophils precisely and effectively deliver TPZ-loaded SiO2 nanoparticles to the brain tumors without invasive surgical resection for amplified inflammation, significantly inhibit tumor growth, and prolong animal survival, representing a targeted and efficacious combinatory chemoimmunotherapy. Notably, Si content measurement suggests >20% of administered nanodrugs are delivered to brain tumor by CAR-neutrophils as compared to 1% by free nanodrugs. Lu *et al*. have successfully developed M@HLPC, a biomimetic targeted drug delivery and synergistically therapeutic system for the effective treatment of GB. Exploiting the strong interaction between CPPO and proteins, they used a one-pot self-assembly strategy for the construction of HLPC, which were then encapsulated with membranes prepared from glioma cells to generate the biomimetic M@HLPC. The result M@HLPC displayed excellent stability and efficient BBB penetration and tumor targeting. Upon reaching tumor sites, the combined activities of the metabolic therapy and chemiexcited PDT agents conferred strong tumor inhibition in murine xenograft tumor model. They extended their study with a successful example of a personalized therapy using hM@HLPC against patient-derived tumor model, thereby highlighting the strong translational potential of our hM@HLPC system to develop clinically relevant GB therapies [57].

CONCLUSION AND FUTURE PERSPECTIVES

Due to infiltrative nature, the presence of the BBB, which restricts entry of therapeutic entities to the tumor area, the paucity of antigen presenting cells, and the immune suppressive nature of the tumor microenvironment, the treatment of GBs is characterized by a high rate of failure. GBs show a complex heterogeneity at the genomic and molecular levels. More, when used EGFR inhibitors, MET and/or PDGFR would maintain activation of downstream pathways, which is a theoretical mechanism of target therapy resistance. Recent research showed the presence of EGFR-VEGF(R) cross-talk in both tumor and tumor-associated endothelial cells and is involved in tumor survival and angiogenesis. GBs are also characterized by genomic instability, which favors gene

mutations and chromosomal alterations, and cytotoxic agents and radiotherapy would accelerate the mutagenesis. Another serious obstacle is represented by the fact that the chosen target can be activated by multiple pathways, in different phases, during tumor progression, thus rendering the treatment ineffective. Considering the clinical resected tumor tissue that carries patient personalized molecules is usually underutilized (except for the biopsy) and membrane materials can be conveniently obtained from resected glioma tissue, it would seem interesting to use the membrane materials from GB to construct personalized nanomedicine. Multiple surface adhesion molecules (such as E-cadherin and epithelial cell adhesion molecules) on the tissue membrane may endow these personalized nanomedicines with the homotypic tumor self-recognition ability. In this context, the infiltrating GBs cells missed by surgery can be targeted and cleared by the personalized membrane-based nanomedicine, thus preventing post-surgical tumor recurrence. Furthermore, the resected GB tissue can be utilized for ex vivo proliferation with culture medium containing specific factors (i.e., Wnt3A, hFGF10, hEGF), which can provide for preparing personalized nanomedicine.

Nanotechnology can be a valuable support in the GB treatments. Thanks to their dimension, the NPs cross the BBB and, by acting as carriers can convey even more therapeutic compounds able to interact with multiple targets. NP-based drug-delivery systems overcome the BBB with high targeted-cell specificity and selectivity. Thus, NPs permits the use of a lower dose of drug efficacious both into the central core of tumor and into the distal foci of tumor cells within areas often characterized from integrity of BBB [58]. Notwithstanding, there are potential risks related with this novel approach. Some cancer cell types could develop drug resistance making ineffective the drugs released from the targeted NPs. In addition, NPs possess low toxicity but degrade quickly and do not circulate in tissues long enough to warrant a sustained drug/gene delivery. Objects of debate are the results about the long-term effects of interactions between NPs and coating of molecules and target cells.

Several molecular biomarkers have been identified, and novel and repurposed drugs, as well as novel drug combinations, have also been included in various studies, being another area of interest of current research. Genomic testing technologies could be useful to identify mutations at the root of a patient's tumor, as well as to predict responsiveness to specific drug through a drug-gene interaction database. Basically, these more personalized strategies could supplement the ongoing efforts of moving GB therapies forward, mainly intended to bridge the gap observed in those patients unresponsive to the current standard therapy. All these new trends in cancer therapy point to a coming era of targeted nanoparticle technology and gene therapy, towards a personalized medicine approach for GB.

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Received on 25-10-2023 Accepted on 22-11-2023 Published on 22-12-2023

https://doi.org/10.30683/1927-7229.2023.12.10