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ADVANCED REVIEW

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Multi-ligand functionalized blood-to-tumor sequential targeting strategies in the field of glioblastoma nanomedicine

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Abstract

Glioblastoma (GBM) is an unmet clinical need characterized by a standard of care (SOC) 5-year survival rate of only 5%, and a treatment mostly palliative. Significant hurdles in GBM therapies include an effective penetration of therapeutics through the brain protective barrier, namely the blood–brain barrier (BBB), and a successful therapeutic delivery to brain-invading tumor cells post-BBB crossing. These hurdles, along with the poor prognosis and critical heterogeneity of the disease, have shifted attention to treatment modalities with capacity to precisely and sequentially target (i) BBB cells, inducing blood-to-brain transport, and (ii) GBM cells, leading to a higher therapeutic accumulation at the tumor site. This sequential targeting allows therapeutic molecules to reach the brain parenchyma and compromise molecular processes that support tumor cell invasion. Besides improving formulation and pharmacokinetics constraints of drugs, nanomedicines offer the possibility of being surface functionalized with multiple possibilities of targeting ligands, while delivering the desired therapeutic cargos to the biological sites of interest. Targeting ligands exploit the site-specific expression or overexpression of specific molecules on BBB and GBM cells, triggering brain plus tumor transport. Since the efficacy of single-ligand functionalized nanomedicines is limited due to the GBM anatomical site (brain) and disease complexity, this review presents an overview of multi-ligand functionalized, BBB and GBM sequentially- and dual-targeted nanomedicines reported in literature over the last 10 years. The role of the BBB in GBM progression, treatment options, and the multiple possibilities of currently available targeting ligands will be summarized.

This article is categorized under:

Nanotechnology Approaches to Biology > Nanoscale Systems in Biology

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KEYWORDS

Blood-brain barrier, Brain drug delivery, Glioblastoma, Nanomedicine, Sequential targeting, Surface functionalization, Targeting ligands

1 | INTRODUCTION

Cancer is a major public health problem and the second leading cause of death in the world, with rapidly growing incidence rates that are projected to double by 2040 (Puyol et al., 2021; Salam et al., 2022). According to the estimates of cancer incidence and mortality of the International Agency for Research on Cancer (IARC) of the World Health Organization (GLOBOCAN), there were approximately 19 million new cases and 10 million deaths related with cancer worldwide in 2020, from which around 300,000 and 250,000, respectively, were due to brain and other central nervous system (CNS) cancers (Sung et al., 2021). Among brain cancers, glioblastoma (GBM) is the most prevalent type, and accounts for nearly half of all CNS malignant tumors (Ostrom et al., 2019). GBM is an aggressive type of brain cancer, with a 15-month median survival from the time of diagnosis. Biologically, GBM is characterized by a high level of cell infiltration, migration and plasticity, as well as inter-patient heterogeneity (Pandey et al., 2022).

Nanomedicines have shown great promise in cancer therapy over the last decades. These systems are versatile nanoscale carriers that offer to cargos protection against harsh biological environments and modulation of their biodistribution, ultimately resulting in enhanced therapeutic outcomes along with lower side toxicity (de Lázaro & Mooney, 2021). More than 50 nanomedicines are currently available in clinics to treat a variety of diseases (Quader et al., 2022). Since nanomedicines were found to access solid tumors mainly through active transport mechanisms (Sindhwani et al., 2020), active targeting-based systems have gained greater visibility in the field of anti-cancer therapies (Gu et al., 2021). Active targeting strategies rely on the interaction between ligand (nanomedicine surface) and receptor (tissue of interest), promoting higher accumulation of cargos loaded into the nanosystems at the targeted site and, thus, an ameliorated therapeutic efficacy. Due to the privileged anatomical location of GBM, the brain, access of therapeutics is hindered by the presence of the most important of all gateways and difficult-to-permeate blood-brain barrier (BBB) (Ma et al., 2021). Moreover, it is still a grand challenge to endow nanomedicines with the ability to target invading tumor cells in the brain, post-BBB crossing. Taking this into consideration, single-ligand functionalized nanomedicines, targeted to either the BBB or GBM, are not sufficient to simultaneously circumvent the BBB restrictive environment and the need to instruct drugs to accumulate at tumor cells (Y Zhu et al., 2018). This, along with the versatility of nanocarriers in regard to the multiple possibilities of surface functionalization, open avenues for the exploitation of multi-ligand functionalized, BBB and GBM sequentially- and dual-targeted nanomedicines.

In this review, we will focus on multi-ligand functionalized, BBB and GBM sequentially- and dual-targeted nanomedicines reported in literature over the last 10 years. Moreover, GBM biological key features will be presented [niches, cell players and tumor microenvironment (TME)], the role of the BBB in the progression of the disease will be discussed, and current treatment options will be analyzed. Lastly, BBB, and GBM biological target opportunities, and their respective ligands, will be scrutinized and summarized.

2 | GBM NICHES, KEY CELL PLAYERS, AND TME

The GBM cell origin is still characterized by lack of consensus among researchers, and grounded two distinct theories, namely the astrocytes de-differentiation and stem cell theory. The astrocytes de-differentiation theory claims that GBM originates from mature astrocytes that suffer driving mutations along the time and return to a more primitive and pluripotent state, with capacity to proliferate and give rise to heterogeneous progeny; on the other hand, the stem cell theory claims that GBM originates from stem cells of the subventricular zone (SVZ) that suffer malignant transformations and possess neurogenesis ability (Habib et al., 2022). The TME of GBM is vast in regard to cell constituents, cell-cell/cell-extracellular matrix (ECM) interactions and function, thus leading to a tumoral organization into three typical niches—perivascular, hypoxic and invasive (Figure 1) (Hambardzumyan & Bergers, 2015). Niches are considered strategic tumor areas of cancer stem cells (CSCs) residency and maximal influence of the TME on GBM malignant features such as angiogenesis (Schiffer et al., 2018).

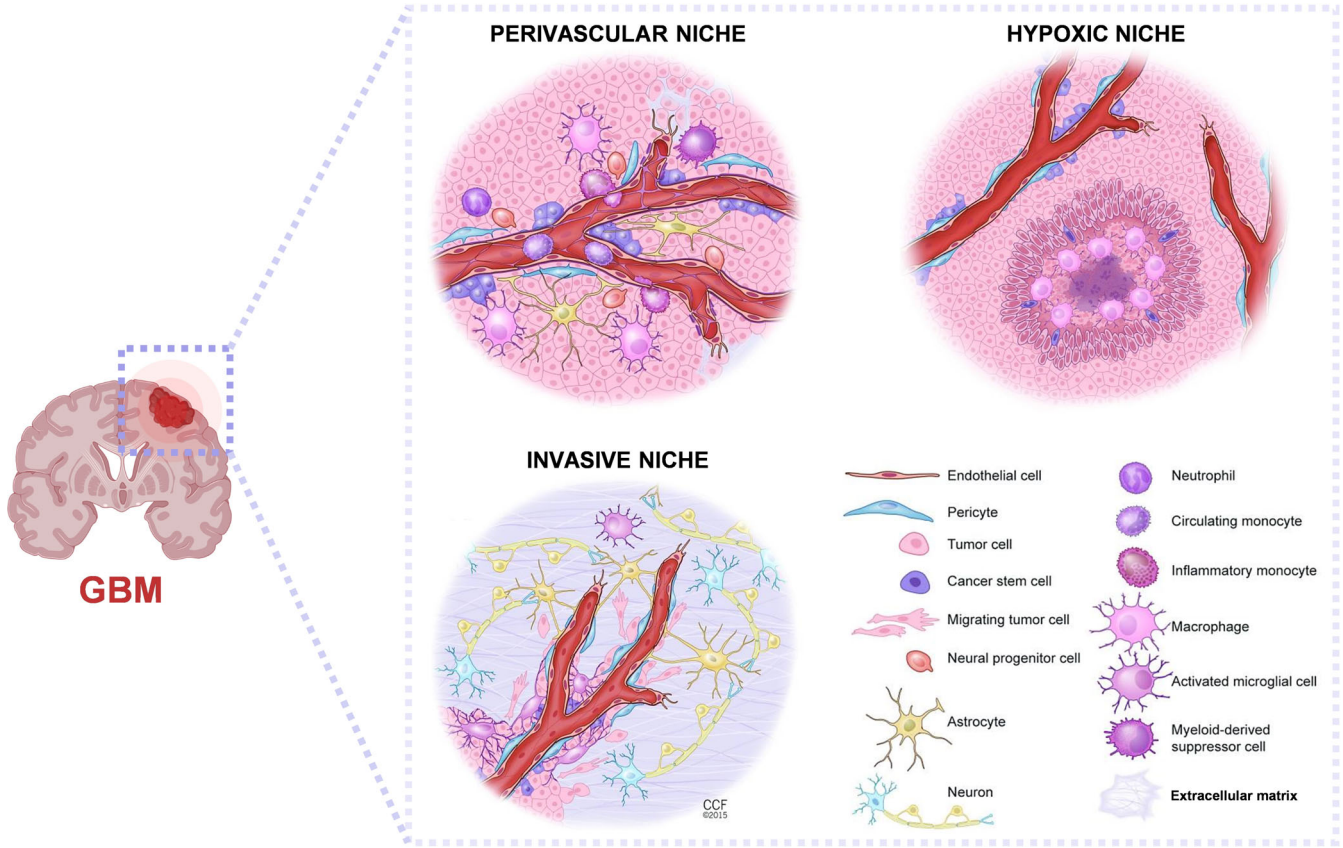

FIGURE 1 GBM TME cell constituents, and inter-cellular and cell-ECM interactions, within each niche—perivascular, hypoxic and invasive. Adapted with permission from (Hambardzumyan & Bergers, 2015) and created with BioRender.com.

The perivascular niche is home for resident or tumor-recruited nonneoplastic [e.g., pericytes, endothelial cells (ECs), macrophages derived from peripheral monocytes, microglia, infiltrating neural progenitor cells (NPCs), neutrophils and myeloid-derived suppressor cells (MDSCs)] and tumor cells, which work together to build an environment supportive of CSC maintenance, survival and growth (Charles & Holland, 2010). Although macrophages can polarize into either a M1 (anti-tumor; pro-inflammatory) or M2 (pro-tumor; anti-inflammatory) phenotype, or present a continuum of phenotypes, macrophages within the GBM TME are known to be mainly M2-like (Andersen et al., 2021). Macrophages, together with brain-intrinsic microglia, constitute the major nonneoplastic GBM cell population—tumor-associated macrophages (TAMs, 30%–50% of the GBM bulk)—being this population key to support tumor phenomena such as stemness, proliferation, angiogenesis and immune suppression (Hambardzumyan et al., 2016; Landry et al., 2020; Morantz et al., 1979). Within TAMs, and compared to microglia, macrophages of peripheral monocyte origin are more abundant ($CCR2^{+}/CX3CR1^{-}$) (Zhou et al., 2015). ECs are another example of a crucial TME nonneoplastic cell type, usually representing 5%–30% of the tumor bulk in GBM patient biopsies (Golebiewska et al., 2013). The main role of ECs within the TME is directly related with tumor angiogenesis, which modulates tumor maintenance and progression, pro-tumor immune responses and therapeutic resistance (Broekman et al., 2018). The hypoxic niche of GBM originates when tumor growth exceeds neovascularization, and is characterized by a necrotic core and pseudopalisading areas (Hambardzumyan & Bergers, 2015). Here, hypoxia inducible factor 1 and 2 (HIF-1 and HIF-2, respectively) are the environmental key players, being responsible for CSCs expansion, and recruitment of innate immune cells such as macrophages (Hambardzumyan & Bergers, 2015; Schiffer et al., 2018). The lack of nutrients and hypoxia lead to the formation of new blood vessels, and adaptive processes such as cell anaerobic metabolism and quiescence (Mosteiro et al., 2022). New blood vessels are formed due to the hypoxic pressure on CSCs, which differentiate into endothelial progenitor cells and, later, endothelium structures. The newly formed blood vessels are typically abnormal, functionally immature and larger in diameter compared to their normal counterparts (Ahir et al., 2020). The invasive niche is characterized by tumor cells that migrate across blood vessels to invade the surrounding brain tissue (Hambardzumyan & Bergers, 2015). Within this niche, major cell components are ECs and endothelium-supportive

pericytes, microglia and neurons. GBM invasive margin cells are responsible for around 85% of tumor relapses, that usually take place within 2 cm^3 of the infiltrative margin (Petrecca et al., 2013; Vasey et al., 2021). Few examples of molecular features that have been reported for GBM invasive margin cells are the consistent upregulation, and expression by tumor core and rim region cells to a significantly lower extent, of the SERPINE1, FGF1 and ALDH1A1 genes, which are associated with primary mechanisms of GBM invasion by ECM degradation via plasmin, angiogenesis promotion and stem-like subpopulations, respectively (Smith et al., 2017; Smith et al., 2020). In the invasive niche, GBM cells ensure the blood supply needs by populating the areas of pre-existing blood vessels and causing the displacement of astrocytic endfeet from ECs, therefore promoting invasion through hijacking of the existing vasculature—process termed vessel co-option (Ribatti, 2022). The ECM, in turn, is tailored by each cell component of the GBM TME, often resulting in conditions favorable to tumor progression (Hambardzumyan & Bergers, 2015). A total of 90% of all GBMs originates in the temporal, parietal or frontal lobe, while only 10% is found in the occipital lobe and, rarely, on the cerebellum, spinal cord or brainstem (Natsis et al., 2021). Primary GBM develops de novo (around 90% of the cases), affects mostly the elderly, and is characterized by epidermal growth factor receptor (EGFR) amplification (36% of cases), p16INK4a deletion (31% of cases), loss of heterozygosity 10q (70% of cases) and PTEN mutations (25% of cases). Whereas, secondary GBM originates from pre-existing low-grade astrocytomas, affects mostly younger patients, and is genetically characterized by TP53 mutations, which are the most common and earliest detectable alterations in approximately 60% of the low-grade precursor tumors (Balachandran et al., 2020; Quader et al., 2022).

2.1 | The BBB and blood-brain tumor barrier (BBTB) role

The brain is a privileged anatomical part of the body due to the presence of the most protective CNS gatekeeper, namely the BBB. First termed as "barrier" by Stern and Gautier in 1918, the BBB is mainly composed by ECs connected by tight and adherens junctions, surrounded by an ECM basal lamina embedding pericytes and astrocytic endfeet and, to a lower extent, microglia and neuron projections (Figure 2a) (Arvanitis et al., 2020; Saunders et al., 2014). The BBB presents a specialized network of transport channels that orchestrate the brain influx of molecules pivotal for CNS homeostasis and surveillance, and efflux of toxic byproducts (Pandit et al., 2020). This barrier is the richest network of blood capillaries in the body, characterized by 10–15 μm average distances between capillaries, and the ability to provide supply to brain cells in a ratio of about one capillary per neuron (Tian et al., 2020). Compared to ECs of other anatomical sites, BBB ECs are non-fenestrated, due to a reduced number of pores in their membrane, and present limited pinocytic and intracellular vesicular trafficking (Arvanitis et al., 2020). Transcellular trafficking across the BBB (Figure 2b) can occur through: (i) carrier-mediated transport, or (ii) vesicle-mediated transcytosis via receptor (RMT)- or adsorptive (AMT)-mediated transcytosis (Arvanitis et al., 2020). Carrier-mediated transport does not involve a vesicular transport, uses either facilitated diffusion or active transport, and is responsible for the transport of molecules such as amino acids, glucose and vitamins (Khan et al., 2019; Sorets et al., 2020). For RMT, characterized by active transport mechanisms, one of the possible BBB trafficking pathways involves clathrin-dependent endocytosis—herein, dynamin regulates the formation of the initial vesicle, the clathrin coat is further shed, and the vesicle fuses with the early endosome (sorting endosome). The endocytosed cargo is afterwards directed to late endosomes (and eventually to the lysosomal degradation pathway) or transcytotic vesicles (Moura et al., 2019). Another trafficking pathway for RMT, shared with AMT, involves the endocytosis of molecules via caveosomes, which are uncoated vesicles based on lipid rafts stabilized by caveolin-1 that are speculated to avoid degradation pathways (Sorets et al., 2020). AMT is based on the interaction of polycationic molecules with negative components of the membrane of BBB ECs, hence triggering the delivery of the cargos into the brain (Zhu et al., 2019). Transferrin (Tf), lactoferrin, insulin, and lipoproteins are examples of molecules that undergo RMT across the BBB, while albumin and other plasma proteins are transported by AMT (Gosselet et al., 2021). BBB trafficking through passive diffusion is practically negligible, since it only applies for highly lipophilic drugs with a molecular weight lower than 500 Da (Lipinski et al., 1997). The transport of substances across the BBB is highly restrictive compared to other tissues and scrupulously controlled due to the brain-protecting nature of this barrier. However, despite restricting the brain permeability of harmful agents, the BBB also prevents 98% and nearly 100% of all small molecule and macromolecular drugs, respectively, to successfully reach the brain tissue (Liang et al., 2022). In fact, the drug-impenetrable BBB is a major responsible for the 50% lower chances of CNS drug candidates to reach de market, compared to other therapeutic areas (Kesselheim et al., 2015). Therefore, the inability to successfully surpass the BBB is a major obstacle in the field of drug delivery to the brain.

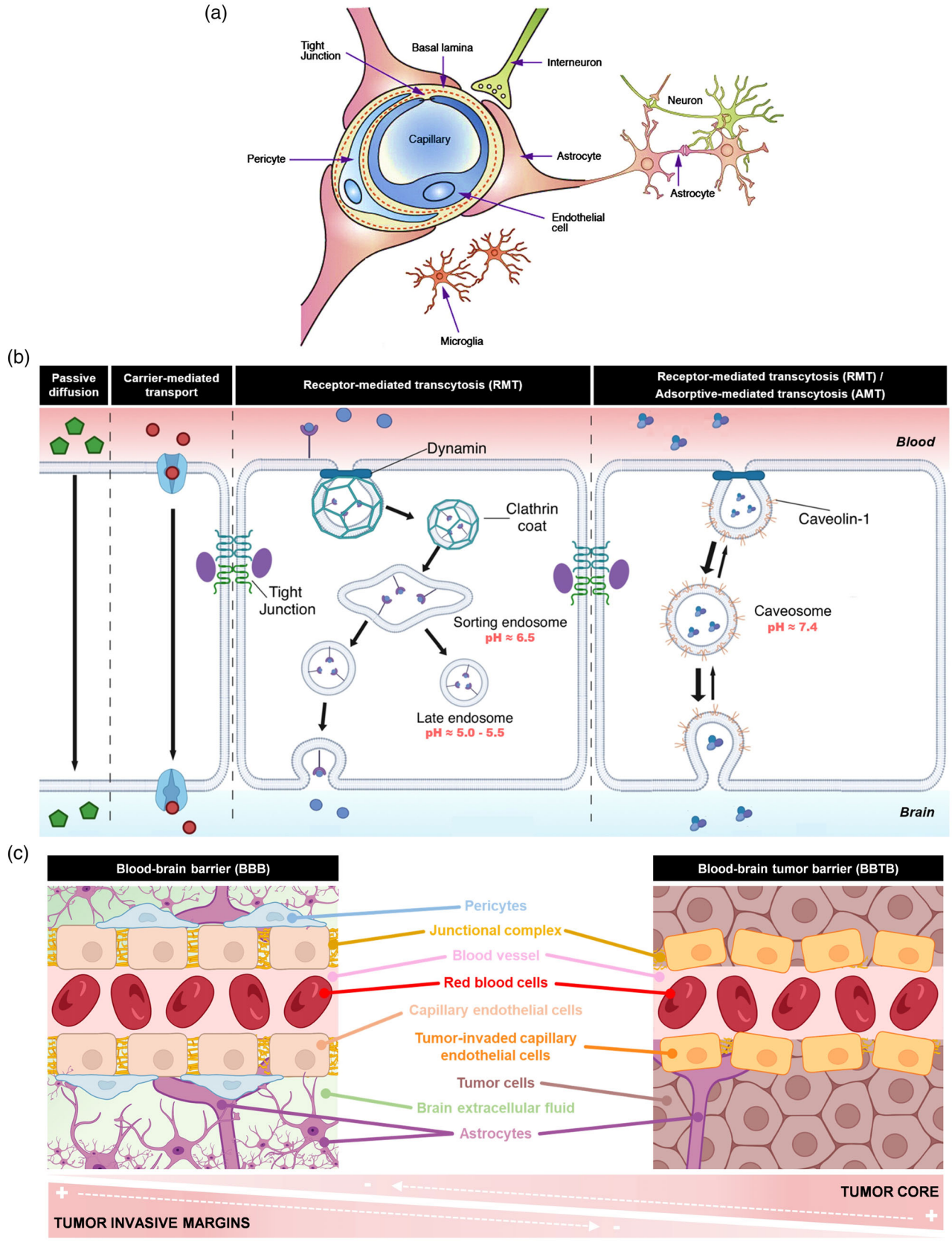

FIGURE 2 (a) Schematic representation of the BBB major components. Adapted with permission from (Chen & Liu, 2012). (b) Main intake transport routes of the BBB. Adapted with permission from (Naqvi et al., 2020; Sorets et al., 2020; Tashima, 2022). (c) Physiological differences between the GBM BBB (tumor invasive margins) and BBTB (tumor core). Created with BioRender.com.

The concept of the blood-brain tumor barrier (BBTB) arose to describe areas of physically altered BBB as a consequence of brain tumor damage and TME biological pressures (Figure 2c). In the case of GBM, certain tumor areas are reported to possess a BBTB, characterized by a leaky and disrupted BBB (Kim et al., 2015). This is associated with the impact of tumor events on the BBB structural integrity, leading to loss or displacement of astrocytic endfeet, lower expression of EC junction proteins, and an abnormal pericytic distribution (Achrol et al., 2019; Dubois et al., 2014; Watkins et al., 2014). However, it is known that the BBTB is localized at the tumor bulky core, while a gross region beyond the magnetic resonance imaging (MRI) contrast-enhancing tumor bulk, consisting of the peritumoral region and, specially, the tumor invasive margins, present an intact BBB (Arvanitis et al., 2020; Kessler & Bhatt, 2018; Sarkaria et al., 2018). The BBTB at the tumor bulky core is surrounded by areas of significant necrosis, where a leaky barrier and, consequently, higher permeation of therapeutics from blood, does not necessarily result in a therapeutic advantage (Kim et al., 2018; Quader et al., 2022). Altogether, the existence of a localized BBTB does not soften the fact that therapeutic delivery to GBM still poses a grand clinical challenge – the tumor invasive margins, encircled by an intact BBB, are highly correlated with tumor recurrence and disease progression. Thus, it is general consensus that drug distribution across an intact BBB is an important validation step in the development of novel therapies for GBM and other brain tumors, not placing too much hope on limited areas of BBTB (Sarkaria et al., 2018).

3 | GBM TREATMENT OPTIONS—STANDARD OF CARE (SOC), LIMITATIONS, AND OPPORTUNITIES

The current standard of care (SOC) for GBM relies on maximally safe surgical resection, followed by (i) concomitant radiotherapy (RT) and temozolomide (TMZ) chemotherapy for 6 weeks, and (ii) adjuvant TMZ chemotherapy for 6 months (Fisher & Adamson, 2021). This RT and TMZ pharmacotherapy regimen was proposed by Stupp et al. in 2005—the so-called Stupp protocol—when findings from a randomized controlled trial revealed that RT plus TMZ significantly improve the overall survival (OS) and progression-free survival (PFS) of newly diagnosed GBM patients compared to RT alone (14.6 vs. 12.1 months OS; 53.9% vs. 36.4% PFS at 6 months) (Stupp et al., 2005). GBM surgical resection is often challenging since the boundaries of the tumor are not easily distinguishable from the surrounding brain tissue, ultimately leading to tumor relapse due to the invasive potential of residual tumor cells (Sales et al., 2022). Apart from the SOC, four drugs and two medical devices are currently approved by the Food and Drug Administration (FDA) for the treatment of GBM (Figure 3): lomustine (1976), intravenous carmustine (1977), wafer implants of carmustine (Gliadel; 1996: recurrent GBM; 2003: newly diagnosed GBM), bevacizumab (2009), intratumoral thermotherapeutic iron oxide NPs (NanoTherm; 2010: recurrent GBM), and tumor treating fields (TTFs; Optune; 2011: recurrent GBM; 2015: newly diagnosed GBM) (de Lázaro & Mooney, 2021). Despite some of them are approved for newly diagnosed GBM, these alternative FDA-approved treatments have been mainly used to manage recurrent GBM and disease symptoms (Fisher & Adamson, 2021). Importantly, among these treatment alternatives, only TTFs have demonstrated ability to significantly improve OS and PFS of newly diagnosed GBM patients when combined with adjuvant TMZ chemotherapy (20.5 vs. 15.6 OS; 56% vs. 37% PFS at 6 months) (Stupp et al., 2015). However, following these findings, worldwide brain cancer experts decided to not yet add TTFs to the SOC due to marginal survival benefits, high costs, and patient compliance issues (Mehta et al., 2017). Nonetheless, over time, TTFs have gained great acceptance, not only because of the above-mentioned OS and PFS numbers, but also due to an increase in the 5-year survival of GBM patients from approximately 5%–13% compared to the SOC arm (Stupp et al., 2017). Other treatment modalities, such as immunotherapy (Ma et al., 2021), gene therapy (Yoo et al., 2021) and phototherapy (Li et al., 2022) are emerging as promising therapeutic approaches for GBM.

Although the Stupp protocol has remained the SOC for the past 20 years, great efforts have been invested in developing new and more effective therapeutic approaches to combat the lethal course of GBM. As already discussed in Section 2.1, a significant limitation is the presence of the BBB—the inability to surpass this barrier in therapeutically relevant concentrations limits the application of the most prospective drugs. Moreover, nearly 50% of the available anticancer drugs are characterized by poor water solubility, which challenges their formulation and compromises drug therapeutic concentration in biological fluids (Ren et al., 2019; Wong et al., 2008). Another challenge to the development of more effective therapeutic approaches is the short half-life of certain therapeutic molecules that are degraded or cleared from bloodstream minutes after their administration, thus requiring frequent dosing (Marqus et al., 2017). To circumvent this, chemical modification of these molecules with polyethylene glycol (PEG) might be a strategy to lengthen the therapeutics half-life, but it is nowadays known that free PEG moieties on drug-PEG conjugates lower

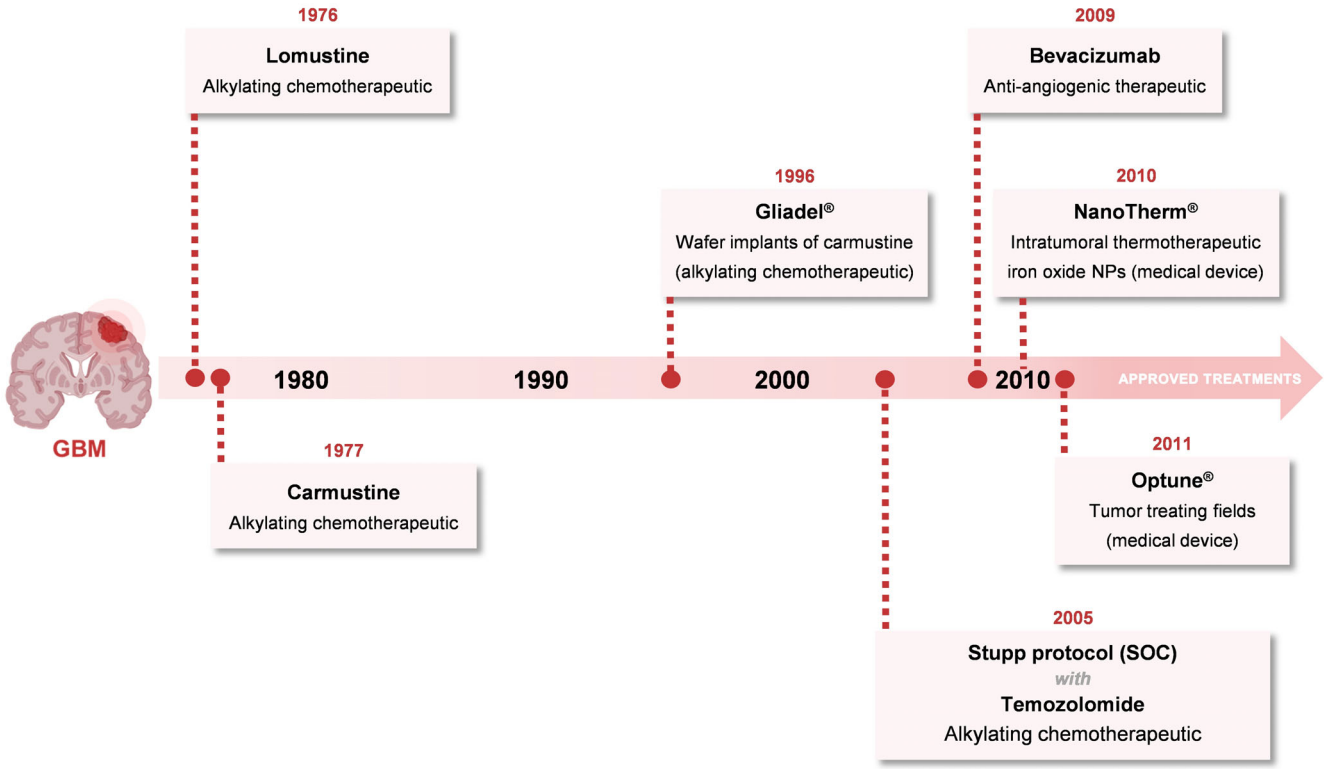

FIGURE 3 Clinically approved drugs and medical devices for GBM treatment.

their interaction with cancer cells (Verhoef & Anchordoquy, 2013). Other modifications include the synthesis of cell-penetrating protein complexes and engineered fc-based proteins, but such direct derivatization of drug molecules deserves careful consideration as it might significantly alter anti-tumor efficacy (Fung & Chan, 2017). Alongside with frequent drug dosing needs, the lack of tumor site targetability of anti-cancer therapeutics results in the emergence of severe side effects. This is particularly critical for chemotherapeutics, since they are able to trigger anti-proliferative mechanisms or cell cycle arrest in any type of rapidly proliferating or dividing cell, independently of its healthy or tumor nature (Mahato et al., 2011). As an example, common TMZ side effects include nausea and vomiting, fatigue, headache and myelosuppression (Cohen et al., 2005; Yung et al., 1999). Still in regard to GBM chemotherapy, TMZ presents an outstanding capacity of crossing the BBB (blood plasma to cerebrospinal fluid ratio of 0.2-0.3), but also well reported mechanisms of drug resistance and poor anti-cancer potency, as demonstrated by a 50% inhibitory concentration (IC_{50}) in U-87 MG GBM cells of 800 μM (Ostermann et al., 2004; Yang et al., 2012). Therefore, this opens a room of opportunities for exploiting other chemotherapeutics, with a higher tumoricidal potency, such as docetaxel (DTX), paclitaxel (PTX), doxorubicin (DOX) or irinotecan-0.041, 1.054, 0.061, and 10.916 μM IC_{50} in U-87 MG GBM cells, respectively (Yang et al., 2012). However, it is crucial to address key pharmaceutical constrains of these drugs, including low aqueous solubility, BBB permeation (blood plasma to cerebrospinal fluid ratio as low as 0.001) and GBM cell targeting post-BBB crossing (only 1% tumor uptake of the total administered dose) (Wilhelm et al., 2016).

4 | NANOMEDICINE TO COMBAT THE LIMITATIONS OF GBM THERAPEUTICS

Over the years, several nanomedicine strategies have been proposed to prevent, diagnose and treat a wide range of diseases. Nanomedicine gained popularity in the late 1990s and, according to the European Technology Platform on Nanomedicine (ETPN), its definition relies on "(...) the application of nanotechnology to health. It exploits the improved and often novel physical, chemical, and biological properties of materials at the nanometric scale." (Boisseau & Loubaton, 2011; Freitas, 1999; Shi et al., 2017). The US National Institutes of Health (NIH) standards, in turn, limit the definition of nanomedicine to nanotechnologies applied to medicine in the size range between 1 and

100 nm (Dolez, 2015). However, this definition is often considered too restrictive since several clinically approved nanomedicines exceed 100 nm, such as Abraxane (130 nm) and others, as reviewed elsewhere (Stiepel et al., 2022).

Nanomedicines are known to possess distinctive features that make them attractive for therapeutic delivery, including: (i) possibility of targeted delivery to a tissue, cell or organelle of interest, (ii) enhancement of formulation constraints of cargo molecules (e.g., aqueous solubility, in vivo half-life), (iii) amelioration of drug therapeutic indexes by increased efficacy and/or reduced toxicity, (iv) molecule release in a sustained or stimulus-triggered manner, (v) easing of delivery of biomacromolecule drugs, (vi) delivery of cocktails of cargos, (vii) facilitation of transcytosis of molecules across difficult-to-permeate biological barriers, and (viii) single-vehicle combination and delivery of therapeutic and imaging cargos (Shi et al., 2017). There are more than 50 clinically-approved nanomedicines currently available as prescription medicine (Quader et al., 2022), and a significant part of them focus on cancer therapy (de Lázaro & Mooney, 2021; He et al., 2019; Souri et al., 2022). The progress of the nanomedicine field has generated diverse classes of systems with several different matrix compositions, shapes, mechanical properties, surface decorations, sizes, among others (Figure 4a) (Domingues et al., 2022). Polymeric nanoparticles (NPs), particularly solid particles, have been focus of considerable research due to their versatile physicochemical properties (e.g., hydrophobicity, size, charge) according to the manufacture protocol (e.g., matrix composition, precursors molecular weight, energy shearing), sustained release of cargo molecules over time, and possibility of encapsulation of cargo molecules with variable characteristics (e.g., from small molecule to biomacromolecule drugs, from practically water-insoluble to highly hydrophilic molecules) (Wibowo et al., 2021). Poly(lactic-co-glycolic acid) (PLGA) is one of the most popular synthetic polymeric matrix used in drug delivery systems, and adds to the list of advantages of polymeric NPs an easy, reproducible and scalable production, as well as FDA approval for human use (Martins et al., 2018; Wan et al., 2021). Actually, PLGA entered the clinics in the early 1970s, as biodegradable surgical sutures, but its unique properties of biodegradability, biocompatibility and mechanical strength received the attention of pharmaceutical scientists, further leading to the emergence of drug delivery applications (Blasi, 2019). A gold standard modification of PLGA NPs relies on the combination of PLGA with PEG, thus endowing the system with multiple possibilities of surface modification by functionalization of the outer arms of PEG (Souri et al., 2022).

4.1 | Targeted GBM nanomedicine

Targeted nanomedicines, using active targeting strategies, are systems typically functionalized with surface moieties complementary to target sites of interest in the human body, such as tumor and vascular cells, intracellular organelles and ECM tags (Wilhelm et al., 2016). Active targeting strategies arose from the need to improve the sub-optimal

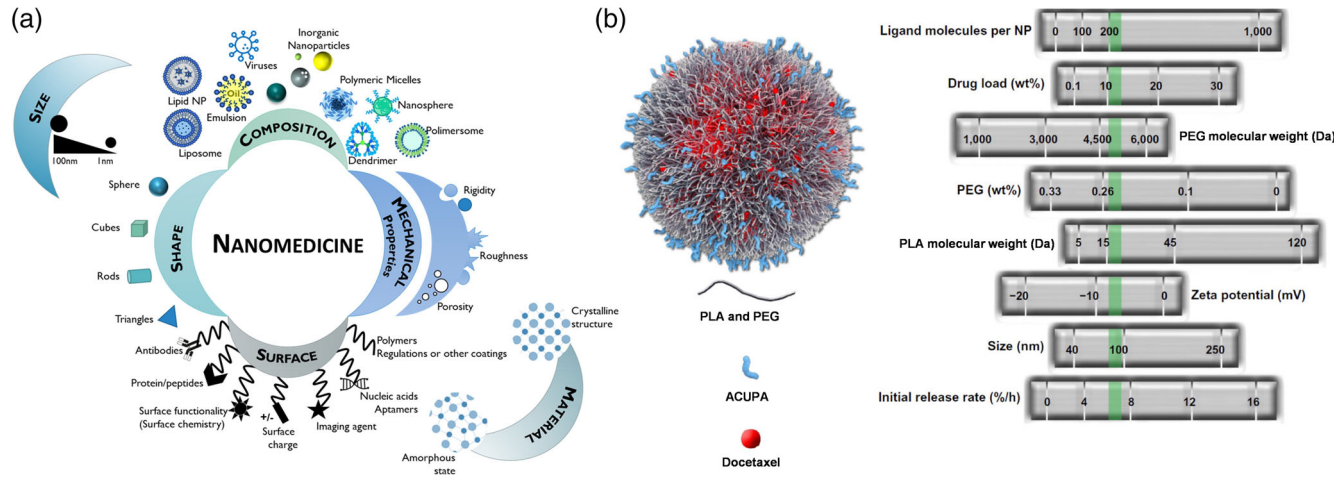

FIGURE 4 (a) Diverse classes of systems resulting from the progress of the nanomedicine field over recent years, leading to the report of several different matrix compositions, shapes, mechanical properties, surface decorations, sizes, among others. Reproduced with permission from (Domingues et al., 2022). Copyright 2022 American Chemical Society. (b) Formulation composition, key parameters and physicochemical characteristics (green range) of the targeted cancer nanomedicine candidate BIND-014. Adapted with permission from (Gu et al., 2021).

biodistribution of nanomedicines, ultimately leading to the accumulation and premature release of therapeutic molecules in off-target healthy tissues and, consequently, a reduced therapeutic efficacy alongside with considerable side effects (Souri et al., 2022). A strong basis for these targeted strategies are the antibody-drug conjugates already approved in clinics, whose antibody counterpart is often used to direct and deliver the conjugated drug to specific cancer cells—there are around 15 of these antibody-drug conjugates currently used in cancer therapy, as extensively reviewed elsewhere (Fu et al., 2022; Schwach et al., 2022; Tong et al., 2021). Although antibody-drug conjugates use antibody moieties to target the tumor, targeted cancer nanomedicines can present a diversity of other target-recognition surface molecules, including proteins, peptides, aptamers and amino acids, attached to the NP surface (Domingues et al., 2022). Although there is currently no clinically-approved targeted nanomedicine in the market (Woythe et al., 2021), the field has experienced a research boom over the past years, and several candidates are undergoing clinical trials (examples presented in Table 1). Perhaps the most appealing targeted cancer nanomedicine candidate over the past decade was BIND-014 (Figure 4b). It consisted of poly(lactic acid) (PLA) and PEG NPs encapsulating DTX, surface decorated with S,S-2-[3-[5-amino-1-carboxypentyl]-ureido]-pentanedioic acid (ACUPA), to target the prostate-specific membrane antigen (PSMA) overexpressed by prostate cancer cells (Hrkach et al., 2012). BIND-014 pioneering nanotechnology resulted in the foundation of BIND Biosciences, and the initiation of Phase I clinical trials in 2007. In the patient cohort of Phase I trials (NCT01300533), BIND-014 was well tolerated, demonstrated dose-dependent linear pharmacokinetics and an improved circulation time. However, in phase II trials (NCT02479178), the low response to treatment observed, along with manufacturer constraints, led to the termination of BIND-014 testing (Jurczyk et al., 2022). Careful analysis of this failure suggests that the inter-patient tumor heterogeneity and lack of patient stratification prior to treatment administration, as often performed for molecularly targeted therapeutics and antibody-drug conjugates, were responsible for the unmet endpoints of BIND-014 (Gu et al., 2021; Sanna & Sechi, 2020).

4.1.1 | BBB/neovasculature and tumor targets

The transferrin receptor (TfR) has been widely exploited for brain-targeted drug delivery since it is significantly overexpressed by ECs of the brain capillaries compared with other tissues (Jefferies et al., 1984; Taylor & Morgan, 1990). The transport of iron to the brain, to maintain iron homeostasis, is the major biological role of TfR and occurs through RMT in a clathrin-dependent pathway that involves the formation of endosomal vesicles (Moura et al., 2019). Besides being overexpressed by BBB ECs, TfR is also overexpressed by tumor cells undergoing rapid proliferation such as GBM cells, therefore making this receptor a promising target for both BBB- and GBM-targeted therapies (Voth et al., 2015). Ligands that recognize and bind TfR include the Tf protein (X. Wang et al., 2019; Zhu, Zhou, et al., 2018; Gao et al., 2013; Lakkadwala et al., 2019; Lakkadwala & Singh, 2018; Zhang et al., 2012) and the T7 peptide (Fu et al., 2019; Zong et al., 2014). Similarly to TfR, the low-density lipoprotein receptor (LDLR) has also been vastly exploited for the purpose of BBB- and GBM-targeted drug delivery, since it presents overexpression in both ECs of brain capillaries and tumor cells (Di & Maiseyeu, 2021). Contrary to brain capillaries, LDLR is often downregulated in large vessels, where its transcytosis rate is considerably lower compared with the brain tissue (Dehouck et al., 1994). LDLR controls the brain homeostasis of cholesterol, and internalizes cargos through RMT in a clathrin- or caveolin-dependent pathway (Moura et al., 2019). The Angiopep-2 peptide (ANG2) is the best-known LDLR-targeting ligand (Bruun et al., 2015; Gao, Zhang, Cao, et al., 2014; Liu et al., 2019; Zhu, Jiang, et al., 2018), but other ligands that recognize and bind the receptor include the Pep-22 peptide (Chen et al., 2017) and, indirectly, Polysorbate 80 (Yang et al., 2022). Nicotine acetylcholine receptors (nAChRs) and choline transporters also present a very high expression at the brain due to their role in modulating the transport of acetylcholine or choline and, an aspect of particular interest, working as a gateway for the entrance of certain viruses in the brain such as the rabies virus (Moura et al., 2019). Although further research is required to better understand the trafficking mechanisms behind these transporters, the nAChR-targeted D8 peptide ligand (Farshbaf et al., 2022) and choline analogues (Wang et al., 2022) have shown a promising BBB-targeting ability. Focus of great attention in the fields of GBM neovasculature and cell targets have been the αvβ3 integrins. These molecules are poorly expressed by quiescent ECs, but highly expressed by ECs of newly formed vascular structures such as the tumor neovasculature (Echavidre et al., 2022). Moreover, αvβ3 integrins are overexpressed by glioma cells compared with normal brain cells (Paolillo et al., 2018). A well-studied ligand for αvβ3 integrins in the context of neovasculature- and tumor-targeted delivery is the RGD peptide (Gao, Xiong, Zhang, et al., 2014; Gao, Yang, et al., 2014; Belhadj et al., 2017; Chen et al., 2017; Shi et al., 2020; Zhang et al., 2012). The RGD peptide is also reported

TABLE 1 Examples of targeted cancer nanomedicines undergoing clinical trials.

Product designation	Carrier type	Cargo	Administration route	Disease indication	Year and region of first approval	Reference
MCC-465	Liposomes (GAH antibody fragment targeting moiety)	DOX	Intravenous	Stomach cancer	Phase I	(Hosokawa et al., 2003; Matsumura et al., 2004)
C225-ILS-DOX	Liposomes (cetuximab antibody fragment targeting moiety)	DOX	Intravenous	Solid tumors (EGFR target)	Phase I	(Mamot et al., 2012)
MBP-426	Liposomes (Tf targeting moiety)	Oxaliplatin	Intravenous	Solid tumors (transferrin receptor (TfR) target)	Phase I	(Gu et al., 2021; Senzer et al., 2009)
CALAA-01	Cyclodextrin and PEG polymeric NPs (Tf targeting moiety)	siRNA against ribonucleotide reductase M2	Intravenous	Solid tumors (TfR target)	Phase I	(Davis et al., 2010)
89Zr-DFO-cRGDY-PEG-Cy5-C′ dots	Silica inorganic NPs (RGD peptide targeting moiety)	Zirconium-89 positron emission tomography label and dasatinib prototype small molecule inhibitor	Intravenous	High-grade gliomas (tumor and tumor vasculature α_v integrins target)	Phase I	(Quader et al., 2022)
2B3-101	Liposomes (glutathione tripeptide targeting moiety)	DOX	Intravenous	Brain tumors (unknown transporter target at the BBB)	Phase II	(Gaillard et al., 2014; Gu et al., 2021)
SGT-53	Liposomes (TfR antibody fragment targeting moiety)	Wild-type p53 DNA	Intravenous	Solid tumors (TfR target)	Phase II	(Senzer et al., 2013)
MM-302	Liposomes (HER2 antibody targeting moiety)	DOX	Intravenous	Breast cancer (HER2 target)	Phase III	(Munster et al., 2018)

to bind the neuropilin-1 receptor (NRP1R) of cancer cells (Shi et al., 2020). The folate receptor, in turn, can also be used for both BBB/neovasculature and GBM targeting since it is overexpressed by glioma cells, tumor-associated vasculature and the intact BBB (McCord et al., 2021). Thus, folic acid-based NP surface moieties have been exploited to shuttle nanosystems across the BBB and target brain tumor cells (Gao et al., 2013; Yang et al., 2022). Other classes of receptors that have been studied for brain- or brain- and tumor-targeted delivery, respectively, are the dopamine and sigma receptors. These receptors present a prominent expression in most parts of the CNS such as the BBB

and, for sigma receptors only, an overexpression by brain tumor cells (Lu et al., 2022). Heparan sulfate residues have also been considered as a potential brain-targeting strategy in brain tumor scenarios since their expression is augmented in neovascular ECs (Järvinen & Ruoslahti, 2007; Lv et al., 2016). For GBM cell targeting, the interleukin-13 (IL-13) α2 receptor is one of the few receptors that is specifically overexpressed by GBM cells but undetectable in normal brain cells, thus making it an attractive target for GBM-targeted therapies with high selectivity for tumor cells along with low toxicity for healthy brain resident cells (Newman et al., 2017). However, in order to understand the failure in clinical trials of therapies molecularly targeted to the IL-13 α2 receptor, several patient specimens were analyzed and it was concluded that the percentage of clinical GBMs that present IL-13 α2 receptor overexpression is actually lower than 50% (Jarboe et al., 2007). Nevertheless, if the strategy is preceded by proper patient stratification to maximize the targeting effect, the Pep-1 peptide (Lv et al., 2016) or IL-13 peptide (Gao, Xiong, Zhang, et al., 2014; Gao, Yang, et al., 2014) are promising choices as IL-13 α2 receptor-targeted ligands. Similarly to the IL-13 α2 receptor, the EGFR might be used as a GBM target, since the receptor is overexpressed by GBM cancer cells and is involved in the process of tumorigenesis (Liu et al., 2015). However, it should be taken into consideration that only approximately 36% of clinical GBMs present EGFR amplification, thus, EGFR-targeted therapies require a patient pre-selection (Balachandran et al., 2020; Quader et al., 2022). The EP-1 peptide is an example of an EGFR-targeted moiety (Liu et al., 2019). The glucose-regulated protein 78 (GRP78) is another option for GBM cell targeting since it is highly expressed in clinical GBMs in comparison with normal brain specimens (Lee et al., 2008). The expression of GRP78 is induced by TME key features, including glucose starvation, oxidative stress and hypoxia (Li & Lee, 2006). The RI-VAP peptide has been used as a NP surface ligand for GBM GRP78 targeting (Farshbaf et al., 2022). Nucleolin is a cell surface protein abnormally expressed in GBM cells, promoting the binding of signaling molecules that stimulate cancer proliferation (Sathiyaseelan et al., 2021). An aptamer with high affinity for nucleolin, AS1411, has been used for GBM targeting purposes (Gao, Yang, et al., 2014; X. Zhu et al., 2018; Gao et al., 2012). The CD13 receptor has also been used as a GBM cell target, since it is overexpressed by glioma cells and, moreover, neovascular ECs (Huang et al., 2017). The NGR peptide has been reported to target the CD13 receptor (Cui et al., 2020; Fu et al., 2019). Amino acid transporters such as the L-type amino acid transporter 1 (LAT1) also offer great promise as GBM targets. LAT1 fulfils the high metabolic demands of GBM cells through amino acid uptake (Häfliger & Charles, 2019), hence evidencing amino acids as potential NP surface moieties to target the transporter. LAT1 presents overexpression by GBM cells, lower expression in low-grade gliomas, and basal expression in the normal cerebral cortex (Häfliger & Charles, 2019; Haining et al., 2012; Nawashiro et al., 2006). A different class of NP surface moieties that potentiate GBM tissue penetration consist of cell penetrating peptides (CPPs). These peptides are usually based on cationic or amphipathic sequences that possess ability to cross cellular membranes, although they cannot be considered tumor-selective (Zhang et al., 2021). Examples of CPPs that have been used for GBM cell accumulation include the R8 (Gao, Zhang, et al., 2014; X. Wang et al., 2019), penetratin (Pen) (Lakkadwala et al., 2019; Lakkadwala & Singh, 2018) and TAT (Zhu, Jiang, et al., 2018; Zong et al., 2014) peptides. Following the same rationale, despite not common, the tLyp-1 peptide has been used as a CPP for BBB cell targeting and accumulation (Jin et al., 2021). Another approach for GBM targeting consists of using ligands directed to TME components, including the programmed death 1 (PD-1) protein on cytotoxic T cells (Wang et al., 2022), and the NKG2A receptor on T and NK cells (Jin et al., 2021).

4.2 | Multi-ligand functionalized blood-to-GBM sequentially targeted nanomedicines

The need to overcome two physiological barriers in the context of GBM intravenous therapies, namely the BBB and tumor barrier, opens avenues for the development of novel BBB and GBM dual-targeted nanosystems presenting multi-ligand surface functionalization. These nanosystems are designed to perform sequential biological roles, as the following: (i) BBB targeting and consequent blood-to-brain transport, and (ii) tumor cell targeting once arrival at the brain parenchyma.

Gao et al. proposed a DTX-loaded polymeric NP system of polycaprolactone (PCL) and PEG, targeted to the BBB LDLR by an ANG2 peptide moiety, and further decorated with a TME matrix metalloproteinase-2 (MMP-2)-activable R8 CPP for enhanced GBM cell accumulation (Gao, Zhang, Cao, et al., 2014). In an in vivo therapeutic efficacy trial, the dual-ligand NP group demonstrated an increase in the median survival of a C6 GBM orthotopic mouse model (32 days), compared to the untreated group (19 days), non-functionalized NPs (20 days), NPs presenting only the MMP-2-activable R8 CPP (26 days), and NPs presenting only the ANG2 peptide moiety (27 days)—of important note, non-

nanoparticulate DTX was not included as a control group in the study. Other authors developed DTX-loaded dithiolane trimethylene carbonate and PEG micelles, using ANG2 peptide for both LDLR BBB and glioma cell targeting, along with a TAT CPP for enhanced glioma cell penetration (Zhu, Jiang, et al., 2018). It was observed an improved median survival in a U-87 MG GBM orthotopic mouse model (53 days), compared with the untreated group (24 days), free DTX (30 days), non-functionalized NPs (33 days), and NPs presenting only the ANG2 peptide moiety (44 days). In another study, liposomes loaded with DOX and erlotinib were decorated with a Tf protein moiety for BBB TfR targeting, and a Pen CPP for enhanced GBM cell accumulation (Figure 5) (Lakkadwala et al., 2019). In vivo, the median survival of a U-87 MG GBM orthotopic mice model increased to 36 days compared with the untreated group (22 days), free drugs cocktail (25 days), non-functionalized NPs (25.5 days), liposomes presenting only the Tf moiety (30 days), and liposomes presenting only the Pen CPP moiety (27.5 days). The same authors previously proposed a similar liposome system for 5-fluorouracil loading, which presented a 20%–30% cell viability decrease in a 3D in vitro tumor model in comparison with the free drug, non-functionalized liposomes, Tf-functionalized liposomes and Pen CPP-functionalized liposomes (Lakkadwala & Singh, 2018).

Liu et al. functionalized DOX-loaded dendrimer nanocarriers with ANG2 and an EGFR-targeting peptide (EP-1) for BBB and GBM cell targeting, respectively (Liu et al., 2019). The system was tested in a U-87 MG GBM orthotopic mice model and provided 34 days median survival, which represented an improvement compared with the untreated group, free DOX, non-functionalized NPs, NPs functionalized with only ANG2, and NPs functionalized with only EP-1 (16, 19, 24, 31, and 27 days, respectively). A more advanced approach focused on the development of a polymeric NP system, surface modified with a BBB choline transporter-targeted molecule and a tumor programmed death-ligand 1 (PD-L1) acid-cleavable antibody (Wang et al., 2022). The BBB-targeted molecule was expected to shuttle the NPs to the brain, while the PD-L1 antibody was attached to the NPs via an acid-cleavable strategy to promote its release from the carrier upon encountering the TME acidic pH – the release from the carrier was expected to allow the antibody to exert its therapeutic role through binding to the PD-1 protein on cytotoxic T cells, ultimately resulting in a T cell-mediated anti-tumor immunity. In a LCPN GBM orthotopic mice model, the median survival of animals was increased to 19 days compared with the untreated group (8 days), free PD-L1 antibody (8 days) and non-functionalized NPs (12 days), respectively—herein, it would have been interesting to unravel the therapeutic benefit of the pH-sensitive properties by testing the control based on BBB-targeted NPs presenting a non-cleavable PD-L1 antibody moiety (Wang et al., 2022). In what concerns highly advanced systems, Fu et al. proposed vincristine-loaded, erythrocyte membrane-enveloped lipid NPs (longer circulation half-life and lower immunogenicity), surface modified with the BBB TfR- and GBM

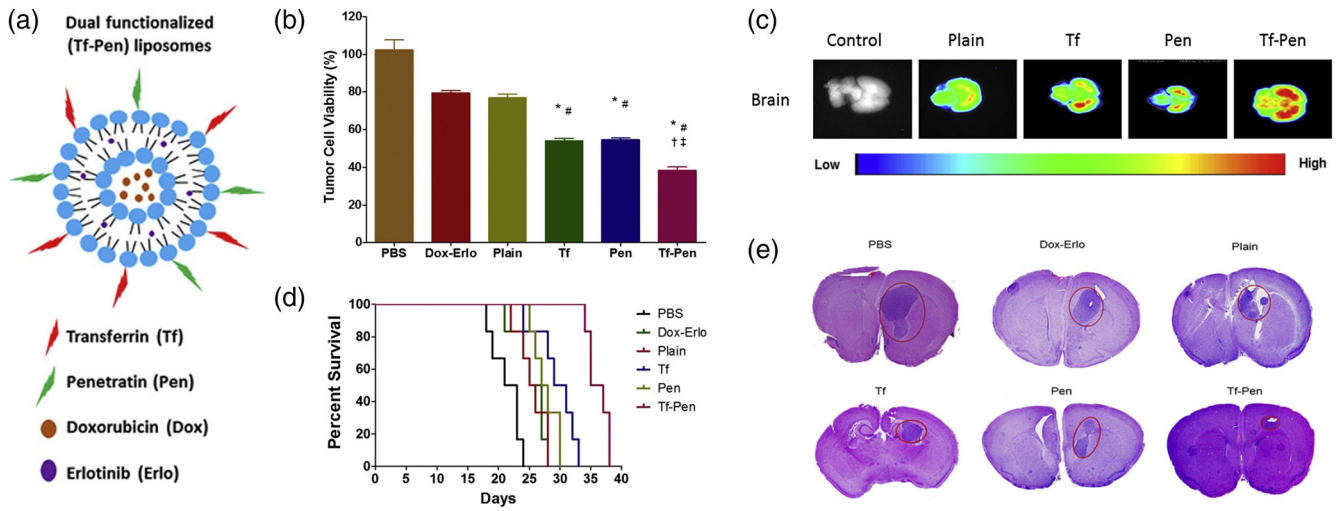

FIGURE 5 Multi-ligand, BBB and GBM dual-targeted nanosystem based on liposomes loaded with DOX and erlotinib, surface decorated with Tf (BBB targeting) and Pen CPP (GBM targeting) moieties. (a) Nanosystem design. (b) In vitro inhibition of tumor cell viability. (c) In vivo brain accumulation of the different formulations. (d) Kaplan-Meier survival curves of mice after treatment. (e) Histological sections of brain displaying tumor regression (red circle) after treatment. The images were taken at 20× magnification. PBS, dox-erlo, plain, Tf, Pen, and Tf-Pen denote the untreated group, free drugs cocktail (DOX and erlotinib), non-functionalized liposomes, liposomes presenting only the Tf moiety, liposomes presenting only the Pen CPP moiety, and liposomes presenting both the Tf and Pen CPP moieties, respectively. Adapted with permission from (Lakkadwala et al., 2019).

CD13-targeted T7 and NGR peptides, respectively (Figure 6) (Fu et al., 2019). In a C6 orthotopic GBM mice model, the system provided an increased median survival of animals (36 days) in comparison with the untreated group, free drug, non-functionalized NPs, NPs presenting only the T7 moiety, and NPs presenting only the NGR moiety (18, 19, 20, 29, and 21 days, respectively). Of important note, despite the selection of a nude mice model, the tumor was based on a rat-based GBM xenograft, instead of the conventional human-based xenografts (e.g., U-87 MG GBM). In the same GBM rodent model, Lv et al. assessed the efficacy of PLGA and PEG polymeric NPs, encapsulating PTX, surface decorated with CGKRK and Pep-1 peptides to target neovascular ECs (heparan sulfate) and GBM cells (IL-13 α2 receptor), respectively (Lv et al., 2016). It was observed a promising improvement in the median survival of animals treated with the developed NPs (61 days) compared with the untreated group, free PTX, non-functionalized NPs, NPs decorated with only CGKRK, and NPs decorated with only Pep-1 (17, 22, 24, 34, and 32 days, respectively).

Gao et al. exploited the use of polymeric NPs to target the GBM IL-13 α2 receptor (IL-13 peptide surface moiety), but using a brain targeting strategy focusing on the BBB αvβ3 integrins (RGD peptide surface moiety) (Gao, Yang, et al., 2014). In a C6 orthotopic GBM mice model, the NPs loaded with DTX provided a superior animal median survival (35 days), compared with the untreated group (17 days), free DTX (20 days), non-functionalized NPs (22 days), RGD peptide-functionalized NPs (27 days) and IL-13 peptide-functionalized NPs (26 days). In a different approach, PEGylated liposomes loaded with DOX were surface decorated with a cyclic RGD peptide to target both neovasculature and GBM cells (αvβ3 integrins), while a second surface decoration based on p-hydroxybenzoic acid (pHA) served the

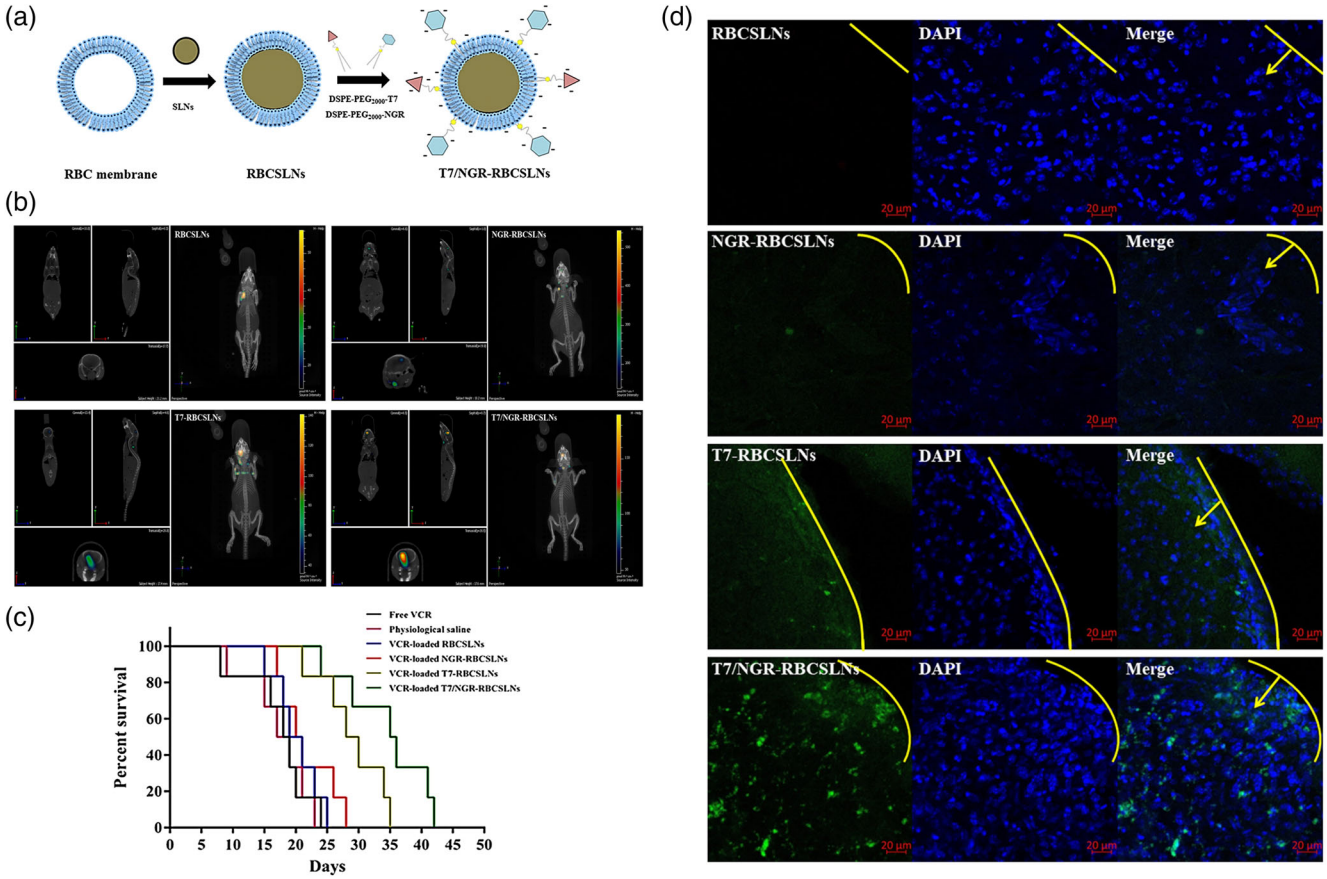

FIGURE 6 Multi-ligand, BBB and GBM dual-targeted nanosystem based on vincristine-loaded, erythrocyte membrane-enveloped lipid NPs, surface modified with the BBB TfR- and GBM CD13-targeted T7 and NGR peptides, respectively. (a) Nanosystem design. (b) Biodistribution of formulations in mice-bearing intracranial C6 GBM. (c) Kaplan-Meier survival curves of mice after treatment. (d) Distribution of NPs in mice brain determined by confocal laser scanning microscopy. Formulations were labeled with cyanine5.5 (red), and cell nuclei with DAPI (blue). The yellow line shows the margin of the intracranial glioma, and the arrow points the location of glioma cells. RBC, SLN, DSPE-PEG_{2000}-T7, DSPE-PEG_{2000}-NGR, T7/NGR-RBCSLNs, RBCSLNs, NGR-RBCSLNs, T7-RBCSLNs and VCR denote red blood cells, solid lipid NPs, NP lipid modified with the T7 peptide, NP lipid modified with the NGR peptide, T7 and NGR NPs, nontargeted NPs enveloped by erythrocyte membranes, NGR-functionalized NPs, T7-functionalized NPs, and vincristine, respectively. Adapted with permission from (Fu et al., 2019).

purpose of BBB targeting (dopamine receptors) (Belhadj et al., 2017). In a U-87 MG GBM orthotopic mice model, the dual-functionalized liposomes provided the longest median survival, 35 days, compared with the other treatment controls (20, 23, 26.5, 30, and 28.5 days for the untreated group, free DOX, non-functionalized NPs, pHA-functionalized NPs and cyclic RGD-functionalized NPs, respectively). Farshbaf et al. proposed bortezomib-loaded lipid NPs, surface functionalized with the D8 and RI-VAP peptides to target the BBB nAChRs and GBM cell GRP78 surface marker, respectively (Figure 7) (Farshbaf et al., 2022). In a GL261 GBM orthotopic mice model, the authors found out a promising increase in the median survival of animals treated with the proposed dual-targeted system (46 days), compared with the untreated group (16 days), free drug (26 days), non-functionalized NPs (30 days), NPs functionalized with only the D8 peptide (36 days), and NPs functionalized with only the RI-VAP peptide (29 days). Bruun and colleagues proposed lipid NPs for siRNA model delivery, surface modified with the ANG2 peptide for both BBB and GBM targeting, and TME matrix metalloproteinase (MMP)-activable glutamic acid residues (Bruun et al., 2015). These glutamic acid residues were expected to cause negative to positive charge switch at the tumor site, favoring GBM cell endocytosis and endosomal escape. The in vitro uptake in U-87 MG GBM cells and bEnd.3 BBB cells was significantly enhanced compared with non-functionalized NPs, but no relevant differences were found between the proposed formulation and the control formulation without ANG2, suggesting the poor effect of ANG2 in regard to BBB and GBM cell targeting (Bruun et al., 2015). In the field of biologic-responsive nanosystems, other authors developed mesoporous ruthenium inorganic NPs attached with $[Ru(bpy)2(tip)]^{2+}$ (RBT) via tumor glutathione-cleavable bonds, surface functionalized with Tf and the AS1411 aptamer to target the BBB TfR and GBM nucleolin, respectively (Zhu, Zhou, et al., 2018). The median survival of a U-87 MG GBM orthotopic mice model was increased to 40 days, which was longer than the untreated group (22 days), free drug (26 days) and NPs presenting only the Tf moiety (34 days)—the effect of NPs functionalized with only the AS1411 aptamer was not assessed. As an adjuvant therapeutic effect, the median survival suffered a proportional increase across all treatment types under laser irradiation, since the irradiated RBT provided photodynamic therapy by the local production of reactive oxygen species that augmented tumor cell apoptosis (Zhu, Zhou, et al., 2018).

Table 2 summarizes the multi-ligand functionalized, BBB- and GBM-dual targeted nanomedicines reported in literature data. A scheme of the main biological targets exploited in the development of multi-ligand functionalized, BBB and tumor dual-targeted nanomedicines for GBM therapies over the past 10 years is presented in Figure 8.

Overall, the data summarized in this review highlighted the great promise of multi-ligand functionalized, BBB and tumor sequential- and dual-targeted nanosystems over the single-ligand nanosystem control counterparts (targeted to either the BBB or GBM) and the free cargo controls. In the revised in vivo findings, and although they are based on

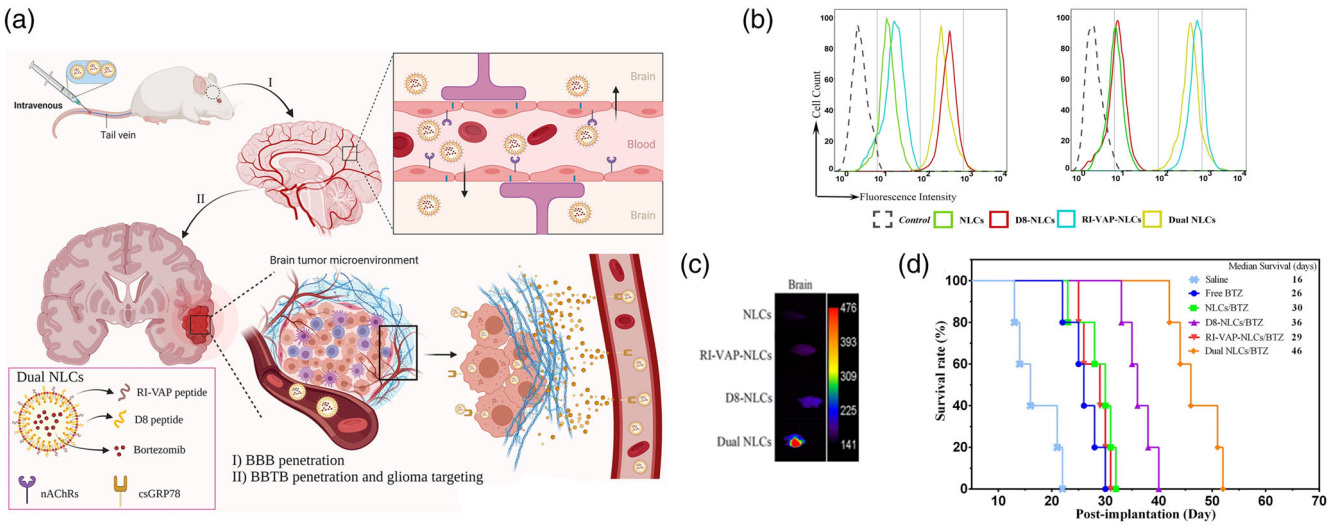

FIGURE 7 Multi-ligand, BBB and GBM dual-targeted nanosystem based on bortezomib-loaded lipid NPs, surface functionalized with the D8 and RI-VAP peptides to target the BBB nAChRs and GBM cell GRP78 surface marker, respectively. (a) Nanosystem design and proposed biological mechanism. (b) Cellular uptake of NP formulations by hCMEC/D3 BBB cells (left) and GL261 GBM cells (right). (c) *Ex vivo* fluorescence imaging of brain of intracranial glioma-bearing mice harvested 24 h after NPs administration. (d) Kaplan-Meier survival curves of mice after treatment. NLCs, csGRP78, D8-NLCs, RI-VAP-NLCs, dual NLCs and BTZ denote nanostructured lipid NPs, cell surface GRP78, D8 peptide-functionalized NPs, RI-VAP peptide-functionalized NPs, NPs functionalized with both D8 and RI-VAP peptides, and bortezomib, respectively. Adapted with permission from (Farshbaf et al., 2022).

TABLE 2 Multi-ligand functionalized, BBB and tumor sequential- and dual-targeted nanosystems reported in literature for the management of GBM. The presented nanosystems were designed for intravenous administration. The table does not include nanosystems presenting multi-ligand functionalization only for the purpose of higher BBB crossing and not envisaging tumor targeting; functionalization moieties can be therapeutic; single-ligand functionalization strategies based on fusion proteins or chimeric peptides were not included; studies presenting in vivo data were only included if providing GBM orthotopic testing models.

Carrier type	Cargo(s)	BBB/ neovasculature target(s)	BBB-/ neovasculature-directed moiety(ies)	GBM target(s)	GBM-directed moiety(ies)	Key findings	Reference
Lipid NPs	Bortezomib	nAChRs	D8 peptide	GRP78	RI-VAP peptide	Median survival of GL261 GBM orthotopic mice model increased to 46 days (16, 26, 30, 36, and 29 days for the untreated group, free drug, non-functionalized NPs, D8-NPs and RI-VAP-NPs, respectively)	(Farshbaf et al., 2022)
Liposomes	-	LDLR	Polysorbate 80 (P80) coating	Folate receptor Cell mitochondria	Folic acid (acid-cleavable) Berberine isoquinoline plant alkaloid	Similar brain accumulation to P80-coated liposomes in a C6 orthotopic GBM mice model, and superior brain accumulation compared with non-functionalized liposomes, berberine-liposomes, folic acid-liposomes, and folic acid plus berberine liposomes	(Yang et al., 2022)
Liposomes	**DOX and lonidamine**	**Dopamine and sigma receptors**	**p-HA (acid-cleavable)**	**Sigma receptors Cell mitochondria**	**p-HA (acid-cleavable) Triphenylphosphonium (TPP, endosomal escape)**	**Median survival of C6 GBM orthotopic mice model increased to 41.5 days (16.5, 22, 23.5, 24.5, and 33.5 days for the untreated group, free drugs cocktail, non-functionalized liposomes, TPP-liposomes, and TPP plus non-cleavable p-HA liposomes, respectively)**	**(Lu et al., 2022)**
PEG monomethacrylatepolymeric NPs	-	Choline transporter	Choline analogue	PD-1 protein (cytotoxic T cells)	PD-L1 antibody (acid-cleavable)	Median survival of LCPN GBM orthotopic mice model increased to 19 days (8, 8, and 12 days for the untreated group, free PD-L1 antibody and non-functionalized NPs, respectively)	(Wang et al., 2022)
Dendrimers	LSINCT5 siRNA	-	tLyp-1 CPP	NKG2A receptor (T and NK cells)	NKG2A antibody (acid-cleavable)	Median survival of U-87 MG GBM orthotopic mice model increased to around 45 days (20, 40, and 30 days for the non-functionalized NPs, tLyp-1-NPs and NKG2A antibody-NPs, respectively)	(Jin et al., 2021)

(Continues)

TABLE 2 (Continued)

				Sigma receptors	p-HA (acid-cleavable)		
Liposomes	**DOX and lonidamine**	**Dopamine and sigma receptors**	**p-HA (acid-cleavable)**	**Cell mitochondria**	**Triphenylphosphonium (TPP, endosomal escape)**	**Median survival of C6 GBM orthotopic mice model increased to 41.5 days (16.5, 22, 23.5, 24.5, and 33.5 days for the untreated group, free drugs cocktail, non-functionalized liposomes, TPP-liposomes, and TPP plus non-cleavable p-HA liposomes, respectively)**	**(Lu et al., 2022)**
Erythrocyte membrane-enveloped PLGA polymeric NPs	Euphorbia factor L1	-	^{D}WSW bacterial quorum sensing peptide	CD13 receptor	NGR peptide	Median survival of C6 GBM orthotopic mice model increased to 36 days (20, 22.5, 24, 27.7, and 30 days for the untreated group, free cargo, non-functionalized NPs, ^{D}WSW-NPs and NGR-NPs, respectively)	(Cui et al., 2020)

		Not mentioned	TGN peptide				
Dendrimers	**Arsenic trioxide**	**αvβ3/αvβ5 integrins**	**RGD peptide**	**NRP1R**	**RGD peptide**	**Median survival of glioma-bearing mice (model unspecified) increased to 24.3 days (14.5, 14.25, 18.5, and 16.4 days for the untreated group, free cargo, RGD-NPs and TGN-NPs, respectively)**	**(Shi et al., 2020)**
Liposomes	DOX	TfR	Tf protein	TfR	Tf protein	Median survival of U-87 MG GBM orthotopic mice model increased to 25 days (20, 22, and 24 days for the untreated group, free drug and non-functionalized liposomes, respectively)	(Wang, Zhao, et al., 2019)
				-	R8 CPP		
Liposomes	DOX and erlotinib	TfR	Tf protein	-	Pen CPP	Median survival of U-87 MG GBM orthotopic mice model increased to 36 days (22, 25, 25.5, 30, and 27.5 days for the untreated group, free drugs cocktail, non-functionalized liposomes, Tf-liposomes and Pen-liposomes, respectively)	(Lakkadwala et al., 2019)
Dendrimers	DOX	LDLR	ANG2 peptide	EGFR	EP-1 peptide	Median survival of U-87 MG GBM orthotopic mice model increased to 34 days (16, 19, 24, 31, and 27 days for the untreated group, free DOX, non-functionalized NPs, ANG2-NPs and EP-1-NPs, respectively)	(Liu et al., 2019)
Erythrocyte membrane-enveloped lipid NPs	Vincristine	TfR	T7 peptide	CD13 receptor	NGR peptide	Median survival of C6 GBM orthotopic mice model increased to 36 days (18, 19, 20, 29, and 21 days for the untreated group, free drug, non-functionalized NPs, T7-NPs and NGR-NPs, respectively)	(Fu et al., 2019)
Liposomes	5-flouoracil	TfR	Tf protein	-	Pen CPP	Lower cell viability (50%–60%) in a 3D in vitro tumor model compared with the free drug (90%), non-functionalized liposomes (80%), Tf-liposomes (70%) and Pen-liposomes (80%)	(Lakkadwala & Singh, 2018)
Dithiolane trimethylene	DTX	LDLR	ANG2 peptide	LDLR	ANG2 peptide	Median survival of U-87 MG GBM orthotopic mice model increased to 53 days (24, 30, 33, and 44 days for the untreated	(Zhu, Jiang, et al., 2018)
				-	TAT CPP		

TABLE 2 (Continued)

carbonate and PEG micelles						group, free DTX, non-functionalized NPs and ANG2-NPs, respectively)	
Mesoporous ruthenium inorganic NPs	RBT (attached via glutathione-cleavable bonds)	TfR	Tf protein	Nucleolin	AS1411 aptamer	Median survival of U-87 MG GBM orthotopic mice model increased to 40 days (22, 26, and 34 days for the untreated group, free drug and Tf-NPs, respectively)	(Zhu, Zhou, et al., 2018)
Liposomes	DOX	Dopamine receptors	pHA	αvβ3 integrins	RGD peptide	Median survival of U-87 MG GBM orthotopic mice model increased to 35 days (20, 23, 26.5, 30, and 28.5 days for the untreated group, free DOX, non-functionalized NPs, pHA-NPs and RGD-NPs, respectively)	(Belhadj et al., 2017)
		αvβ3 integrins	RGD peptide				
Liposomes	DOX	LDLR	Pep-22 peptide	αvβ3/αvβ5 integrins	RGD peptide	Median survival of U-87 MG GBM orthotopic mice model increased to 39.5 days (27, 30.5, 31.5, 34.5, and 36 days for the untreated group, free DOX, non-functionalized NPs, RGD-NPs and Pep-22-NPs, respectively)	(Chen et al., 2017)
		αvβ3/αvβ5 integrins	RGD peptide				
PLGA and PEG polymeric NPs	PTX	Heparan sulfate	CGKRK peptide	IL-13 α2 receptor	Pep-1 peptide	Median survival of C6 GBM orthotopic mice model increased to 61 days (17, 22, 24, 34, and 32 days for the untreated group, free PTX, non-functionalized NPs, CGKRK-NPs and Pep-1-NPs, respectively)	(Lv et al., 2016)
Lipid NPs	Model siRNA	LDLR	ANG2 peptide	LDLR	ANG2 peptide	U-87 MG GBM cells and bEnd.3 BBB cells in vitro uptake similar to non-functionalized NPs and MMP-activable glutamic acid residues-NPs	(Bruun et al., 2015)
				-	MMP-activable glutamic acid residues (endosomal escape)		
PCL and PEG polymeric NPs	DTX	LDLR	ANG2 peptide	-	MMP-activable R8 CPP	Median survival of C6 GBM orthotopic mice model increased to 32 days (19, 20, 26, and 27 days for the untreated group, non-functionalized NPs, R8-NPs and ANG2-NPs, respectively)	(Gao, Zhang, Cao, et al., 2014)
PCL and PEG polymeric NPs	-	αvβ3 integrins	RGD peptide	IL-13 α2 receptor	IL-13 peptide	Slightly higher brain accumulation in a C6 orthotopic GBM mice model compared with non-functionalized NPs, RGD-NPs and IL-13 peptide-NPs	(Gao, Xiong, Zhang, et al., 2014)
PCL and PEG polymeric NPs	DTX	αvβ3 integrins	RGD peptide	IL-13 α2 receptor	IL-13 peptide	Median survival of C6 GBM orthotopic mice model increased to 35 days (17, 20, 22, 27, and 26 days for the untreated group, free DTX, non-functionalized NPs, RGD-NPs and IL-13 peptide-NPs, respectively)	(Gao, Yang, et al., 2014)
Liposomes	DOX	TfR	T7 peptide	TfR	T7 peptide	Median survival of C6 GBM orthotopic mice model increased to 43 days (17, 22, 26, 35, and 28 days for the untreated group,	(Zong et al., 2014)
				-	TAT CPP		

(Continues)

TABLE 2 (Continued)

						free DOX, non-functionalized NPs, T7-NPs and TAT-NPs, respectively)	
Liposomes	DOX	TfR	Tf protein	Folate receptor	Folate	Median survival of U-87 MG GBM orthotopic mice model increased to 30 days (20, 24, and 27 days for the untreated group, free DOX and non-functionalized NPs, respectively)	(Gao et al., 2013)
PCL and PEG polymeric NPs	DTX	Not mentioned	TGN peptide	Nucleolin	AS1411 aptamer	Median survival of C6 GBM orthotopic mice model increased to 32 days (17, 18, 17, 25, and 25 days for the untreated group, free DTX, non-functionalized NPs, TGN-NPs and AS1411-NPs, respectively)	(Gao, Yang, et al., 2014; Gao et al., 2012)
PCL and poly(ethyl ethylene phosphate) micelles	PTX	TfR	Tf protein	αvβ3 integrins	RGD peptide	Median survival of U-87 MG GBM orthotopic mice model increased to 42.8 days (34.5, 33.5, 34.8, and 39.5 days for the untreated group, free PTX, non-functionalized NPs and Tf-NPs, respectively)	(Zhang et al., 2012)

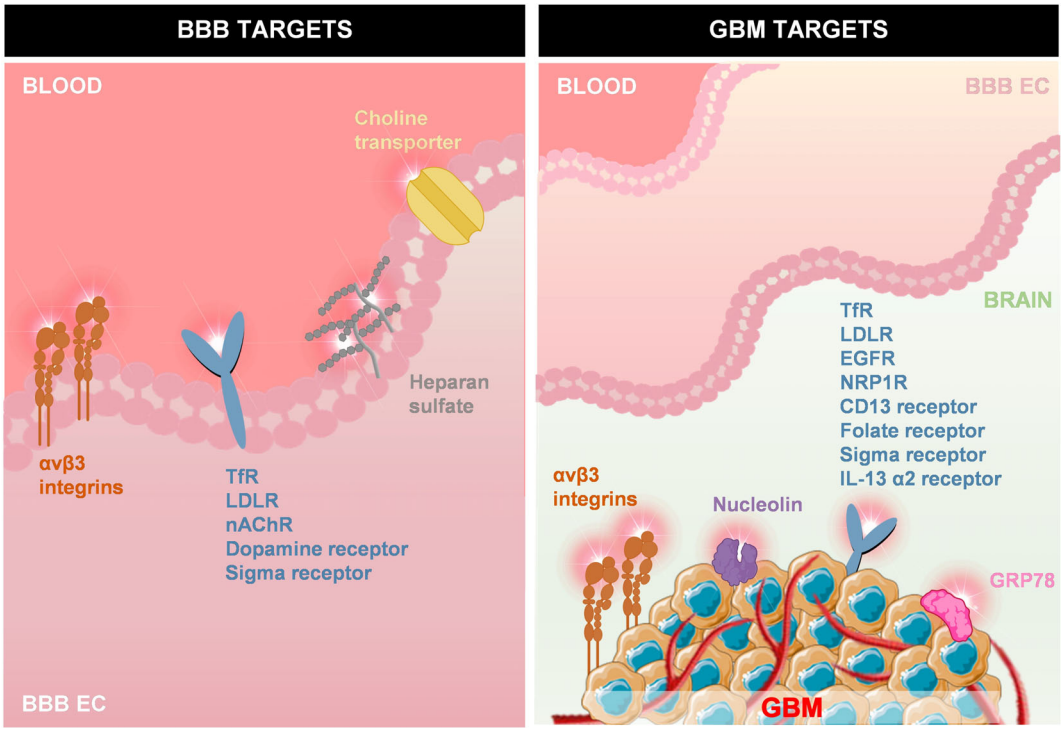

FIGURE 8 Main biological targets exploited in the development of multi-ligand functionalized, BBB and tumor dual-targeted nanomedicines for GBM therapies over the past 10 years.

different animal models which limits direct comparison of results, the median survival of animals was improved by an average of 10 days (multi-ligand vs. single-ligand functionalized nanosystems) (Gao, Yang, et al., 2014; Zhu, Jiang, et al., 2018; Farshbaf et al., 2022; Fu et al., 2019; Jin et al., 2021; Lakkadwala et al., 2019; Lu et al., 2022). In the best-case scenario, the median survival of animals could even be doubled by the use of multi-ligand functionalized nanosystems (Lv et al., 2016). In order to advance the development of these multi-ligand functionalized nanosystems, consequently achieving continuously better therapeutic outcomes, key aspects should be taken into consideration. A significant part of the revised multi-ligand functionalized nanosystems reported the inclusion of different CPPs for GBM cell accumulation. However, alternatives to CPPs should be considered as these peptides present lack of tumor cell selectivity and their cellular permeation depends on the type of cell membrane, therefore giving rise to off-target toxicity and inefficient delivery, respectively (Khan et al., 2021; Kim et al., 2021). In what concerns the ligand responsible for BBB targeting and transport, alternatives should focus on pathways that privilege transcytosis and avoid lysosomal degradation, such as based on caveolin-mediated transport through the use of the ANG2 peptide (Papademetriou & Porter, 2015; Sorets et al., 2020). This would allow to increase the success rate of blood-to-brain transport and, afterwards, nanosystem availability at the tumor site. There is great evidence that drug delivery from blood to brain can be enhanced through the use of BBB targeting ligands that suffer cleavage upon encountering the acidic pH of BBB endosomal vesicles (Wiley et al., 2013). The pH-triggered cleavage of these ligands during BBB transport avoids ligand-receptor high avidity, which is responsible for a faster degradation of the transported nanosystems within BBB endothelial cells. Whereas, the pH-triggered cleavage of the ligands favors ligand-receptor mid avidity, which increases the formation of syndapin-2 tubular structures and leads to a faster shuttling process across the BBB (Tian et al., 2020). As a final consideration, multi-ligand functionalized nanosystems that focus on pH-sensitive mechanisms to promote GBM cell accumulation should be carefully designed. This is due to the fact that GBM presents high heterogeneity regarding the areas of acidity within the TME (Akbari et al., 2021). Apart from inter-patient heterogeneity, local heterogeneity of tumor acidity within individual tumors has been observed in gliomas (Wang, Yao, et al., 2019).

5 | CONCLUSION AND OUTLOOK

Multi-ligand functionalized, BBB and GBM sequentially- and dual-targeted nanomedicines have shown promising capacity to precisely target (i) BBB cells, inducing transport form blood to brain, and (ii) GBM cells, leading to a higher

tumor therapeutic accumulation. The BBB and GBM sequentially- and dual-targeted nanomedicines herein revised demonstrated the ability to successfully penetrate the BBB and lower tumor cell viability in different in vivo testing models that emulate the GBM clinical scenario. Moreover, the anti-GBM therapeutic outcomes of these nanomedicines were generally superior compared with each of the single-ligand nanosystem control counterparts (targeted to either the BBB or GBM) and the free cargo controls. However, there is significant room for improvement, and the road to translation is still long and winding. Since GBM is characterized by a high level of molecular heterogeneity, thus leading to dynamic variations in the percentage of expression of the envisaged targets, it is necessary to ensure patient stratification prior to the treatment administration in order to make sure that the dual-targeting properties of the nanosystems are maintained. In addition, priority should be given to receptor or transporter targets that are less prone to saturation and rapidly recycled at the cell surface, which will lead to higher rates of transport across the BBB and tumor barriers. The development of the nanocarriers should be based on a careful design since the presence of more than one surface targeting ligand might cause phenomena of inter-steric hindrance, thus compromising the individual targetability of each one of the ligands. Herein, it should be of interest to play with polymer outer arms (e.g., PEG) to endow the nanocarrier surface with different length layers of each ligand or, as another alternative, develop stimulus-sensitive systems that eliminate the BBB-targeting ligand during blood-to-brain transport, in order to surface expose further ligands responsible for GBM targeting without hindrance constraints. Lastly, it is foreseen that future clinical approval of the first targeted nanomedicines will help to speed up the process of translation of more complex systems, such as these multi-ligand functionalized, dual-targeted nanomedicines.

AUTHOR CONTRIBUTIONS

Cláudia Martins: Conceptualization (equal); data curation (lead); investigation (lead); writing – original draft (lead).
Bruno Sarmento: Conceptualization (equal); supervision (lead); writing – review and editing (lead).

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CONFLICT OF INTEREST STATEMENT

The authors have declared no conflicts of interest for this article.

DATA AVAILABILITY STATEMENT

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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