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An EGFRvIII-targeted bispecific T-cell engager overcomes limitations of the standard of care for glioblastoma

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Abstract

While advanced surgical techniques, radiation therapy and chemotherapeutic regimens provide a tangible benefit for patients with glioblastoma (GBM), the average survival from the time of diagnosis remains less than 15 months. Current therapy for GBM is limited by the nonspecific nature of treatment, prohibiting therapy that is aggressive and prolonged enough to eliminate all malignant cells. As an alternative, bispecific antibodies can redirect the immune system to eliminate malignant cells with exquisite potency and specificity. We have recently developed an EGF receptor variant III (EGFRvIII)-targeted bispecific antibody that redirects T cells to eliminate EGFRvIII-expressing GBM. The absolute tumor specificity of EGFRvIII and the lack of immunologic crossreactivity with healthy cells allow this therapeutic to overcome limitations associated with the nonspecific nature of the current standard of care for GBM. Evidence indicates that the molecule can exert therapeutically significant effects in the CNS following systemic administration. Additional advantages in terms of ease-of-production and off-the-shelf availability further the clinical utility of this class of therapeutics.

Keywords

antibodies; antigens; cancer; CNS; immunotherapy

Glioblastoma (GBM), the most common primary malignant brain tumor, is an especially difficult-to-treat malignancy. The 5-year survival rate for patients with newly diagnosed

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GBM is less than 5% [1]. Despite an aggressive therapeutic approach including surgical resection, radiotherapy and chemotherapy, the median overall survival (OS) for patients with newly diagnosed GBM is less than 15 months [2].

Therapy for GBM is complicated by the highly malignant characteristics of the tumor and the delicate nature of the CNS, where the tumor occurs. Characterized by the WHO as a grade IV neoplasm [3], these tumors consist of diffusely infiltrating, poorly differentiated astrocytic cells. Regions of endothelial proliferation and necrosis, essential diagnostic features, further contribute to the devastating nature of the lesion. Unfortunately, these high-grade lesions most often occur in regions of the brain providing functionally critical connections, namely in the subcortical white matter of the cerebral hemispheres.

Since tumor cells spread out from the center of the tumor, implant into surrounding areas and intermingle with functionally important healthy cells, with conventional antineoplastic treatment, it is impossible to eliminate all cells of malignant potential while preserving functionally critical nontransformed tissue. Off-target toxicity due to the nonspecific nature of the current standard of care is a major limitation leading to premature withdrawal of treatment and disease progression.

In contrast, antitumor immunotherapy provides an exquisitely precise method to selectively eradicate cancer cells. By exploiting endogenous immune mechanisms, immunotherapy can target and destroy individual malignant cells while preserving intermixed and surrounding normal tissue. With the detailed mechanistic understanding of tumor immunology that researchers continue to unveil, this approach is now able to achieve great success. Recently, the US FDA approved the first immune-based therapeutics for tumors, sipuleucel-T and ipilimumab, for the treatment of hormone-refractory prostate cancer and metastatic melanoma respectively [4, 5]. Antitumor immunotherapy continues to garner much enthusiasm; clinical trials have demonstrated that forms of metastatic melanoma, renal cell carcinoma and non-small-cell lung cancer that are severely refractive to conventional treatment demonstrate extensive tumor shrinkage with immune modulatory therapeutics designed to antagonize PD-1 protein, a T-cell coinhibitory receptor [6, 7]. While no FDA-approved immune-based approaches currently exist for the management of GBM, antitumor immunotherapy stands to significantly improve the standard of care for patients with GBM.

Among the many hypothetical immunotherapeutic approaches for the management of GBM, one approach, the use of engineered bispecific antibodies, offers unique advantages. Bispecific antibodies can tether immune effector cells to tumor cells, allowing for a safe and highly effective cytotoxic response with exquisite neoplastic specificity. Evidence indicates that bispecific antibodies orchestrate a substantial antitumor response in the CNS following intravenous administration, allowing for minimally invasive therapy. Additionally, advantages in terms of ease-of-production and off-the-shelf availability further enhance the appeal of this class of drugs for the treatment of GBM. Taken together, these advantages place bispecific antibodies in a prime position for clinical implementation, potentially overcoming the limitations associated with the current standard of care for GBM.

Standard of care for glioblastoma

Long established as essential components of therapy, surgery followed by radiotherapy plays a critical role in the currently employed multimodal approach for the management of newly diagnosed GBM. In addition to allowing for histopathologic diagnosis and characterization, surgical resection alleviates devastating mass effect symptoms associated with the expanding space-occupying lesion located within the ridged cranium. Novel fluorescence-guided surgical techniques have proven beneficial; evidence suggests that more complete surgical resection made possible by 5-aminolevulinic acid-derived tumor fluorescence results in a significant progression-free survival (PFS) benefit [8]. Likewise, radiotherapy is an indispensable component of therapy long known to provide a significant survival benefit versus supportive care alone [9]. Advances in the ability to tailor the distribution of radiation to the irregular contours of a tumor with intensity-modulated or image-guided techniques allows physicians to minimize the dose of radiation to non-neoplastic tissue surrounding the tumor [10].

Chemotherapy also plays a prominent role in the currently employed multimodal standard of care for newly diagnosed GBM. In a landmark study, Stupp *et al.* reported the outcome of a multi-institutional, Phase III trial in which radiotherapy with concomitant and adjuvant temozolomide resulted in a significantly higher 2-year survival rate (26.5%) versus radiotherapy alone (10.4%) [11]. The orally active alkylating agent temozolomide exhibits a favorable toxicity profile in comparison with older chemotherapeutic agents. Unfortunately, despite an aggressive, multifaceted approach consisting of extensive surgical resection, state-of-the-art radiotherapy and multiple cycles of temozolomide chemotherapy, the median OS for patients with newly diagnosed GBM remains less than 15 months [2].

With the current standard of therapy consisting of surgery followed by radiotherapy with concomitant and adjuvant temozolomide, tumors invariably recur. Treatment upon recurrence is prescribed with consideration to previous therapy, performance status, time to recurrence and quality of life. In addition to the option of reemploying previously attempted modalities, bevacizumab, a humanized monoclonal antibody that targets VEGF, can be used alone or in combination with irinotecan or other agents. While bevacizumab therapy is attractive due to the vascular nature of GBM, the median OS documented in Phase II trials is only 10 months or less and considerable thromboembolic toxicity can occur [12, 13].

Currently employed standard-of-care treatment modalities provide a limited survival benefit due to their nonspecific nature. Although advances in therapy have resulted in some tangible benefit for patients with GBM, treatment-induced damage to normal tissue prohibits therapy that is aggressive and prolonged enough to achieve a cure. Adverse reactions induced by treatment further complicate the management of GBM. By contrast, a safe therapeutic that specifically eliminates tumor cells, leaving nontransformed tissue unharmed, could significantly improve outcomes for patients with GBM.

Antibodies as therapeutic molecules

Although long hailed as attractive candidates for biotherapeutics, antibodies required many developments in order to achieve clinical utility. Today, clinical application of antibody-

based biologics is burgeoning; in 2008, the total US sales of therapeutic monoclonal antibodies (mAbs) were approximately US\$15.5 billion, making this therapeutic class the highest selling class of biologics [14].

In the context of therapeutics, antibodies serve as highly useful biomolecules due to the exquisite specificity and strength of binding imparted by antigen recognition domains. The advent of hybridoma technology by Kohler and Milstein in 1975 paved the road for the advancement of this class of molecules [15]. Prior to this breakthrough, antibodies were obtained from the sera of animals immunized with an antigen. While mAbs of interest could be produced in this manner, other proteins (antibodies and otherwise) also present in the sera elicited allergic reactions and prohibited therapeutic use. Through the use of hybridoma technology, however, B-lymphocytes from the spleen of immunized mice could be immortalized, resulting in the production of unlimited quantities of mAbs [16].

While hybridoma technology allows for the production of high-purity mAbs, patients treated with murine antibodies produced in this fashion often develop a human antimouse antibody response, severely limiting therapeutic utility due to rapid clearance from the patient's serum [17]. Multiple strategies reviewed by Lonberg exist to circumvent this problem [18]. These include the production of chimeric and humanized antibody molecules through various molecular biology techniques designed to replace segments of murine antibodies with human antibody counterparts and, more recently, the production of fully human antibodies using phage display technology and transgenic mouse platforms. Panitumumab and adalimumab, derived from phage display technology and a transgenic mouse platform respectively, represent the first fully human mAbs to receive FDA approval [19, 20]. In a Phase III clinical study, none of the 231 patients treated with panitumumab developed detectable levels of antidrug antibodies [21]. More recent advancements allow for high-throughput automation and direct selection of fully human antibodies *in vitro* through the use of mRNA and ribosome display [22].

Despite success in production of fully human mAbs, their use in cancer therapy still faces challenges. The Fc portion of mAbs can bind to Fc γ RIIb on monocytes, macrophages and neutrophils, resulting in signals inhibitory to effector function. Likewise, Fc binding to Fc γ RIIIb on neutrophils does not trigger cytotoxicity. Furthermore, human IgG1 is known to bind to noneffector cells including platelets and B cells, which can act as an antibody sink and perhaps contribute to adverse therapeutic effects [23].

For the treatment of GBM, if intended for systemic administration, mAbs face further issues with delivery to neoplastic tissue located behind the blood–brain barrier (BBB), especially delivery to the most invasive margins of the tumor which are thought to reside behind a fully intact BBB. Generally, the rate of protein diffusion into the CNS varies inversely with the molecular weight of the protein. Accordingly, the cumbersome size of mAbs severely precludes efficient delivery. Further impeding delivery, the neonatal Fc receptor (FcRN) is highly expressed in the CNS endothelium and choroid plexus [24] and likely mediates reverse transcytosis of antibodies from the CNS back into circulation [25].

In order to circumvent these problems, scientists can piece together a wide variety of antibody fragments which, although smaller, maintain exquisite antigen-binding strength and specificity (Figure 1). The smallest fragment imparting antigen specificity, the Fv fragment, consists of an association between the variable heavy (V_H) and variable light (V_L) chains, although this structure lacks stability since the hydrophobic interactions between these two fragments are weak [26, 27]. In order to increase stability, a covalent peptide linker of 15–20 residues can be introduced between V_H and V_L , resulting in what is termed a single-chain Fv fragment (scFv) [28]. The introduction of disulfide bonds between V_H and V_L is an additional approach that also increases stability [29]. A larger fragment, termed the Fab fragment, consists of an entire single light chain and the Fd chain (N-terminal half of the heavy chain) [27]. Advantages of this larger fragment include longer time to clear from the blood, increased stability and presence of a conserved CH1 domain that can be used for detection [30]. Finally, fusing a CH3 domain to a scFv or Fab fragment can restore bivalency in molecules termed minibodies [31]. However, just as with full mAbs, in the context of systemic administration for the treatment of intracerebral tumors, larger constructs are more likely to face issues with efficient tumor penetration.

Engineering bispecific antibodies

In addition to chiseling away superfluous or undesirable fragments, molecular engineers can couple mAb fragments of different specificities in what are termed bispecific antibodies. This class of molecules holds great potential to enhance therapeutic functionality in comparison with standard single antigen-binding antibodies or fragments thereof. In the most general sense, a bispecific antibody consists of a class of constructs in which two antibody-derived antigen-specific binding sites are joined within one molecule [32].

In order to promote an antitumor immune response, bispecific antibodies are designed to simultaneously bind to receptors on the surface of immune effector cells (effector-binding arm) and to transmembrane molecules on the surface of cancer cells (target-binding arm). Such an engineered bispecific antibody consequentially redirects immune effector cells to neoplastic cells, and if the proper targets are selected, directs the activity of effector cells only while tethered to a neoplastic cell.

Bispecific scFvs (bi-scFvs), a highly successful bispecific antibody format, consists of two scFv fragments translated in tandem and linked with a short flexible peptide linker (Figure 1) [33]. For example, as in the initial description of the construct, a single amino acid chain translated in tandem can encode for a scFv imparting specificity for CD3, the signal transduction element of the T-cell receptor (TCR), a peptide linker composed of 5 flexible residues (usually G_4S) and a scFv imparting antigen specificity for a transmembrane protein found on the surface of cancer cells. This highly potent molecule creates an immunological synapse between $CD3^+$ T cells and cancer cells containing the targeted transmembrane protein. T-cell-induced cytotoxicity ensues, only upon binding to both targets and formation of an immunological synapse, avoiding activity against any cell without the targeted transmembrane protein.

Bispecific diabodies (Figure 1), another bispecific antibody format to achieve success, consists of a V_H fragment from one monoclonal antibody (e.g., mAb A) translated in tandem with a short linker (five amino acids) and a V_L fragment from another monoclonal antibody (e.g., mAb B). The short linker prevents dimerization. The V_H segment from mAb B is similarly translated in tandem with the V_L segment from mAb A. Coexpression of these two fragments results in hydrophobic interactions between V_H and V_L fragments from the same monoclonal antibody and the formation of a heterodimeric bispecific molecule [34]. Although different in construction, bispecific diabodies can induce cellular responses similar to those induced by bi-scFvs.

While few studies have directly compared bi-scFvs and diabodies, it is clear that functional differences exist as a result of differences in construction. While the tightly packed diabody format may exhibit increased protein stability, rotation at the linkers is limited as a result of noncovalent interactions between V_H and V_L segments. In contrast, bi-scFvs consists of two binding sites free to rotate relative to each other, offering an advantage in allowing increased access to epitopes of interest at cell surfaces. In one side-by-side comparison, both bispecific antibody formats designed to target carcinoembryonic-antigen-expressing tumor cells resulted in indistinguishable biological activity and binding kinetics *in vitro* [35]. On the other hand, another study found that for bispecific antibodies targeting EGF receptor (EGFR), bi-scFvs resulted in equivalent binding capability but drastically increased cytotoxic function in comparison with diabodies [36]. It is likely that the ultimate therapeutic activity of a given format would vary based on the specific targets and the context of administration.

In order to realize clinical utility of a given bispecific antibody construct, it is also important to consider the potential for scale up in production. In the first preclinical iterations, bispecific antibodies were produced in *Escherichia coli*, likely owing to the historically low cost and ease of producing proteins in bacterial systems. However, in bacterial expression systems, bispecific antibodies often fail to fold properly, precipitate in inclusion bodies, are consequentially must be subjected to complex renaturing protocols in order to attain functional, soluble proteins. Some researchers have attempted varying the order of V_H and V_L segments in the bi-scFv bispecific antibody format, finding that functional protein can be attained with some arrangements but not others [37]. Still, the amount of functional bispecific protein produced in a bacterial system is usually exceedingly low, precluding cost- and time-effective clinical production.

As a practical solution, use of a mammalian expression system, such as the commonly used *Cricetulus griseus* (CHO) expression system, results in greater yield of functional protein and ultimately a more cost- and time-effective method for clinical production. In one study of a bi-scFv targeting CD3 and the epithelial 17-1A antigen, a bacterial expression system failed to produce functional protein while the same construct was easily produced in CHO cells [38], a system in which soluble, fully folded protein was secreted directly into protein-free cell culture media. In addition to allowing for production of functional protein when bacterial systems fall short and avoiding the need for tedious renaturing protocols, the CHO system results in greater yield of protein and a more cost-effective method of production.

With a mammalian expression system one easily scale up manufacturing to produce large amounts of protein in bioreactors.

Bispecific antibodies require access to tumor & immune effector cells for a therapeutic effect

In the context of treating intracerebral tumors, successful application of bispecific antibody immunotherapy faces unique challenges. In order to induce an effective antitumor response, bispecific antibodies require access to both tumor and effector immune cells. Historically, effector immune cells and proteins in the intravascular compartment were thought to have limited access to the CNS. Recently, however, much evidence has accumulated indicating that these essential components do in fact routinely gain access to the CNS from the intravascular compartment and once within the CNS play a functionally significant role.

Functionally active T cells traffic to the CNS

In the absence of inflammatory disease, activated T cells routinely enter the CNS. This finding was initially surprising given the anatomical structure of the BBB. The BBB consists of a complex network of interendothelial tight junctions, similar to those between epithelial cells, and also of associated pericytes and basal lamina. In the CNS parenchyma, tightly apposed astrocyte foot processes provide a further restrictive barrier. This highly regulated association between specialized endothelial cells, pericytes and astrocytes serves to patrol and often, from a therapeutic perspective, restrict access to the CNS from the intravascular compartment. Regardless, in the absence of inflammation, a factor known to alter BBB physiology, activated T cells routinely traffic to the CNS.

First demonstrated in animal models, activated, radiolabeled T cells specific for myelin basic protein (MBP), an antigen found in the CNS, trafficked to the CNS following intravenous injection. Naive radiolabeled T cells with the same specificity for MBP did not enter the CNS, however, suggesting that BBB T-cell penetration is possible only after T-cell activation [39]. In a more recent study, the capacity for T cells to enter the CNS was less dependent on activation status and attained only after transient passage through the lung tissues. Before reaching the CNS, investigators observed T cells moving along the airways to bronchus-associated lymphoid tissues and lung draining mediastinal lymph nodes prior to reaching systemic circulation from where they reach the CNS. In the lungs, these CNS-destined T cells drastically altered their gene-expression profile, downregulating their activation program and upregulating cellular locomotion molecules and chemokine and adhesion receptors. Ninjurin 1 for example, an adhesion receptor which mediates intravascular T-cell crawling on cerebral blood vessels was significantly upregulated in the lung tissues. Interestingly, after passage through the lung tissues, both resting and activated T cells proliferated, assumed migratory properties, and ultimately entered the CNS where they induced significant effects [40].

Alternate routes for T-cell access to the CNS are possible. Activated memory T cells likely enter the CNS cerebrospinal fluid (CSF) directly from systemic circulation via the large venules in the choroid plexus and subarachnoid space. These cells are thought to play an

important role in routine CNS immuno-surveillance. Between 1000 and 3000 leukocytes per ml can be found in the CSF of healthy individuals, the majority of which are lymphocytes. Given that T cells represent over 80% of the leukocytes normally found in the CSF, but only 5% of the circulating leukocytes in blood, this suggests that a mechanism exists to preferentially traffic T cells from the periphery to the CNS. Upon characterization of the surface phenotype of CSF cells in healthy individuals, the vast majority of cells within the CSF consist of activated central memory T cells ($CD4^+/CD45RA^-/CD27^+/CD69^+$) expressing high levels of P-selectin glycoprotein ligand 1. The presence of P-selectin immunoreactivity in large venules of the choroid plexus and subarachnoid space but not in CNS parenchymal microvessels is indicative of a potential mechanism for selective trafficking [41]. Thought to play a critical role in the CSF, activated memory T cells retain their capacity to initiate local immune reactions.

Although new evidence continues to indicate that, in the absence of inflammation, cellular components of the immune system traffic into the CNS and can be exploited therapeutically, it has long been known that in the setting of frank inflammation, such as that which occurs in cancer, the BBB undergoes changes that alter its ability to block migration of immune effectors into the CNS [42]. Perhaps the strongest evidence for this comes from a number of recent studies demonstrating that GBM lesions are infiltrated by effector immune cells and that the degree of intratumoral infiltration of $CD8^+$ cytotoxic T cells predicts favorable outcomes in patients with GBM [43, 44]. It is likely that in disease states such as GBM, changes to normal physiology lead to an increased T-cell infiltrate into the CNS, leading to the possibility of increased T-cell-mediated immune responses in the CNS.

Peripherally administered antibodies gain access to the CNS & mediate therapeutically significant functions

In addition to evidence of cellular immunity in the CNS, evidence continues to accumulate supporting the notion that therapeutic antibodies can gain access to the CNS from the intravascular compartment and mediate therapeutically significant functions once in the CNS. Conventionally, antibodies are thought to poorly penetrate the BBB into the CNS; in healthy individuals, antibody titers measured in the CSF are relatively low in comparison with those measured in peripheral blood. For example, the physiologic CSF to serum ratio for IgG ranges from 0.16 to 0.32%. Despite this relative paucity, antibodies can be detected and are functionally important within the CST and CNS parenchyma after either passive or active immunization.

Many studies document significant therapeutic responses despite relatively small amounts of antibody infiltration into the CNS. Important for the treatment for GBM, research indicates that in the context of intracerebral tumors, peripherally administered antibodies access the intracerebral environment and exert therapeutic outcomes. In preclinical GBM models, the antitenascin antibody 81C6, an antibody directed against a stromal component of GBM, demonstrated therapeutic activity following systemic administration, and in patients with intracranial malignancy, peripheral administration of ^{131}I -labeled 81C6 resulted in selective tumor localization [45]. Still, tumor-specific uptake of the antibody was low (less than 5×10^{-3} of the injected dose per gram) and the antibody accumulated in other tissues including

the liver, spleen and bone marrow. These findings may be in part due to the physiologic presence of tenascin in non-neoplastic peripheral tissue. In contrast, clinical assessment of the ch806 antibody against epidermal growth factor receptor variant III (EGFRvIII), an antigen found exclusively on the surface of GBM, demonstrated a higher percentage of BBB penetration following systemic administration (Figure 2) [46], suggesting that in the absence of crossreactivity with systemic epitopes, an effective ‘antibody sink’ located within the CNS results in enhanced antibody BBB penetration.

EGFRvIII provides an ideal bispecific antibody target-binding arm for treating GBM

Among the many genetic alterations present in GBM samples, modifications to the *EGFR* proto-oncogene are of particular therapeutic relevance. In GBM samples, the *EGFR* gene is amplified in up to 50% of the cases and overexpressed in over 90% of the cases [47, 48], leading to disruption of ordinarily strictly controlled cell growth pathways. Amplification and overexpression of *EGFR* in GBM is considered a poor prognostic indicator. Furthermore, structurally rearranged aberrant forms of the *EGFR* gene play a significant role in GBM pathophysiology. Mutations to the *EGFR* gene are widely reported in the literature and are typically associated with extensive amplification of the *EGFR* locus [49, 50].

EGFRvIII, a rearranged variant of EGFR, is frequently expressed on the surface of GBM specimens [51], approximately 31% and up to 50% in some studies [52], as well as other common neoplasms including breast and lung carcinoma [53], but entirely absent from normal tissue. Among GBM samples expressing EGFRvIII, 37–86% of the cells express the rearranged receptor [52], suggesting that the aberrant EGFRvIII receptor is translated with at least some level of homogeneity. The *EGFRvIII* mutation results in an in-frame deletion of 801 base pairs coding for the extracellular portion of the wild-type receptor. Upon translation, the deletion produces a novel glycine residue at the fusion junction of the rearranged protein. The novel glycine residue and proximity of ordinarily distant parts of the receptors extracellular domain produce a cell-surface, highly immunogenic, tumor-specific epitope [54]. Importantly, anti-bodies directed against EGFRvIII do not crossreact with the wild-type receptor.

EGFRvIII plays a significant role in the pathobiology of GBM. As a consequence of the deletion, the EGFRvIII tyrosine kinase receptor is constitutively active, and in patients, EGFRvIII results in tumorigenic signaling and a more aggressive tumor phenotype linked to poor survival [55]. In a study investigating the effect of EGFRvIII expression among 196 patients with GBM, expression of the rearranged receptor was an independent negative prognostic indicator in patients surviving 1 year or longer [56]. EGFRvIII rearrangement heightens cell growth and migration [57, 58] and confers enhanced resistance to chemotherapy [59, 60] and radiation [61]. Imparting additional aggressive characteristics, the rearranged tyrosine kinase receptor enhances cell growth of neighboring EGFRvIII-negative tumor cells via IL-6 family cytokine-mediated paracrine signaling [62]. These malignant characteristics propagate further through the release of lipid-raft-related microvesicles which contain a functional EGFRvIII receptor. These lipid-rafts often merge with the plasma membranes of EGFRvIII-negative tumor cells resulting in the transfer of an

oncogenic, functionally active receptor [63]. Recent research has also found that EGFRvIII is expressed in glioma stem cell lines [64], an important consideration given the paradigm that tumor stem cells represent a subpopulation of cells that give rise to all cells in a differentiated tumor [65]. As a result of its exquisite tumor-specificity, its clonal expression on the surface of transformed cells, and its importance in the pathobiology of GBM, the EGFRvIII mutation provides an ideal target for bispecific antibody immunotherapy for GBM.

A bi-scFvs directed against CD3 & EGFRvIII induces potent & specific T-cell-mediated cytotoxicity

A bi-scFv that binds to CD3 and a tumor antigen, also known as a bispecific T-cell engager (BiTE), serves as a small molecular tether capable of inducing immunological synapses between CD3⁺ T cells and targeted tumor cells that are indistinguishable in size, composition and subdomain arrangement from native cytolytic synapses [66]. Following BiTE-mediated synapse formation, tumor-cell apoptosis follows via T-cell release of perforin and granzyme proteases [67]. Upon binding to both targets, BiTEs also induce T-cell proliferation/cell cycling, secretion of inflammatory cytokines including INF- γ , TNF- α , IL-2, IL-4, IL-6 and IL10 and T-cell expression of CD69 and CD25 activation markers [68]. While proliferation is most prominent among the CD8⁺ T-cell compartment, CD4⁺ T cells also contribute a delayed response by dramatically upregulating granzyme B expression [68, 69].

BiTEs are highly potent molecules that can trigger specific lysis of tumor cells even at low picomolar concentrations [70]. Video-assisted microscopy has demonstrated that BiTEs alter the motility and activity of T cells from scanning mode to killing mode, allowing individual T cells to sustain serial rounds of target cell lysis. Within a 24-h period, complete elimination of target cells was observed at effector-to-target cell ratios as low as 1:5 [71]. Importantly, however, BiTE induced T-cell activity displays exquisite specificity. Even at concentrations 3–6 orders of magnitude greater than concentrations for half maximal T-cell activation, no cytolytic activity, T-cell proliferation/cell cycling, secretion of inflammatory cytokines or induction of activation markers was observed unless cells expressing the target antigen were present [72].

By globally interacting with the invariant CD3 signaling complex, BiTEs circumvent ordinary clonotypic T-cell specificity, potentially allowing any T cell, regardless of endogenous specificity or phenotype, to exert an antineoplastic effect. *In vivo* experiments show that BiTEs can reactivate potentially unresponsive, anergic T cells, such as those frequently encountered among tumor infiltrating lymphocyte populations [73]. It is possible that even Tregs, known to play an immunosuppressive role in GBM, could be redirected to kill cancer cells. Furthermore, by interacting with tumor cells via scFv induced specificity to an antigen expressed on the surface of tumor cells, ordinary MHC presentation requirements are circumvented [66], providing a mechanism to overcome tumor immune escape via loss or downregulation of MHC.

As an example of the clinical utility of BiTE technology, blinatumomab, a CD3 engaging bi-scFv that targets CD19 receptors on B cells, is currently in late-stage clinical development for patients with non-Hodgkin's lymphoma and acute lymphoblastic leukemia. In patients with non-Hodgkin's lymphoma, doses as low as 0.005 mg per square meter per day resulted in elimination of malignant cells in blood. Slightly higher doses led to partial or complete tumor regression and at a dose of 0.05 mg per square meter per day all patients (n = 7) experienced tumor regression in the blood as well as bone marrow and liver [74]. The exceedingly low-dose used to demonstrate clinical efficacy exemplifies the high potency of bispecific antibodies. Interestingly, although the target CD19 is not known to be expressed in the CNS, some patients treated with blinatumomab experienced transient and reversible CNS related side effects, which in the context of bispecific antibody therapy for intracranial tumors is supportive of the concept that bispecific antibodies, activated T cells, or both may enter the CNS parenchyma and exert clinically significant effects.

For the treatment of GBM, our group has developed a bi-scFv that binds to the invariant CD3 signaling complex on T cells and EGFRvIII on GBM cells (bscEGFRvIIIxCD3) [75]. While BiTEs against tumor-associated antigens including CD19, EpCAM and EGFR have proven therapeutically effective due to the ability to potently and specifically redirect T cells as described above, since these targets are not strictly found only in tumors, these constructs have led to unwanted destruction of normal, healthy cells [74, 76, 77]. In contrast, the absolute tumor specificity of EGFRvIII entirely averts these complications. Furthermore, the extracellular domain of EGFRvIII is relatively small, providing an ideal target for the BiTE platform since cells expressing smaller target surface antigens are generally better lysed by BiTE technology in comparison to cells expressing larger target surface antigens [78].

In vitro, the bscEGFRvIIIxCD3 construct bound to CD4⁺ and CD8⁺ human T cells known to express CD3, as well as to EGFRvIII-positive glioma cells, but did not bind to cells bearing only the wild-type EGFR. Upon binding to both targets, the construct resulted in potent tumor cell lysis, T-cell proliferation, secretion of Th1-type cytokines and upregulation of T-cell activation markers. Importantly, cells expressing only the wild-type EGFR failed to induce any of these findings.

In vivo, the bscEGFRvIIIxCD3 construct showed exquisite efficacy, specificity and potency (Figure 3). Systemic administration of the bi-scFv produced durable, complete cures in up to 75% of mice with established EGFRvIII-expressing intracerebral tumors, while no effect was observed among treated mice with intra-cerebral tumors lacking EGFRvIII expression. Dose-dependent effects indicative of the high potency of the construct were also observed *in vivo*; a cumulative dose that is roughly equivalent to 0.02 mg/kg for the average 60-kg adult significantly prolonged survival. Significant antitumor effects were also observed following treatment of very late-stage, well-established tumors, just days before death of controls. The ability to effectively treat established intracerebral tumors in preclinical models with a systemically administered therapeutic represents a significant therapeutic advancement.

While formal toxicity studies are currently underway in preparation for clinical trials, no apparent toxicity has been detected to date. The absolute tumor specificity of the bscEGFRvIIIxCD3 construct eliminates the potential for any crossreactivity with normal,

healthy cells. We have also demonstrated an effective ‘antidote’ for any potential toxicity that may result from administration of the bscEGFRvIIIxCD3 protein in a clinical setting. By administering a short peptide that spans the EGFRvIII mutation (PEPvIII), we have effectively blocked bispecific antibody function both *in vitro* and *in vivo*, providing a tool highly likely to aid in safe clinical administration of any EGFRvIII-targeted bispecific antibody.

Expert commentary & five-year view

While advances in surgical techniques, radiation therapy and chemotherapeutic regimens have provided tangible benefits for patients with GBM, the nonspecific nature of these therapies prohibits treatment prolonged and aggressive enough to achieve a cure. By effectively inducing lysis of only EGFRvIII-expressing cells, EGFRvIII-targeted BiTE therapy offers an attractive solution.

The finding that a systemically administered bispecific antibody effectively treats established intracranial tumors in preclinical models represents a significant advancement in the field and, if confirmed in the clinical setting, will allow for highly effective, minimally invasive treatment for GBM. Evidence supports the notion that systemically administered EGFRvIII-specific antibodies have the ability to accumulate in EGFRvIII-expressing tumors within the brain, a result which may be due in part to exclusive expression of the target antigen within the malignancy creating an effective ‘antigen sink’. The significantly reduced molecular weight of BiTEs in comparison with full mAbs also enhances their ability to localize to intracerebral tumors. Moreover, given the relative potency of BiTE technology, it is likely that only a small amount needs to reach the tumor to mediate significant therapeutic effects.

Future work will focus on safe clinical implementation of EGFRvIII-targeted BiTE therapy for the treatment of GBM. The absence of EGFRvIII-targeted BiTE crossreactivity with the unmutated receptor and the strict need for the therapeutic to bind to both target antigens to induce any effect on T cells, coupled with the absolute tumor cell specificity of EGFRvIII expression, will avert any drug induced damage to healthy tissue. While preclinical assessment of bispecific constructs in immunocompromized mice offered a distinct advantage as molecules identical to those destined for the clinics were used to assess responses against human tumors, studies with newly available transgenic mice expressing functional human CD3 receptors will likely provide an additional indication of safety as well as efficacy in preparation for clinical trials. Furthermore, soluble PEPvIII peptide can be administered to selectively block the effects of the EGFRvIII-targeted BiTE, offering clinicians a tool to further enhance safety.

Advances in construct design and production will also likely facilitate clinical implementation. For example, fully human antibodies will allow for the production of an EGFRvIII-targeted BiTE that is much less likely to produce antidrug antibodies, increasing therapeutic efficacy. Additionally, manufacturing the drug in a mammalian expression system will increase production yield and allow for straightforward integration with commonly used manufacturing infrastructure.

Further investigation will also be required to determine whether EGFRvIII-targeted BiTE-induced therapeutic effects are maintained in the context of standard-of-care therapy for GBM. Radiation and temozolomide chemotherapy, proven to provide a survival benefit for patients with GBM, carry a major side effect of lymphopenia. Still, given the low effector-to-target cell ratios required to observe therapeutic responses, it is likely that BiTE therapy would maintain efficacy in the context of standard-of-care therapy for GBM. Multiple immunotherapeutic approaches have proven efficacious in the context of lymphopenia [79–81], likely a result of lymphopenia-induced homeostatic cytokines that reduce the activation threshold and induce the proliferation of T cells [82].

We believe that the use of an EGFRvIII-targeted BiTE offers unique advantages which allow for safe and effective clinical implementation. While other EGFRvIII-targeted immunotherapeutic approaches including dendritic cell vaccines or genetically modified T cells have demonstrated that immunologic targeting of EGFRvIII is safe and effective [64, 83], the technically difficult and laborious process required to produce autologous vaccines greatly enhances the appeal of EGFRvIII-targeted BiTE therapy in comparison. It is further possible that by using a therapeutic to directly engage cellular components of the immune system with EGFRvIII-positive tumor cells, an endogenous immune response against other tumor antigens may ensue, mitigating tumor recurrence. Taken together, the safety, efficacy and off-the-shelf availability of EGFRvIII-targeted BiTE therapy is likely to offer patients with EGFRvIII-positive GBM an attractive therapeutic approach.

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- of interest
- of considerable interest

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Key issues

- Advances in state-of-the-art surgical techniques, tailored distribution of radiotherapy and clinically verified chemotherapeutic regimens provide tangible benefits to patients with glioblastoma (GBM). Despite implementation of these advances, patients with newly diagnosed GBM survive an average of less than 15 months.
- Current standard of care for GBM is limited by the non-specific nature of treatment, which causes damage to healthy cells in addition to tumor cells, preventing therapy that is aggressive and prolonged enough to achieve a cure.
- Functionally active T cells routinely traffic to the CNS and are capable of mediating therapeutically significant effects in the CNS. GBM lesions are highly infiltrated by effector immune cells.
- Evidence suggests that systemically administered antibodies gain access to the CNS, a finding that is more pronounced when the target antigen is absent from peripheral tissue.
- EGFRvIII is commonly expressed on the surface of GBM (approximately 31% of cases) as well as on the surface of breast and lung carcinomas. The mutation contributes to increased malignant characteristics and a worse prognosis. The absolute tumor specificity of the mutation and the absence of immunologic crossreactivity with the wild-type receptor make EGFRvIII an ideal candidate for antitumor immunotherapy.
- Bispecific antibodies form a molecular tether by combining two antibody-derived antigen-specific binding sites within one molecule. BiTEs represent a subset of bispecific antibodies capable of inducing immunologic synapses between CD3⁺ T cells and a targeted antigen.
- Upon synapse formation, BiTEs induce highly effective target cell cytotoxicity, T-cell proliferation, secretion of inflammatory cytokines and upregulation of T-cell activation markers.
- BiTEs fail to exert any effect on T cells in the absence of the targeted tumor antigen.
- Systemic administration of an EGFRvIII-targeted BiTE safely produced durable, complete cures in murine models with established intracerebral EGFRvIII-expressing tumors.
- Antitumor effects with an EGFRvIII-targeted BiTE were observed even following treatment of late-stage, well-established intracerebral tumors in murine models.
- The relative ease of production and off-the-shelf availability enhance the appeal of this class of therapeutics.

- Clinical trials are necessary to continue to verify the safety and efficacy of EGFRvIII-targeted BiTEs for the treatment of GBM.

Figure 1. Schematic representation of several different formats of Ig fragments

The IgG is composed of two identical heavy chains (VH+CH1+CH2+CH3) and two identical light chains (VL+CL). Both chains are organized as domains containing about 110 amino acids with each domain possessing an intradisulfide bond (not shown). Interdisulfide bonds (red lines) link the light chain to the heavy chain and the two heavy chains together. The variable domains (VH and VL) contain the complementary determining regions which bind to the antigen (complementary determining region represented by small dots). The Fv fragment (VH and VL domains) possesses the binding activity. The scFv corresponds to the VH linked to the VL by a flexible peptide linker (black lines). Bi-scFvs and bispecific diabodies can be obtained by using short linkers (represented by black lines). Blue: heavy chain originating fragments; purple: light chain originating fragments; yellow and green: variable heavy and light chains imparting second specificity. Reproduced with permission from [30].

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Figure 2. ch806 targeting of glioma

(A–C) Planar images of the head and neck of patient 8 obtained on day 0 (A), day 3 (B) and day 7 (C) after infusion of ^{111}In -ch806. Initial blood pool activity is seen on day 0, and uptake of ^{111}In -ch806 in an anaplastic astrocytoma in the right frontal lobe is evident by day 3 (arrow) and increases by day 7. (D–F) Specific uptake of ^{111}In -ch806 is confirmed in SPECT image of the brain (D) (arrow), at the site of tumor (arrow) evident in ^{18}F -FDG positron emission tomography (E) and MRI (F). FDG: Fluorodeoxyglucose; SPECT: Single-photon emission computed tomography. Reproduced with permission from [46].

Figure 3. Antitumor response produced by bscEGFRvIIIxCD3 is specific to EGFRvIII-expressing tumors *in vivo*

NSG mice ($n = 8$) were implanted i.c. with 1×10^5 tumor cells and unstimulated human PBMCs at a ratio of 1:1. Mice implanted with U87MG (A) or U87MG. EGFR (B) were treated with bscEGFRvIIIxCD3 or control bscAbxCD3 by daily intravenous infusion (arrows). (C) (Upper) To assess dose-response, bscEGFRvIIIxCD3 was administered to mice at indicated doses. (C) (Lower) For delayed treatment, mice were implanted i.c. with U87MG. EGFR alone, and treatment was started on day 10 after tumor implantation. These

mice were immune-reconstituted peripherally with 2×10^7 unstimulated human PBMCs by intraperitoneal injection on day 10, followed by infusion with bscAb. **(D)** (Upper left) Representative section of i.c. tumor and local invasion surrounding an adjacent vessel from an animal receiving PBS. **(D)** (Upper right) In contrast, in mice treated with bscEGFRvIIIxCD3, PBMCs are seen to exit peritumoral i.c. vessels toward tumor tissue (box). **(D)** (Lower) Tumor tissue in mice treated with bscEGFRvIIIxCD3 and receiving PBMCs intraperitoneally shows diffuse tumor-associated mononuclear infiltrate and areas of necrosis (arrows).

EGFR: EGF receptor; i.c.: Intracerebral; NSG: NOD *scid* gamma; PBMC: Peripheral blood mononuclear cell; PBS: Phosphate buffered saline. Reproduced with permission from [75].