A Novel Antibody-Drug Conjugate to Inhibit Glioblastoma Multiforme Progression

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01. Abstract

Glioblastoma multiforme (GBM) is an incurable brain tumor characterized by aggressive progression and frequent relapses. The average life expectancy for GBM patients is 9 months (Brown et al., 2022), with only 3 to 5 percent of patients surviving longer than 3 years (Mohammed et al., 2022). Although multiple chemotherapies and immunotherapies are available to potentially prolong the life of patients with GBM, the prognosis remains extremely poor. The two biggest challenges in treating glioblastoma multiforme are eliminating dormant, cancerous glial cells left behind after surgery and transporting therapeutic molecules across the highly selective blood-brain barrier.

The constantly evolving field of nanomedicine has shown great promise in addressing these challenges. Antibody-drug conjugates, or ADCs, consist of a combination of a monoclonal antibody (mAb) covalently linked to a cytotoxic payload molecule (Fu et al., 2022). When used to inhibit cancer progression, the antibody specifically binds to antigens overexpressed on the surface of tumors, selectively destroying cancer cells and preserving healthy tissue. While ADCs have been approved to treat breast cancer (Mark et al., 2023), there is minimal research on how they can be used to safely combat GBM. In this paper, we discuss the design of a novel antibody-drug conjugate targeting IL13Rα2 and suggest a method of delivery specific to GBM.

02. Introduction

2.1 What is Glioblastoma?

GBM, also known as grade IV astrocytoma, is a malignant, aggressively spreading tumor of the brain. GBM is the most common primary malignant brain tumor, accounting for 16% of all primary brain and central nervous system (CNS) tumors. The estimated prevalence of GBM is 3.19 cases in 100,000 people, and the median age of diagnosis is 64 years old (Thakkar et al., 2014).

GBM arises primarily due to genetic mutations in stem cells destined to differentiate into astrocytes, a subtype of glial cells that provide structure and support for the brain's neurons. Risk factors for GBM include exposure to ionizing radiation, which induces point mutations, frameshift mutations, double-strand breaks, or other chromosomal anomalies. Currently, there is no solid epidemiological evidence that exposure to carcinogens or chemicals correlates with an increased risk of GBM (Smith et al., 2024). Additionally, evidence suggests that genetic factors may influence the risk of familial gliomas, unlike GBM which is mostly sporadic (Choi et al., 2023).

2.1.1 Subtypes of GBM

The Cancer Genome Atlas (TCGA) classifies GBM into four subtypes based on their genetic and epigenetic markers: mesenchymal, classical, proneural, and neural (Verhaak et al., 2010). Mesynchenal GBM is characterized by mutations in the neurofibromin 1 (*NF1*) gene, as well as frequent mutations in the *PTEN* and *TP53* tumor suppressor genes. Classical GBM is

characterized by *EGFR* amplification but the absence of *TP53* mutations. Proneural GBM is uniquely associated with *IDH1* and *PDGFRA* mutations, as well as mutations in the *TP53* gene. Recent studies suggest that more than one of these subgroups can coexist within the same tumor (Qazi et al., 2017).

2.2 Current Therapies

There are a variety of currently approved therapies for GBM treatment. However, current multimodal treatments only marginally prolong a patient's life. In this section, we will review the 5 major modalities of cancer treatment: surgery, radiation, chemotherapy, targeted therapy, and immunotherapy. The current standard of care for GBM is surgical removal of the primary tumor followed by concomitant radiation and the chemotherapeutic temozolomide (TMZ) to remove residual tumor cells.

2.2.1 Surgical Resection

Maximal surgical resection of GBM has been correlated with an increase in life expectancy and quality of life. Recent advancements in image-guided surgeries have assisted in more precise removal of the primary tumor (Rong et al., 2022). Robotic surgeries have also achieved a large volume of tumor removal, with patients experiencing side effects similar to those of traditional surgery (Baron et al., 2020).

2.2.2 Chemotherapy

Chemotherapy is a cancer treatment that utilizes drugs or pharmaceuticals to inhibit cancer cell proliferation and tumor multiplication. Chemotherapy agents primarily target macromolecular synthesis or protein synthesis processes in cancerous cells, ultimately leading to their apoptosis (Amjad et al., 2025).

2.2.2.1. Temozolomide Accompanied by Radiation

Discovered more than two decades ago, TMZ remains the first line of treatment against GBM, irrespective of histology. TMZ is an oral alkylating agent with effective CNS penetration (Jia et al., 2023). TMZ works by methylating the purine bases of DNA in tumor cells (Syro et al., 2018). In addition, it increases the likelihood of radiation-induced DNA double-strand breaks and cell death when administered concomitant to radiation (Miermeister et al., 2015).

2.2.3 Targeted Therapies

Targeted therapies are cancer treatments that utilize small molecule agents or therapeutic monoclonal antibodies to precisely target and eliminate cancerous cells. Compared to chemotherapy, targeted therapies are reputed for their effectiveness and minimal side effects on healthy cells (Min & Lee, 2022).

2.2.3.1. Bevacizumab

Bevacizumab is a monoclonal antibody (mAb) targeting vascular endothelial growth factor A (VEGF-A) with anti-angiogenic effects. Bevacizumab is an attractive therapeutic target since levels of VEGF-A are approximately 30 times higher in GBM than in low-grade astrocytomas. Bevacizumab does not significantly improve overall survival for patients with newly diagnosed GBM, so it is predominantly used to treat recurrent GBM (Wu et al., 2021). It is intravenously administered and can be used on its own or with adjuvant medications. However, multiple negative side-effects of bevacizumab have been recorded, including bleeding, gastrointestinal

perforation, delayed wound healing, and intensification of the cytotoxic effects of chemotherapy (Angom et al., 2023).

2.2.4 Immunotherapies
In immunotherapy treatment, immune cells play a primary role in targeting and eliminating tumor cells (Tan et al., 2020).

2.2.4.1 Personalized Peptide Vaccines
A newly developed personalized neo-antigen targeting peptide vaccine has shown great promise in treating GBM. Ongoing clinical trials of peptide vaccines suggest their safety and efficacy in treating GBM, with infrequent adverse events limited to grades I and II. The vaccines used in clinical trials are based on tumor specific somatic mutations unique to each patient. Computational methods are used to determine which neoantigens are differentially expressed on the surface of the patient's tumor cells and can be recognized by T-cells. Then, the amino acid sequence of patient-specific neoantigens is synthesized into a personalized peptide vaccine. Administration of a personalized peptide vaccine can trigger an immune response against the tumor and amplify tumor-specific T-cell responses (Latzer et al., 2024).

2.2.4.2 CAR-T Cell Therapy
Chimeric antigen receptor (CAR)-T cell therapy in GBM recognizes tumor associated antigens (TAAs) and attacks GBM cells in a targeted manner. In CAR-T therapy, the patient's T-cells are removed, genetically modified in the lab to express chimeric antigen receptor targeting neoantigens expressed on the tumor, then readministered to the patient. The modified T-cells are typically delivered intravenously or intracranially. Following binding and recognition, intracellular signaling pathways initiate downstream signalling cascades to trigger a primary immune response that leads to T-cell proliferation, survival, and cytotoxic activity. CAR-T cell therapy that targets antigens highly expressed in GBM tumors such as B7-H3, EGFRvIII, and IL13Rα2 have demonstrated promising results in clinical and preclinical trials (Agosti et al., 2024).

2.3 Ongoing Challenges of GBM Treatment

2.3.1 Rapid Infiltration
GBM tumors grow rapidly and are nearly impossible to completely surgically resect due to their location in the fragile brain tissue. Approximately 80% of GBM patients undergo an initial surgery, and another 20% undergo a second resection after experiencing a relapse (Smith et al., 2024).

2.3.2 Intertumor and Intratumor Heterogeneity
GBM is thought to be monoclonal in origin but acquires significant genetic and histological heterogeneity by the time of diagnosis (Smith et al., 2024). Intratumor heterogeneity is a notable feature of GBM. A study by Sottoriva et al. found that subclones of cells exist within even a fragment of a GBM tumor, with each of these often belonging to a different cell lineage (Sottoriva et al., 2013). Tumor heterogeneity makes GBM treatment especially challenging by preventing the elimination of areas of the tumor that are unresponsive to targeted therapies and permitting the renewal of therapy-resistant subpopulations.

2.3.3 Blood-Brain Barrier

The blood-brain barrier (BBB) is a semipermeable vascular boundary between the circulatory system and extracellular space of the nervous system. The walls of the BBB are lined with tightly packed endothelial cells that greatly limit the biopharmaceuticals that can enter the parenchyma. Even if a therapeutic drug manages to penetrate into the tumor tissue, it is often unable to reach effective levels due to the upregulation of efflux pumps in GBM cells (Wu et al., 2021).

2.3.4 Immunosuppressive Microenvironments

GBM tumors are characterized by a lack of tumor antigens, defects in antigen presentation, and a high accumulation of immunosuppressive cells (Lim et al., 2018). GBM is a “cold tumor”, meaning that it lacks pre-existing T-cell infiltration, which results in tumor resistance to immunotherapeutic drugs like immune checkpoint inhibitors. In contrast, “hot tumors” are infiltrated by tumor-reactive T-cells and are therefore more immunogenic (Wu et al., 2021).

2.3.5. Cellular Dormancy

Cellular dormancy plays an important role in fatal GBM relapses. Chemotherapies are designed to target cancer cells with an overactive metabolism or high rates of cell division, but studies suggest that some GBM subtypes are able to evade therapy by entering a dormant state. These dormant, or quiescent, cells can then give rise to new cancer cells later on, serving as a type of cancer stem cell (Adamski et al., 2017).

2.4 Antibody Drug Conjugates

Antibody drug conjugates (ADCs), nicknamed the “biological missiles” of targeted cancer therapy, consist of a monoclonal antibody (mAb) chemically linked to a cytotoxic payload molecule (Fu et al., 2022). ADCs combine the accurate targeting of an mAb with the toxicity of a payload molecule to achieve a high rate of specific cancer cell death. After an ADC is administered, it circulates as an inactive assembly until it is eventually catabolized via endogenous cleavage mechanisms in the intracellular environment of the target cell (Theocharopoulos et al., 2021). Since the first ADC, Mylotarg™ ***(Figure 1)***, was developed in 2000 to treat acute myeloid leukemia (AML), only 15 ADCs have been approved by the Food and Drug Administration (FDA) (Maecker et al., 2023).

ADC	Registered Trademark	Company	Disease	Antigen	Linker	Payload	Approval
Mirvetuximab soravtansine	ELAHERE	ImmunoGen (Waltham, MA, USA)	Platinum-resistant epithelial ovarian	FRα	Sulfo-SPDB	DM4	2022
Tisotumab vedotin-tftv	Tivdak	Seagen Inc (Copenhagen, Denmark)	Recurrent or metastatic cervical cancer	Tissue factor	MC-Val-Cit-PAB C	MMAE	2021
Loncastuximab tesirine-lpyl	Zynlonta	ADC Therapeutics (Epalinges, Switzerland)	Diffuse large B-cell lymphoma	CD19	Val-Ala dipeptide	PDB dimer	2021
Belantamab mafodotin-blmf	Blenrep	GlaxoSmithKline (London, UK)	Relapsed or refractory multiple myeloma	BCMA	MC	MMAF	2020; withdrawn 2022
Sacituzumab govitecan	Trodelvy	Immunomedics (Foster, CA, USA)	Metastatic triple-negative breast cancer	Trop-2	Carbonate	SN38	2020
Trastuzumab deruxtecan	Enhertu	AstraZeneca/Daiichi Sankyo (Cambridge, UK)	Unresectable or metastatic HER2-positive breast cancer	HER2	Tetrapeptide	DXd	2019
Enfortumab vedotin	Padcev	Astellas/Seagen Genetics (Tokyo, Japan)	Advanced or metastatic urothelial carcinoma	Nectin-4	MC-Val-Cit-PAB C	MMAE	2019
Polatuzumab vedotin-piiq	Polivy	Genentech, Roche (Basel, Switzerland)	Relapsed or refractory diffuse large B-cell lymphoma	CD79	MC-Val-Cit-PAB C	MMAE	2019
Moxetumomab pasudotox	Lumoxiti	Astrazeneca (Cambridge, UK)	Relapsed or refractory hairy cell leukemia	CD22	MC-Val-Cit-PAB C	PE38	2018
Inotuzumab ozogamicin	Besponsa	Pfizer/Wyeth (New York, NY, USA)	B-cell acute lymphocytic leukemia	CD22	Hydrazone	N-acetyl-γ calicheamicin	2017
Trastuzumab emtansine	Kadcyla	Genentech, Roche (Basel, Switzerland)	HER2-positive breast cancer	HER2	SMCC	DM1	2013
Brentuximab vedotin	Adcetris	Seagen Genetics, Millennium/Takeda (Tokyo, Japan)	Anaplastic large-cell lymphoma	CD30	MC-Val-Cit-PAB C	MMAE	2011
Gemtuzumab ozogamicin	Mylotarg	Pfizer/Wyeth (New York, NY, USA)	Acute myeloid leukemia	CD33	Hydrazone	N-acetyl-γ calicheamicin	2000; reapproved 2017

Fig.1 List of commercially approved ADCs. The figure was reproduced from “Trends in the Development of Antibody-Drug Conjugates for Cancer Therapy” with permission from the author (Song et al., 2023).

2.5 Design of Antibody-Drug Conjugates

ADCs function optimally when target antigens are tumor-specific, homogeneously expressed, and rapidly internalized. Most antigens are tumor-associated rather than tumor-specific, making ADCs vulnerable to off-target effects. Tumor antigens should preferably be located on tissues developing resistance to the payload molecule or on highly proliferative tissues (Bhardwaj et al., 2018). In solid tumors, the level of antigen expression is nearly linearly correlated with the

therapeutic efficiency of the ADC, as it determines the amount of cytotoxic payload that will be internalized into the cell (Sharma et al., 2020). Minimal antigen shedding is also beneficial as the secreted epitopes can disable immunoconjugates in local circulation. Core features of the ideal mAb include a high binding affinity to target antigens, a low incidence of immunogenicity, and favorable pharmacokinetic properties. Payload molecules should be highly toxic to the target cell and typically fall under one of two categories: DNA-damaging agents and microtubule-disrupting agents (Theocharopoulos et al., 2021).

2.6 Purpose of study

As of now, there are no FDA approved ADCs for glioblastoma. Researchers have found that ADCs have a high tumor-killing efficacy in early clinical trials (Liu et al., 2024). As illustrated by the evidence above, there is an urgent and unmet need for effective therapies for GBM. Based on the success of ADCs in other cancers, we propose to design a novel ADC capable of specifically binding to tumor-associated antigens expressed on a majority of GBM tumors. We used computational approaches to identify a target and payload compound predicted to exert selective cytotoxicity against GBM cells.

03. Materials and Methods

3.1 Assaying Gene Expression in GBM Cell Lines

In this project, we utilized DepMap Data Explorer 2.0 to identify an antigen receptor that would provide a suitable target for a novel ADC. The Cancer Dependency Map (DepMap) is a portal created by the Broad Institute that contains clinical genetic data from thousands of cancer cell lines from real patients. To visually examine RNA-sequencing data of protein coding genes, we generated a scatter plot comparing gene expression in the *24Q2* dataset between healthy brain tissue and GBM cell lines. For the control and experimental models, we selected specific “Disease Subtypes” that would be representative of healthy brain tissue and GBM tissue respectively. The control model included 1 sample of immortalized neuronal progenitor cells and 1 sample of immortalized adult astrocytic cells. The experimental model included 52 GBM cell lines.

3.2 Assaying Gene Expression in GBM Tumors

We examined expression of genes of interest in GBM tissue using the Gene Expression Profiling Interactive Analysis (GEPIA) database. GEPIA is a newly developed web server for analyzing RNA sequencing expression data from thousands of tumor and normal samples from the TCGA and GETx databases (Tang et al., 2019).

3.3 Identifying Candidate Payload Molecules from Public Datasets

The next experimental step involved identifying a payload molecule for the ADC using the Cancer Therapeutics Response Portal (CTRP) by the Broad Institute. CTRP is an extensive database that links genetic, lineage, and other cellular features of a cancer cell to small-molecule sensitivity to accelerate drug discovery (*Cancer Therapeutics Response Portal*, n.d.). We used the “Features” tool to obtain a total of 842 cancer cell lines with gene expression (GEX) data for the target gene. The cell lines were further filtered to solely include 25 Grade IV Astrocytoma samples. We then used the “Correlation Analysis” page to generate a boxplot with z-scores and linear regression of multiple screening hits’ efficacies in comparison to expression

of the target genes. The “interquartile multiplier” was set to 1 and the “outlier radius” was set to “medium”.

We then visually confirmed the inverse correlation between candidate molecules using DepMap. Once in the DepMap Data Explorer, we selected a *scatter plot* as the “Plot Type”, *models* for “Points”, *drug screen* for the “X Axis data type” and *expression* for the “Y Axis data type”. We chose candidate compounds for the “X Axis feature” and expression of target genes for the “Y Axis feature”. For this particular scatter plot, we utilized the same experimental model of 52 glioblastoma cell lines (titled Glioblastoma) as was used in DepMap analysis.

04. Results

4.1. IL13Rα2 is a Promising Target for a Novel ADC

Using DepMap, we identified genes of interest as potential targets for the ADC. Genes of interest included those with high expression in GBM cell lines and low expression in control cell lines (increased y-value relative to x-value). We reviewed numerous genes with Y:X value ratios of greater than 3:1 log scale. For example, potential candidates included *MTZ2B* (Y:X ratio of 3.44292601:1), *SCAND1* (Y:X ratio of 3.10278615:1), *CCDC85B* (Y:X ratio of 6.84678034:1), *MGAT2* (Y:X ratio of 6.25581368:1), among several others. Interleukin 13 receptor subunit alpha 2 (IL13Rα2) had a Y:X ratio of 3.15364637:1 ***(Figure 2)***. A literature search suggested that IL13Rα2 is already under investigation as a biomarker of GBM - a study by Jaén et al. confirmed that IL13Rα2 is a GBM-restricted receptor commonly associated with invasion (Jaén et al., 2022).

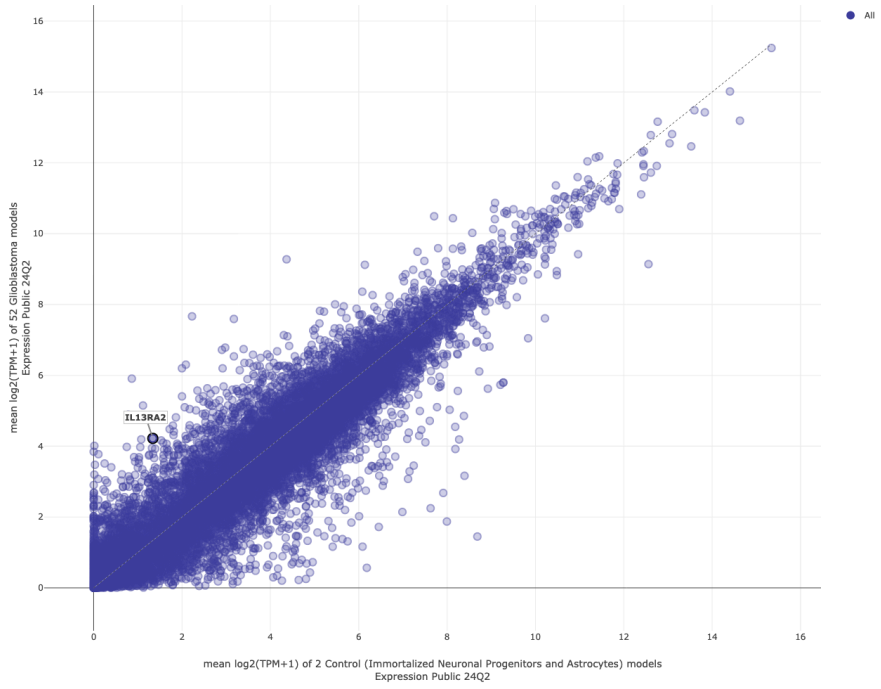


Fig.2 The figure above displays a scatter plot of gene expression in immortalized neuronal progenitor models and glioblastoma models. The plot was created using DepMap. The selected gene of interest IL13Ra2 is labeled on the plot.

Interleukin 13 (IL-13) is a cytokine ligand with a low binding affinity for interleukin 13 receptor alpha 1 (IL13Rα1), which is ubiquitously expressed in normal human astrocytes. The binding of IL-13 to IL13Rα1 via the E13 moiety allows for the binding of the IL13Rα1/IL13 complex to IL4Ra on healthy cells. This heterodimeric complex activates the STAT6 signaling pathway, which drives the translocation of transcription factors to the nucleus and induces expression of pro-apoptotic genes.

In glioblastoma cells, IL13Rα2 is highly expressed with up to 30,000 binding sites per cell. IL-13 binds to IL13Rα2 with high affinity via mutations of the moieties K105 and R109, thus sequestering the IL-13 ligand away from IL13Rα1. This sequestration of the ligand IL-13 allows the tumor cell to impede the downstream activation of apoptosis through the STAT6 pathway (Thaci et al., 2014). Recent studies suggest that IL13Rα2 has its own separate function upregulating AP-1 transcription factors in human glioma samples in situ (Bhardwaj et al., 2018). Activator protein 1 (AP-1) is an important transcription factor that regulates a wide range of cellular processes including cell proliferation, differentiation, cell migration, and transformation (Ye et al., 2014).

4.2 IL13Rα2 is Overexpressed in GBM Tumors Relative to Healthy Tissue
We used GEPIA to validate elevated expression of IL13Rα2 in human tumor samples. Notably, IL13Rα2 showed differentially increased expression in GBM, pheochromocytoma, and paraganglioma tumors compared with healthy brain tissue. It was also highly expressed in normal testicular tissue, which could potentially result in off-target effects ***(Figure 3)***.

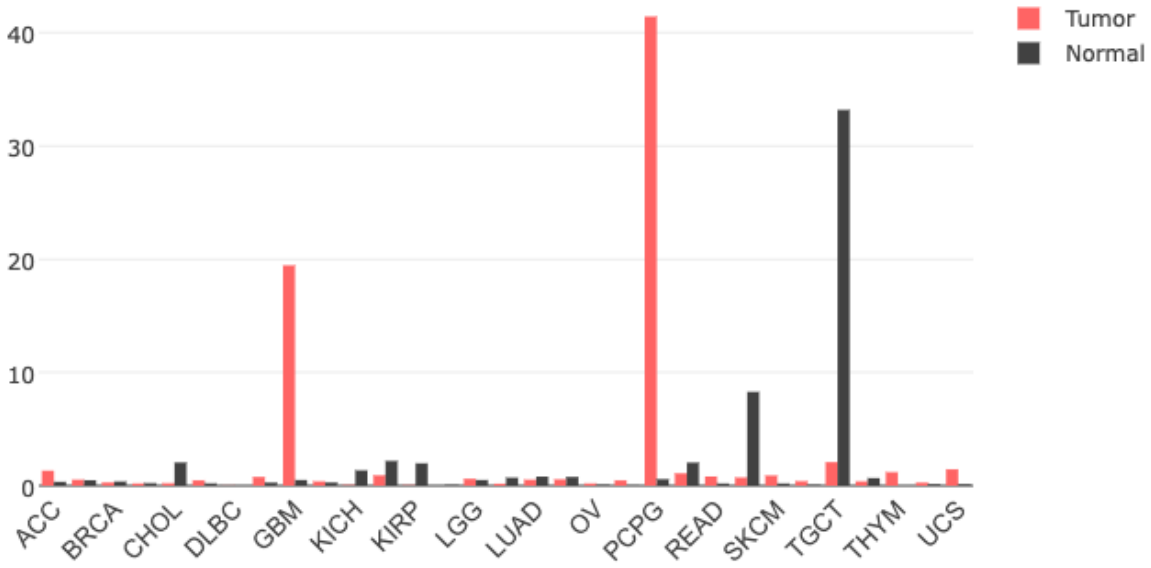


Fig.3 The bar plot above displays a comparison of IL13Ra2 expression among various cancerous and normal tissue types. The plot was created using GEPIA. As seen in the figure above, IL13Ra2 is highly expressed in cancerous GBM tissue, cancerous pheochromocytoma and paraganglioma tissue (PCPG), and healthy testicular germ cell tissue (TGCT).

4.3. Clone 47 is a Promising Antibody Candidate

IL13Rα2 is a glioblastoma-restricted receptor that is abundantly expressed in over 75% of GBMs but absent in normal brain tissue. It may serve as an attractive therapeutic target against GBM. However, its corresponding ligand, IL-13, binds to both IL13Rα2 and the ubiquitously expressed IL13Rα1 (Sattiraju et al., 2017). The usage of an anti-IL13Rα2 mAb would direct ADCs directly to GBM cells and minimize off-target effects in cells that express IL13Rα1.

In 2012, Balyasnikova et al. developed Clone 47, a humanized mAb specific to IL13Rα2. Clone 47 bound specifically and with high affinity to the native conformation of IL13Rα2 but not to IL13Rα1. Additionally, competitive binding assays revealed that Clone 47 significantly inhibited the interaction between IL-13 and the IL13Rα2 receptor, allowing IL13Rα1 to continue its normal signalling pathways. In vivo trials discovered that the survival of mice implanted with a human U251 glioma xenograft was notably improved (Balyasnikova et al., 2012). Based on the results of the study above, we believe Clone 47 is a favorable candidate for an anti-IL13Rα2 ADC.

4.4 BRD1812 is a Promising Candidate Payload

We used the CTRP database to identify a candidate payload drug, BRD1812. Of the tens of screening hits identified, BRD1812 was found to have the lowest z-score of -2.38 and strongest inverse correlation of -0.487 with IL13Rα2 gene expression (GEX). The resulting linear regression line of the scatter plot produced in DepMap had a moderate negative correlation (Pearson score = -0.421) between *IL13Rα2* GEX and the mean area under the curve (AUC) of BRD1812. Based on recent drug sensitivity analyses, CTRP data, and DepMap data, we concluded that the drug BRD1812 would be a promising payload molecule for an anti-GBM ADC ***(Figure 4)***.

Fig. 4a

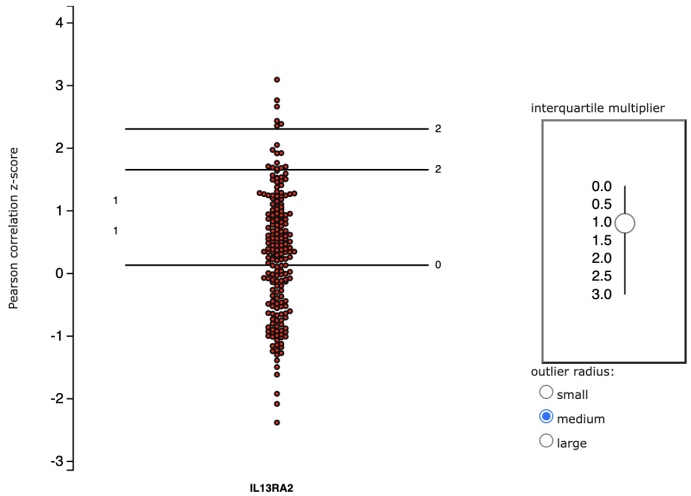


Fig. 4b

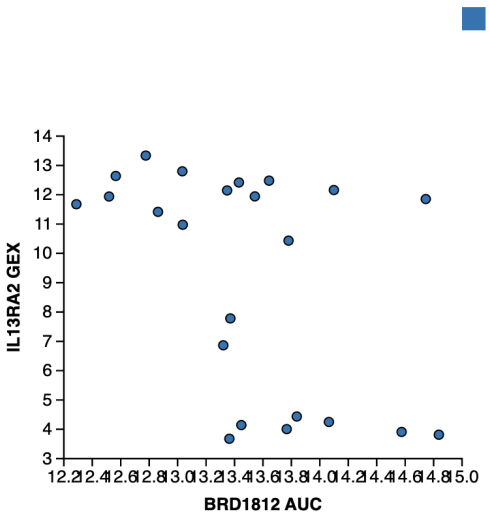
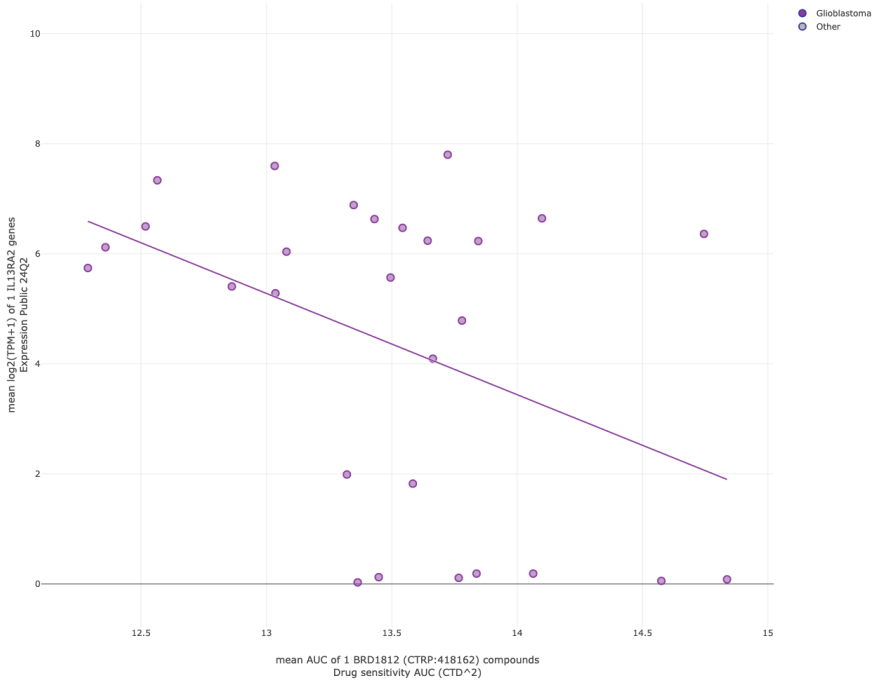


Fig. 4c



Group	Points	Pearson	Spearman	Slope	Intercept	p-value (linregress)
Glioblastoma	28	-0.421	-0.313	-1.84E+0	2.92E+1	2.58E-2

Fig.4 (a) This boxplot shows the tens of high throughput drug screening hits provided by CTRP. **(b)** The accompanying scatter plot provided by CTRP showed a downward trend in IL13Ra2 gene expression in comparison to BRD1812 AUC. **(c)** The downward trend was confirmed by a scatter plot produced in DepMap that showed a moderate negative correlation between IL13Ra2 GEX and the AUC of BRD1812.

Finally, we searched the literature for studies on cancer cell sensitivity to BRD1812. A drug sensitivity analysis by Han et al. in 2022 found that BRD1812 expression was inversely correlated with mRNA expression of IL-17B (Han et al., 2022). IL-17B is part of the interleukin-17 family, a subset of cytokines involved in acute inflammatory responses. This chronic inflammation has been shown to trigger a series of molecular events that lead to malignant transformation of differentiated cells and antitumor immunosuppression (X. Song et al., 2021). Another drug sensitivity study conducted by Lv et al. in 2024 found that BRCA cell lines with high risk genes were most sensitive to 13 small molecules including BRD1812 (Lv et al., 2024). The CTRP database additionally tagged BRD1812 as an effector of neuron differentiation and activator of apoptosis.

4.5 Proposed Linker Design

A linker is the chemical molecule that bridges the mAb to the cytotoxic agent in an ADC. Linkers play a major role in ADC target affinity and side-effects, and ultimately determine its therapeutic efficacy. The ideal linker should remain stable in the circulatory system and selectively release its payload at the tumor site (Su et al., 2021). In addition to circulation, linkers maintain an ADC's stability during the preparation and storage stages. The main challenge in current linker technology is to refine catalysts for linker cleavage between extracellular and intracellular environments (Lu et al., 2016).

Linkers are classified into two primary categories based on their mechanism of release and the stability in blood circulation: cleavable and non-cleavable. Cleavable linkers rely on the physiological environment, such as low pH or special enzymes, to enable biochemical reactions such as hydrolysis or proteolysis (Chari, 2008). On the other hand, non-cleavable linkers rely on the degradation of the mAb after the ADCs' internalization within lysosomes or endosomes to generate metabolites containing cytotoxic drugs with or without a portion of the linkers (McCombs & Owen, 2015). The optimal linker should account for the various properties of the cytotoxic drug, the characteristics of the monoclonal antibody, and the particular disease of focus (Lu et al., 2016).

Lysosomal proteases, such as cathepsin B, are generally overexpressed in cancer cells, allowing for selective drug release in the vicinity of the tumor (Gondi & Rao, 2013). In healthy cells, cathepsins maintain the homeostasis of the cellular environment through antigen-processing during immune responses and by degrading proteases. Despite their versatility in physiological functions, cathepsin upregulation can manifest in a host of clinical disorders. Cathepsins are known to activate and degrade several important neuronal proteins, leading to neurodegenerative and inflammatory diseases such as Parkinson's and Niemann-Pick type C disease. In cancers, tumors metastasize via extracellular matrix (ECM) degradation, and cathepsins aid in breaking down epithelial membranes and cell-cell junctions (Yadati et al., 2020).

Multiple studies suggest that cathepsins are differentially elevated in GBM cells. In 2005, Fukuda et al. found that expression of aspartic cathepsin D in GBM patients significantly correlated with a decrease in overall lifespan (Fukuda et al., 2005). A recent study in 2022 found that lysosomal cysteine carboxypeptidase cathepsin X activity and expression is upregulated in human GBM cells as well as in tumor-associated macrophages and microglia (Majc et al.,

2022). Based on these studies, we concluded that a linker cleaved by the cathepsin enzymes that are differentially overexpressed in GBM cells would be ideal.

The proposed linker for the ADC falls under the major category of enzyme sensitive linkers. The specific linker, a valine-citrulline (VCit) dipeptide linker, is a standard cleavable linker that has been employed in many successful ADCs. In VCit linkers, the payload drug is connected to a *p-aminobenzyloxycarbonyl* (PBAC) group (Anami et al., 2018). VCit linkers are cleaved by cathepsin enzymes upon internalization of the ADCs by target cancer cells, resulting in the traceless release of the payload molecule (Hamblett et al., 2004). Additionally, they remain stable in cynomolgus monkey and human plasma (Anami et al., 2018). While research studies have supported the efficacy of VCit linkers in various cancers, further testing needs to be done in vivo to determine if it is optimal for GBM cells.

4.6 Summary of the Final Proposed ADC Structure

The following is a summary of the final proposed ADC structure ***(Figure 5)*** based on the receptor, mAb, linker, and payload molecule detailed in the experimental steps above. The mAb is Clone 47, which selectively and effectively binds to the IL13Rα2 receptor differentially expressed by glioblastoma cells. The payload molecule is BRD1812, a compound found to negatively correlate with IL13Rα2 GEX. Finally, the linker is a VCit dipeptide linker which remains stable in human blood circulation and can be cleaved by cathepsins overexpressed at the tumor site.

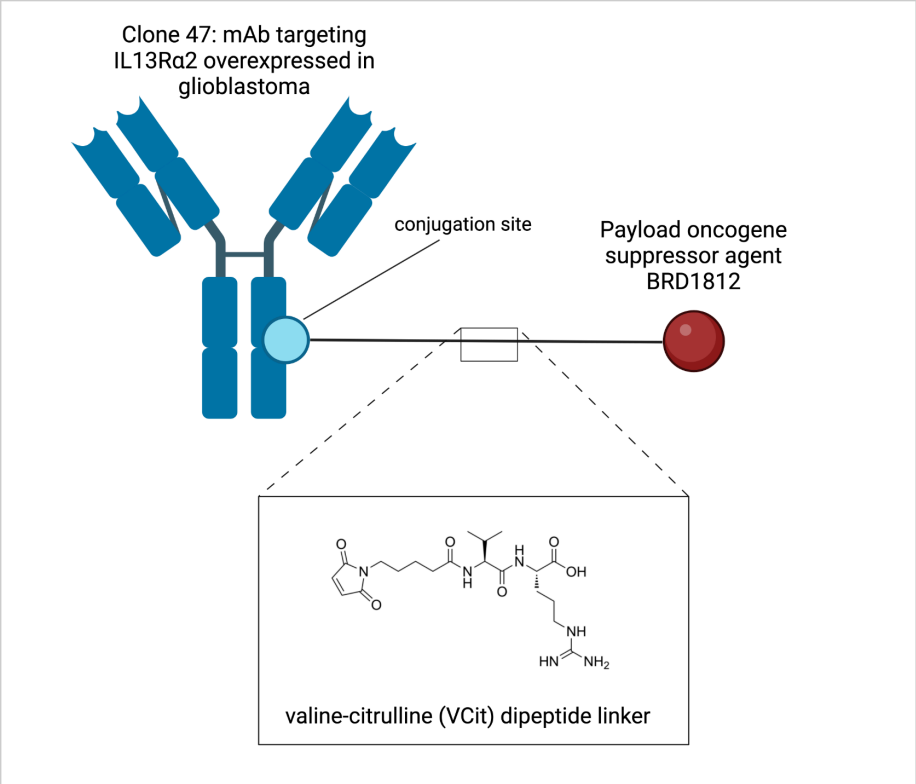


Fig.5 The figure above depicts the final proposed ADC design based on computation analysis. The ADC includes an IL13Rα2 targeting monoclonal antibody (Clone 47), a valine-citrulline chemical linker, and the cytotoxic agent BRD1812. The image of the VCit dipeptide linker was reproduced from medchemexpress.com (*MC(C5)-Val-Cit | ADC Linker | MedChemExpress*, n.d.).

05. Discussion

The following section will detail anticipated drawbacks of this novel ADC as well as future steps for its validation.

5.1 Anticipated Drawbacks

Three anticipated drawbacks of this treatment plan are transportation of the ADC across the BBB, heterogeneous expression of IL13Rα2, and off-target binding.

The capillary wall of the BBB has a thickness of 50-100 nm, while the size of an ADC is about 150 kDa (Hobson, 2024), which translates to roughly 225 nm. A potential approach to transporting a large ADC across the BBB would be the use of focused ultrasound (FUS) and microbubbles to noninvasively open the BBB. In FUS and microbubble therapy, MRI-guided acoustic waves are directed at the tumor site while 0.1 to 10 μm phospholipid microspheres (microbubbles) are intravenously administered. When interacting with the acoustic waves during circulation, the microbubbles oscillate at high frequencies, briefly stretching and compressing the capillary walls of the BBB. Concurrently administered ADCs would feasibly be able to bypass the BBB during this time (Chen & Konofagou, 2014). A study by Song et al. in 2018 found that the time in which BBB took to close was dependent on the size of the microbubbles used, ranging from 24 hours to a few days (C. H. Song et al., 2023) ***(Figure 6)***.

Furthermore, this ADC would solely benefit patients who express the IL13Rα2 antigen on their GBM cells. In a study by Zeng et al. in 2020, the expression of IL13Rα2 was significantly higher in GBMs ($p<0.001$), especially in those with wild type isocitrate dehydrogenase (IDH) and a mutated TERT promoter (Zeng et al., n.d.). Another study found that IL13Rα2 expression is upregulated in 79% of all primary GBM tumors (Newman et al., 2017). Although multiple studies suggest that increased IL13Rα2 is strongly correlated with poor prognosis, the remaining 20-30% of GBM patients with normal expression levels of IL13Rα2 may not benefit as significantly from the treatment.

Finally, IL13Rα2 is expressed at high levels on healthy testicular tissue (Jakobsen & Gjerstorff, 2020), which was further confirmed by our analysis in GEPIA. Since Clone 47 binds to IL13Rα2 with high affinity, the cytotoxic compound BRD1812 may inadvertently be released into the testis. Unintended side-effects may include infertility and tissue damage in the form of inflammation. Fortunately, the blood-testis barrier (BTB) is one of the tightest tissue barriers in the mammalian body (Cheng & Mruk, 2012) and should be able to prevent the ADC from non-specific binding. Regardless, the concerns presented above may make this ADC specifically suitable to female patients or elderly male patients.

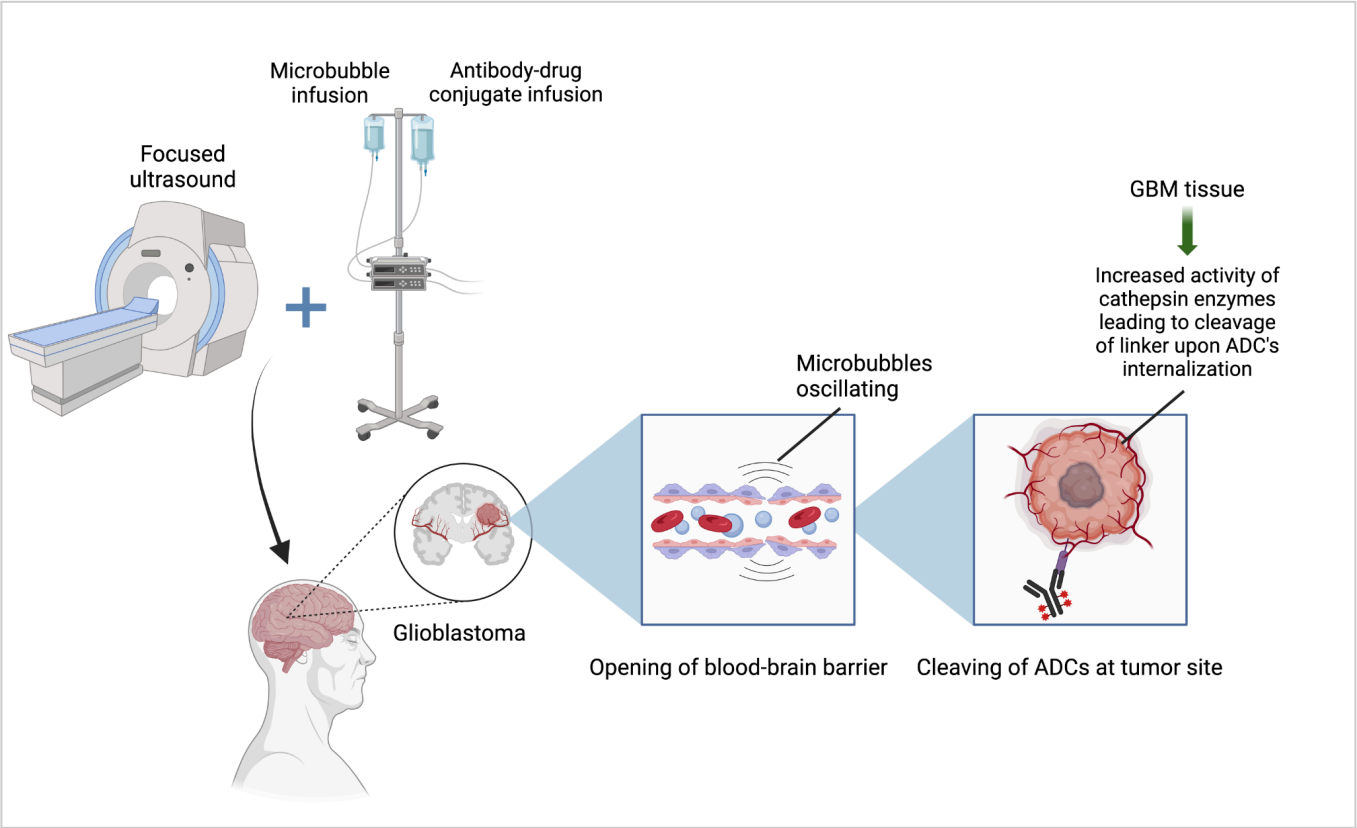


Fig.6 A potential method of ADC administration. The proposed ADC will be administered intravenously during simultaneous focused ultrasound and microbubble therapy. ADCs can then bypass the blood-brain barrier and its linker will be cleaved by cathepsin enzymes upon internalization.

5.2 Future Steps

Future steps to this study would involve creating and testing the ADC in a laboratory setting. For in vitro testing, immortalized non-cancerous brain cell lines and GBM cell lines that express IL13Rα2 at a range of levels would be used. For example, IL13Rα2 mRNA is significantly expressed in GBM cell lines A172 and U251, but barely expressed in GBM cell line T98G (Bhardwaj et al., 2018). Cell viability tests such as MTT assays would be used to compare cytotoxic effects between GBM cell lines with different expression levels of IL13Rα2, including cells that do not express the gene at all. Cell viability assays would also be used for various other immortalized cell lines—such as HEK293 or Neu41—to determine if off-target cytotoxic effects are present. As a positive control, we would administer the standard of care chemotherapy temozolomide, as its cytotoxic effect on GBM cells is well validated. As a negative control, we would treat cells with a vehicle control such as saline or DMSO. Furthermore, it is important to consider a scenario in which the ADC is unable to be activated in culture—in such a case, adding cathepsins to the media would likely allow cleavage of the linker.

If in vitro testing results suggest that the ADCs solely target and kill GBM cells, further testing can be done in vivo. Patient-derived xenografts (PDX) have been identified as a superior method for recreating spatial and heterogeneous characteristics of a tumor in an animal model (Liu et al., 2024). Under ethical guidelines, the PDX of a GBM sample can be surgically implanted into an immunocompromised mouse, allowed to grow, and then be treated using the ADC. In this way, we can safely analyze the cytotoxic effects of the ADC in the presence of a blood-brain barrier.

Finally, if pre-clinical studies support the efficacy of this ADC, the efficacy and safety of the ADC in patients could be tested in phase 1 and 2 clinical trials using healthy participants and patients diagnosed with GBM.

These early clinical studies would help to identify whether the ADC produces any unintended autoimmune responses or adverse events, and determine the optimal dosage of the ADC. During clinical testing, patient demographics such as age and gender, lifestyle factors, the level of IL13Rα2 expression in each patient, and the location of the tumor should be carefully considered.

06. Conclusion

GBM is a malignant and aggressive tumor of the CNS characterized by an extremely poor prognosis. Current therapies have succeeded in minimally prolonging a patient's overall life expectancy, but remain ineffective in the long-term. ADCs have revolutionized the field of cancer therapy through targeted drug delivery, yet no FDA-approved ADCs currently exist to treat GBM. In this paper, we designed a novel ADC for GBM treatment based on computational analysis (DepMap, GEPIA, and CTRP). The objective of this ADC is to selectively kill GBM cells and preserve healthy cell function. Usage of this ADC could help prevent recurrence after the primary tumor has been excised via surgery. Future research should focus on evaluating the efficacy of this ADC on GBM cell lines, animal models, and human volunteers.

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08. References

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