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*Review*

# Virus-Based Therapies for the Treatment of Recurrent High-Grade Glioma

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**Abstract:** As new treatment modalities are being explored in neuro-oncology, viruses are emerging as a promising class of therapeutics. Virotherapy consists of introduction of either wild-type or engineered viruses to the site of disease, where they exert anti-tumor effect. These viruses can either be non-lytic, in which case they are used to deliver gene therapy, or lytic, which induce tumor cell lysis and subsequent host immunologic response. Replication-competent viruses can then go on to further infect and lyse neighboring glioma cells. This treatment paradigm is being explored extensively in both preclinical and clinical studies for a variety of indications. Virus-based therapies are advantageous due to the natural susceptibility of glioma cells to viral infection, which improves therapeutic selectivity. Furthermore, lytic viruses expose glioma antigens to the host immune system and subsequently stimulate an immune response that specifically targets tumor cells. This review surveys the current landscape of oncolytic virotherapy clinical trials in high-grade glioma, summarizes preclinical experiences, identifies challenges associated with this modality across multiple trials, and highlights potential to integrate this therapeutic strategy into promising combinatory approaches.

**Keywords:** glioblastoma; high-grade glioma; refractory glioma; virotherapy; oncolytic viruses; neuro-oncology; refractory glioblastoma; chimeric viruses; clinical trials

## 1. Introduction

### *1.1. Background*

Primary brain tumors are classified by the World Health Organization into Grades I-IV. Of these, high-grade gliomas (Grades III and IV) are associated with high morbidity and mortality, highlighting the need for novel therapeutic approaches[1]. Glioblastoma (WHO Grade IV) continues to be one of the most formidable cancer diagnoses for several reasons. It is highly invasive and its infiltrative growth pattern poses a challenge when attempting complete surgical resection. Even after tumor resection, parenchymal tissue surrounding the resection cavity is highly infiltrated with glioblastoma cells, facilitating recurrence of disease. Current standard of care for glioblastoma includes maximally safe surgical resection, radiation, and chemotherapy[2]. Development of therapeutic resistance to standard chemotherapy is inevitable. Despite standard of care treatment, prognosis remains poor. The median survival for patients diagnosed with glioblastoma is 15 months and the 2-year relative survival rate is 26%[3]. There is currently no standard of care for recurrent glioblastoma, which warrants the investigation of novel treatment strategies. Additionally, systemic delivery of therapeutics into the CNS is hampered by the blood-brain barrier (BBB), which excludes

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many intravenously delivered agents from reaching effective concentrations in the brain. The integrity of the BBB is largely maintained by tight junctions between endothelial cells of cerebral capillaries. This barrier functions both to keep neuro-antigens out of the systemic circulation where they may be immunogenic and to keep large molecules out of the brain, where they can cause toxicity or loss of function.

Recent developments to overcome this therapeutic challenge include modified direct delivery methods such as convection-enhanced delivery (CED), in which a specialized catheter is stereotactically placed into the targeted region of brain and therapeutics can be infused directly into parenchymal tissue. Another direct delivery approach is to infuse therapeutics intra-arterially with an osmotic agent such as mannitol, which dehydrates endothelial cells and transiently disrupts tight junctions that form the BBB, thereby allowing drugs to enter the brain[4]. Additionally, focused ultrasound can be used to enhance the delivery of systemically administered drugs that would otherwise be excluded by the BBB. In this approach, lipid-encased perfluorocarbons are administered intravenously. Under local stimulation with low frequency ultrasound, these microbubbles oscillate and create mechanical forces that transiently and reversibly disrupt endothelial tight junctions, thereby allowing therapeutics to enter the brain[5]. These methods are warranted to reliably deliver a variety of therapeutics, including oncolytic viruses (OVs) into the CNS.

Finally, the immune- privileged status of the CNS is speculated to prevent robust activation of T lymphocytes, dampening the anti-neoplastic activity of the immune system. Due to these challenges, prognosis remains poor and there is an unmet need for additional therapies for glioblastoma. As additional therapeutic areas are explored, the use of oncolytic viruses in glioblastoma shows promise and warrants further investigation.

### *1.2. Historical Context*

The utility of viruses to induce tumor cell death was initially observed by DePace in 1912[6]. In this case report, a woman with cervical cancer was bitten by a dog and treated with Pasteur's attenuated rabies vaccine. Subsequently, regression of her cervical tumor was noted. This incidental finding prompted deeper inquiry into the use of viruses to treat solid tumors. The first preliminary clinical trial using an oncolytic virus to treat neoplasm was conducted when rabies vaccine was given to 30 patients with melanomatosis, of which 8 showed regressive changes[7]. These early findings paved the way for more sophisticated oncolytic virotherapies using engineered viruses that exhibit selectivity tropism for cancer cells (Figure 1). Although there are many sub-types of oncolytic viruses, they can broadly be divided into *replication deficient* or *replication competent* viruses. The former group is used as a viral vector to deliver gene therapy to malignant cells.

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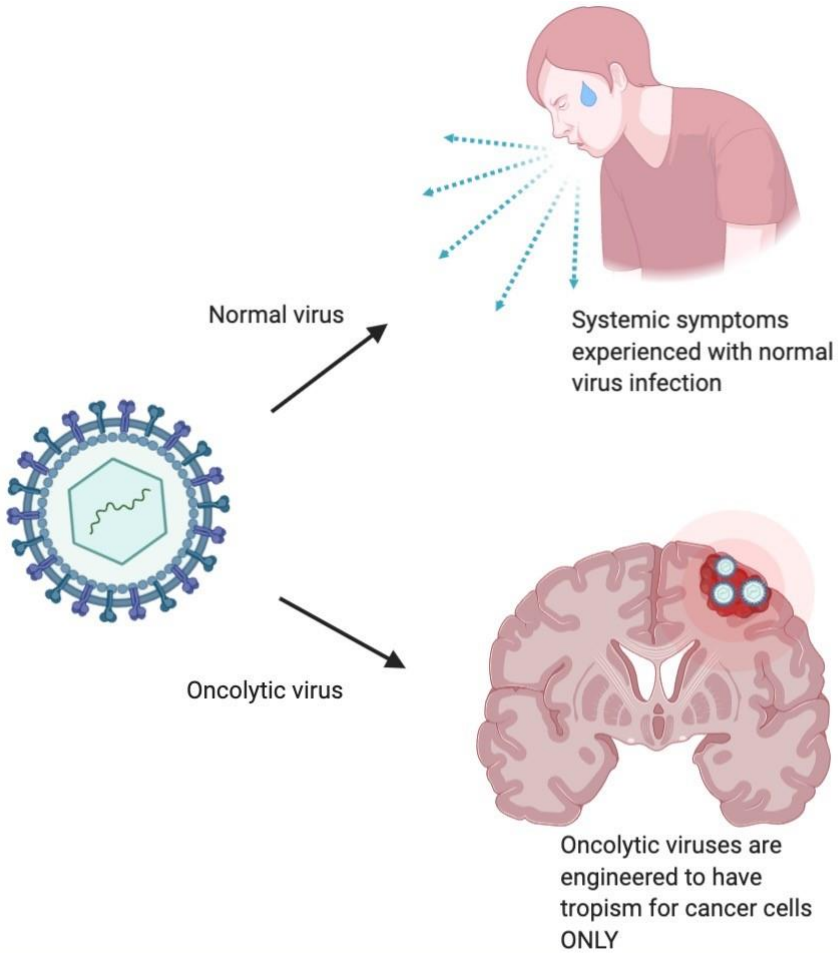


**Figure 1.** Oncolytic viruses have been engineered to exhibit selective tropism for cancer cells. Mechanistically, this entails that they rarely infect normal tissues therefore reducing the systemic signs and symptoms that would be normally experienced with parental strains.

*1.3. Mechanism of Anti-Tumor Effect of Oncolytic Viruses*

Replication deficient viruses can be used functionally as viral vectors to deliver genes that, when expressed, cause tumor cell death and subsequent immune response (gene mediated cytotoxic immunotherapy). This method shows promise as a tool to deliver cytotoxic gene therapy and is worth exploring in combination with existing therapies, such as checkpoint inhibitors.

In contrast, replication competent viruses selectively infect tumor cells and continue to replicate until the cell lyses. Their tendency to preferentially infect tumor cells is partially due to the loss of antiviral mechanisms in the malignant phenotype[8]. The anti-tumor effect of a replication competent virus is two-fold: first, cell death of the infected cancer cell occurs with lysis. Second, the lysed cell releases cytokines and viral antigens, which attract antigen presenting cells and facilitate their maturation into cytotoxic T lymphocytes that are specific to persisting tumor cells **(Figure 2).** After lysis, viral progeny continue to selectively infect neighboring tumor cells and the cycle continues. There has been evidence to show that when replication competent oncolytic viruses are injected into a tumor, their anti-tumor effect can be exerted on neighboring non-injected tumors[9,10].

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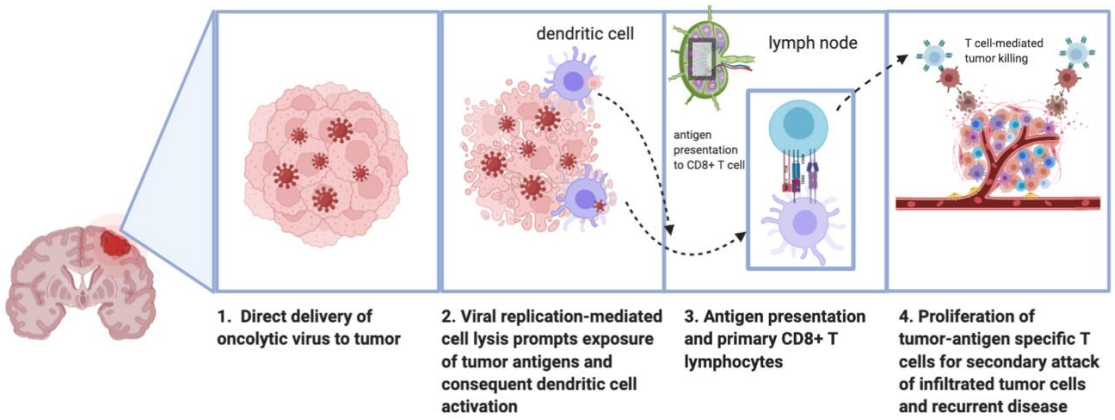


**Figure 2.** Mechanism of action of oncolytic viruses in the treatment of high-grade glioma.

Today, a wide range of oncolytic viruses from multiple viral families are being explored for therapeutic potential in CNS malignancies **(Figure 3).** Viral therapies include those with modifications made to the Herpesviridae, Adenoviridae, Paramyxoviridae, and Reoviridae families, as well as chimeric viruses that are engineered with transgenes to augment anti-tumor effect **(Figure 4)**. This review will 1) survey the current landscape of replication competent oncolytic virotherapy used in the treatment of HGG, 2) provide clinicians with an adequate framework to assess outcomes of clinically tested virotherapies, and 3) identify future directions and potential areas of investigation in this emerging therapeutic field.

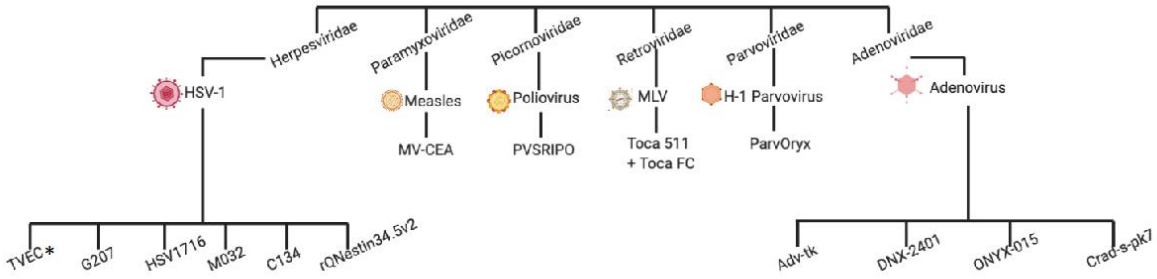


**Figure 3.** Family tree of oncolytic viruses being explored in the high-grade glioma setting. *TVEC has not been used in this setting.

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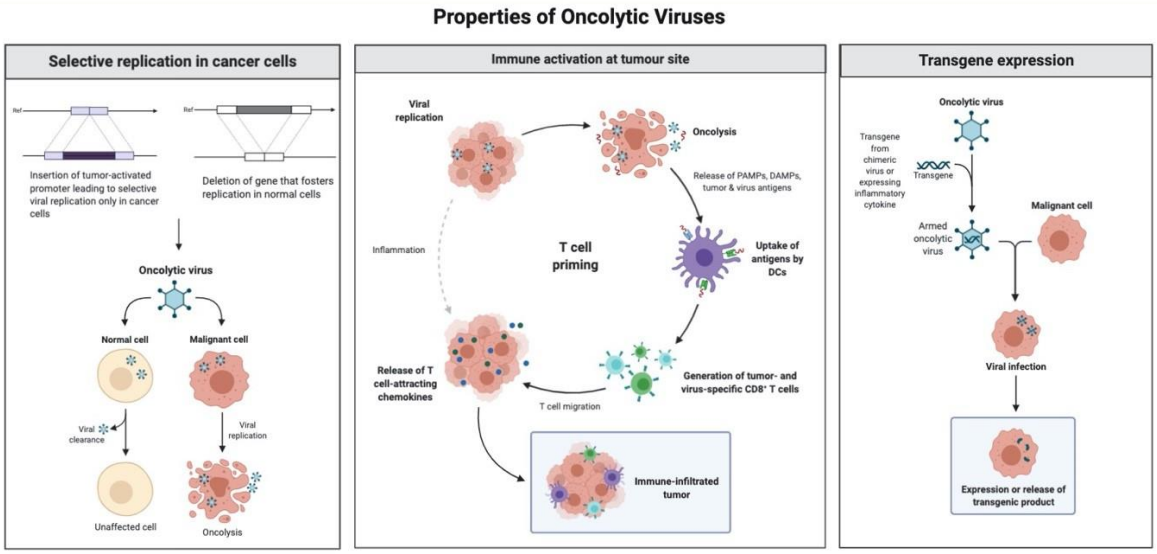


**Figure 4: This depicts the various strategies adopted in the engineering of oncolytic viruses so as to grant selective tropism for malignant cells.** Image adapted from *Groeneveldt et al, Trends Immunol 2020.*

## 2. Clinical Experiences with Virotherapy in High-Grade Glioma

### *2.1. Herpesviridae*

Multiple members of the herpes virus family (HSV-1 subtype) have been modified and studied in clinical trials. Herpesviridae are double stranded DNA viruses that are highly lytic, a property that renders them ideal for oncolytic virotherapy[8].

#### 2.1.1. Talimogene laherparepvec (TVEC, OncoVex$^{GM-CSF}$, or IMLYGIC)

In 2015, TVEC became the first FDA approved oncolytic virotherapy and was initially indicated for metastatic melanoma. TVEC is one of the most widely studied oncolytic viruses, with multiple clinical trials completed and in progress. TVEC was engineered by modifying the HSV-1 virus to improve replication competence, decrease virulence, and improve its profile as an oncolytic agent[8-12]. Notably, the addition of granulocyte- macrophage colony-stimulating factor (GM-CSF) increased the immunogenicity of TVEC by attracting neutrophils to the site of viral infection and stimulating stem cells to differentiate into granulocytes and monocytes, thereby augmenting the anti-tumor response[8].

To date, TVEC has not been used in the setting of glioblastoma. A recent search of the national clinical trials database using the terms "TVEC" and "cancer" reveals 21 active trials which are currently recruiting patients to continue testing this virotherapy in a variety of indications including melanoma, breast, pancreatic, liver, and colorectal cancers. TVEC is now being tested in combination with other therapeutic agents, most notably, checkpoint inhibitors. There are currently 3 active trials recruiting participants to test TVEC in combination with pembrolizumab (KEYTRUDA), an anti-PD1 IgG4 (NCT03069378, NCT02965716, NCT02509507), and 3 trials that are active but not yet recruiting (NCT02626000, NCT02263508, NCT03842943). Of note, there are also 3 active trials currently recruiting participants to test TVEC in combination with nivolumab (Opdivo), another anti-PD1 IgG4 (NCT03597009, NCT02978625, NCT03886311).

#### 2.1.2. HSV G207

Similar to TVEC, HSV G207 is a modified HSV-1. It was also engineered to demonstrate decreased neurovirulence and improved the safety profile through distinct modifications, which can

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be referenced in Markert et al 2014. Of note, G207 has an *E.coli* LacZ gene insertion, which renders this virus unable to replicate in non- dividing cells and restricts its lytic activity to actively dividing cancer cells. Furthermore, the E.Coli LacZ acts as a reporter gene that can be tested for using histochemical assay, indicating whether or not viral replication was successful, which gives this therapy additional clinical utility[11,12,13]. Finally, G207 retains susceptibility to antiviral therapy, which can be initiated if the need to control viral replication arises[12].

The safety of G207 was demonstrated by a phase I trial (NCT00157703) and described by Markert et al 2014. In this trial, 9 patients with recurrent malignant glioma underwent tumor biopsy followed by injection of G207 into 5 sites. Within the next 24 hours, a single dose of 5 Gy radiation was administered. Six of the nine patients showed stable disease or partial response. Three patients showed radiographic response to treatment. Median survival (from time of G207 inoculation) was 7.5 months[12]. A phase Ib/II study (NCT00028158) was completed and described by Markert et al 2000[12]. In the phase Ib portion of the study, 21 patients were given intratumoral G207 and observed for safety. Four out of 21 patients remained alive at the time of submission with a mean of 12.8 months post inoculation (range 7-19 months). Mean survival time from inoculation to death of the remaining 17 patients was 6.2 months following inoculation (range 1-13 months). Mean survival from date of diagnosis for 13 glioblastoma patients was 15.9 months (range 12-22 months). There was no evidence of HSV encephalitis or toxicities exclusively attributed to administration of G207.

Currently, there are two ongoing phase I clinical trials investigating the use of G207 when combined with a single dose of radiation in pediatric patients with recurrent supratentorial brain tumors (NCT02457845) and cerebellar brain tumors (NCT03911388). In these trials, G207 is infused intratumorally and is followed by a subtherapeutic 5 Gy dose of radiation within 24 hours of virus inoculation to enhance viral replication.

#### 2.1.3. HSV1716

Like G207, HSV1716 was also engineered to have reduced neurovirulence while maintaining the ability to replicate in actively dividing cells[14]. Unlike G207, HSV1718 retains the ability to replicate in nondividing cells. Although disruption of this property may improve the safety profile of G207, it may account for the fact that HSV1716 demonstrates greater replication competence and can be administered at comparatively lower doses[15].

Safety of HSV1716 has been demonstrated in early clinical trials. In a phase I trial conducted in the UK, 9 patients were given intratumoral injections of HSV1716. Of these, 4 patients remained alive 12-24 months after treatment. There was no incidence of herpes induced encephalitis or adverse events attributed to HSV1716[15]. In a subsequent phase I study, 12 patients were given intratumoral HSV1716 followed by tumor resection[9]**.** In a third study, 12 patients underwent surgical resection of high- grade glioma followed by injection of HSV1716 into the resection cavity in an effort to target residual tumor cells. Ten out of 12 patients were positive for HSV DNA in tumor tissue surrounding the injection site and four were positive for HSV DNA in tissue that was spatially distinct from the original site of inoculation. At the time of publication, 3 patients remained alive at a range of 18-22 months[16].

There was recently a phase I trial (NCT02031965) initiated in which pediatric patients with refractory/ recurrent HGG were to be HSV1716 peritumorally after maximal tumor resection. As of November 2016, VIRTTU Biologics reported that the trial was terminated due to lack of recruitment.

#### 2.1.4. rQNestin34.5v.2

As previously discussed, although some gene modifications can improve the safety profile of oncolytic HSVs, engineering such viruses can hamper their replication competence in a clinical setting. To circumvent this, rQNestin34.5v.2 (herein rQNestin) was engineered to conditionally express replication competence in malignant glioma cells. Nestin, an intermediate filament, is a molecular marker of malignant glioma (expression was confirmed in 6 out of 6 human glioma lines and 3 out of 4 primary glioma cells). This viral genome was engineered such that the gene that controls replication competence is located downstream from a synthetic Nestin promotor. Thus,

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rQNestin exhibits restricted replication competence in Nestin expressing cells and robust viral replication is only seen in glioblastoma cells[21].

There is currently one active and recruiting phase I clinical trial in progress (NCT03152318) to evaluate rQNestin in 108 adults with recurrent malignant glioma. In Arm A, a single dose of rQNestin is to be administered intratumorally in escalating doses until a maximum tolerated dose (MTD) or highest tolerated dose (HTD) has been established, at which point patients will be enrolled into Arm B. Here, patients will receive pretreatment with cyclophosphamide (CPA), an immunomodulating agent, in a single IV infusion 2 days prior to one intratumoral dose of rQNestin.

#### 2.1.5. M032

M032 is another conditionally replication competent HSV-1 that is lytic in tumor cells. It is distinguished from other oncolytic viruses because it was engineered to express human IL-12 prior to host cell lysis, which stimulates an immune response against remaining tumor cells and propagates the anti-tumor effect of M032[17,18]. Furthermore, IL-12 exerts an anti-angiogenic effect, which may further contribute to the efficacy of M032[19,20].

There is currently one active and recruiting phase I trial (NCT02062827) in which 36 adults with recurrent malignant glioma will receive a single intratumoral dose of M032.

#### 2.1.6. C134

C134 is a second generation chimeric oncolytic virus derived from HSV-1. In an effort to improve viral replication and therapeutic efficacy, C134 was engineered to express the IRS1 gene from human cytomegalovirus (HCMV). This modification allows for ICP34.5 (a neurovirulence factor) to be expressed in malignant cells, thereby improving its antitumor effect[21,22].

There is currently one active phase I trial (NCT03657576) in which 24 adults with recurrent glioblastoma will receive C134 inoculation into 1-5 sites within their tumor.

### *2.2. Adenoviridae*

#### 2.2.1. Aglatimagene besadenovec (AdV-tk)

This is a non-replicating adenoviral vector modified to contain the herpes simplex thymidine kinase gene and can be administered in combination with valacyclovir to elicit an anti-tumor effect. When administered locally into the tumor bed following surgical resection, the viral vector infects remaining cancer cells and causes them to express the viral thymidine kinase gene. This is followed by oral administration of valacyclovir, an anti-herpetic nucleoside analog. Thymidine kinase phosphorylates valacyclovir, which is incorporated into cancer cell DNA and inhibits further DNA synthesis or repair, causing cell death. This effect can be more pronounced when given in combination with radiation, as this causes strand breaks in glioma cell DNA, causing a greater degree of incorporation of phosphorylated valacyclovir, thereby inducing selective cell death. Following this, the adaptive immune system is triggered and immune effector cells amplify the anti-tumor effect[23]

In a phase II multicenter study described by Wheeler et al, (NCT00589875), 48 patients completed therapy with AdV-tk. Patients underwent surgical tumor resection and AdV-tk was subsequently injected into 10 sites within the tumor bed. Valacyclovir was initiated 1-3 days after this and radiation therapy was initiated 4-13 days post vector injection. Patients were also given temozolomide following injection of AdV-tk. No DLTs were observed and the treatment group showed a 3.6 month increase in median OS[23].

#### 2.2.2. DNX-2401 (tasadenoturev, formerly Delta-24-RGD)

DNX-2401 is an oncolytic adenovirus engineered to selectively replicate in malignant cells. It was granted both fast track and orphan drug designation by the US FDA. DNX-2401 was produced with two critical modifications: the first restricts its replication to malignant cells that display a

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dysfunctional retinoblastoma (RB) pathway, which improves the safety profile of this virotherapy[24,25]. The second modification is an insertion of an Arg-Gly-Asp (RGD) peptide motif, which increases interactions with tumor integrins at the cell surface. This is thought to augment viral gene transfer and increase the efficacy of DNX-2401[26].

In a completed phase I study (NCT00805376), DNX-2401 was administered intratumorally to 37 patients with recurrent malignant glioma. In study arm A, 25 patients received a single dose of DNX-2401 intratumorally. In study arm B (treat-resect-treat), 12 patients received an intratumoral injection of DNX-2401 followed by tumor resection 14 days later with DNX-2401 injection into the resection cavity. 20% of the patients in study arm A survived over 3 years after treatment and 12% showed durable complete responses. Analysis of post treatment samples from study arm B showed the immunogenic effect of DNX-2401, as there was evidence of viral replication and spread within the tumor following initial inoculation[27]. Following this, a phase 1b study (NCT02197169, TARGET-I) randomized 27 patients with recurrent glioblastoma to receive either DNX-2401 alone or with interferon-gamma (IFN). Notably, IFN was poorly tolerated and did not provide clinical benefit over DNX-2401 alone. Among both arms, OS-12 was 33% and OS-18 was 22%. Reported adverse events included fatigue, headache, and seizures consistent with existing disease[28]. Another phase I trial completed in Spain combined DNX-2401 with two 28 day cycles of temozolomide in patients with recurrent glioblastoma (NCT01956734)[29]. The preliminary results for this trial were presented at the American Association for Cancer Research annual meeting in 2017. At that time, 31 patients underwent tumor resection and intraparenchymal injection of DNX-2401 followed by 4 cycles of TMZ . Adverse events recorded were attributable to TMZ. Interestingly, seropositive patients who had neutralizing antibodies prior to treatment showed more favorable outcomes.

A recently completed phase 2 trial (NCT02798406, CAPTIVE/KEYNOTE-192) combined DNX-2401 with pembrolizumab (KEYTRUDA) in 49 patients with recurrent glioblastoma. Results were presented at the 2020 Society of Neuro-Oncology annual meeting and demonstrated this combination to be safe. The most commonly reported treatment-related adverse events included headache, brain edema, and fatigue. Efficacy endpoints included mOS (12.5 months), OS-12 (54.5%), and OS-18 (20.8)[30]. A phase 3 trial is planned, but not yet registered through the national clinical trials database.

There is currently one active clinical trial using DNX-2401 in the setting of high-grade glioma. This phase I trial (NCT03896568) will enroll 36 patients with recurrent high-grade glioma. In this treat-resect-treat design, patients will receive bone-marrow derived human mesenchymal stem cells (BM-hMSCs) loaded with DNX-2401 through arterial injection. After 2 weeks, patients will undergo tumor resection and receive another course of BM-hMSCs loaded with DNX-2401.

#### 2.2.3. ONYX-015

Similarly to DNX-2401, ONYX-015 is a selectively replication competent adenovirus. In this case, it was engineered with a gene deletion that renders it unable to exert effects on cells with a functional P53 pathway, which tumor cells lack[31,32]. Although the exact mechanism of selective replication of this virus has not yet been elucidated, it has been used in clinical trials to demonstrate safety.

ONYX-015 has been used in a range of trials demonstrating its safety, most notably in a phase I dose escalation trial in recurrent malignant glioma[33]. In this study, 24 patients were enrolled (6 per dosing cohort) and underwent tumor resection that was immediately followed by injection of ONYX-015 into 10 sites within the tumor resection cavity. There were no serious adverse events reported that could be definitively attributed to ONYX-015 administration. Although administering the virus at the time of tumor resection was shown to be safe, there was no definite antitumor activity. Median time to progression was 46 days (range 13-452 days) and median survival was 6.2 months (range 1.2-28 months). ONYX-015 warrants further study in the setting of malignant glioma.

#### 2.2.4. CRAd-S-pk7

A newly emerging approach to delivering virotherapy involves loading neural stem cells with an oncolytic adenovirus that is delivered locally. CRAd-S-pk7 is a conditionally replicating adenoviral vector. “S-pk7” refers to the addition of a survin promotor and a polycysteine, which

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together enhance tumor specific viral replication and improve transduction efficacy of the viral vector[34,35]. Neural stem cells are used as a delivery mechanism for virotherapy due to their tendency to migrate towards neoplastic tissue and aid in more direct delivery of therapeutics[36].

There is currently one active clinical trial in which neural stem cells loaded with CRAd-S-pk7 will be administered to 36 patients with recurrent malignant glioma in a phase 1 study (NCT03072134). In the first study arm, patients with unresectable tumors will undergo a biopsy followed by injection of NSC loaded with CRAd-S-pk7 into the tumor. In the second study arm, patients with resectable tumors will undergo tumor resection followed by injection of NSC loaded with CRAd-S-pk7 into the resection cavity. Following injection, both arms will receive standard of care chemoradiation. Tumor response is to be assessed on MRI.

### *2.3. Retroviridae*

#### 2.3.1. Vocimagene amiretrorepvec + [5-fluorocytosine(6-amino-5fluoro-1H-pyrimidin-2-one)]- (Toca 511 +Toca FC)

This is a dual agent combination. The first agent (Vocimagene amiretrorepvec or Toca 511) is a modified, nonlytic retroviral vector engineered from the murine leukemia virus (MLV) to include the yeast cytosine deaminase (CD) gene[37]. Toca 511selectively infects cancer cells, causing the CD gene to be integrated into the genome of actively dividing cells and produce the CD enzyme[37,32]. Subsequently, Toca FC (a prodrug) is given orally and is converted to 5-FU (a cytotoxic agent that induces cell death) in cells expressing CD. Preclinical models have also show that 5-FU may induce death in neighboring myeloid derived suppressor cells and further stimulate the body's immune reaction[38]. Furthermore, 5-FU was shown to be a radiosensitizing agent both in vivo and in vitro when tested in radioresistant glioma cell lines, which highlights the potential for concomitant radiotherapy as a possible therapeutic combination[39].

Clinical trials using the Toca 511+ Toca FC combination were conducted under breakthrough designation awarded by the US FDA. In an initial phase I trail (NCT01470794), 58 patients with recurrent HGG underwent tumor resection followed by Toca 511 injection into the resection cavity and Toca FC dosing throughout the course of the 30 week study. Following this, two separate cohorts also received bevacizumab or lomustine. Preliminary results from 45 patients showed the overall survival in patients with HGG to be 13.6 months and in glioblastoma to be 11.6 months[40]. In a post-hoc analysis of 56 enrolled patients (53 of whom were evaluable), the objective response rate was found to be 11.3% and mOS was 11.9 months. At the time of study conclusion, all 6 responders remained alive and in complete remission 33.9 to 52.5 months after treatment initiation[41]. In another phase I study (NCT01156584), 54 patients with recurrent HGG were recruited into cohorts that received one of the following interventions: 1) intratumoral injection of Toca 511, 2) IV injection of Toca 511 daily for 3 days, or 3) IV injection of Toca 511 daily for 5 days. All patients subsequently received oral Toca FC. In a third phase I study (NCT01985256), 17 patients were given an IV bolus of Toca 511. After 11 days, patients underwent surgical resection and intracranial injection of Toca 511 into the resection cavity followed by Toca FC.

Two ongoing trials using Toca 511 and Toca FC were recently discontinued by the sponsor. The first, "Toca 5" (NCT02414165), was a randomized phase II/III trial in which the Toca 511 and Toca FC combination was tested against a standard of care control arm in patients with recurrent glioblastoma or anaplastic astrocytoma. This study enrolled 403 patients (201 were randomized into the experimental arm and 202 into the control arm). The experimental arm intervention included injection of Toca 511 into the resection cavity at the time of surgery followed by oral Toca FC six weeks later. In patients who underwent treatment, the Toca 511/FC combination did not demonstrate efficacy, as there was no improvement in overall survival[42]. The second discontinued trial, dubbed "Toca 7" (NCT02598011), planned to combine Toca 511 and Toca FC with standard of care therapy in patients with newly diagnosed HGG. This phase Ib study planned to enroll 18 patients and conclude in 2022. Patients were to undergo intracranial injection of Toca 511 followed by Toca FC and TMZ/ radiation.

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*2.4. Picornoviridae*

2.4.1. PVSRIPO

PVSRIPO is a replication competent recombinant poliovirus in which the internal ribosome entry site (IRES) is replaced with that of human rhinovirus, effectively abolishing neurovirulence in non-malignant cells[43]. Poliovirus recognizes and binds to CD155, a tumor antigen that is widely expressed in solid tumors. Cytotoxic replication of PVSRIPO initiates malignant cell death, generates inflammation, and primes the immune system to recognize tumor cells[44,45].

In a phase I trial (NCT01491893), 61 adult patients with recurrent grade IV HGG were treated with 1 intratumoral infusion of PVSRIPO via CED at 7 escalating dose levels. Patients were given a booster of the poliovirus immunization 2 weeks prior to infusion. Overall survival of 21% was observed at 24 months and was sustained at 36 months. Median overall survival was 12.5 months[46].

There are currently three clinical trials investigating treatment of HGG with PVSRIPO. A phase Ib trial (NCT03043391) is enrolling 12 pediatric patients with malignant glioma who will receive one intratumoral infusion of PVSRIPO and be monitored for one year after treatment. A phase 1b/II trail (NCT03973879) planned to enroll 31 adults with recurrent grade IV glioma to receive intratumoral infusion of PVSRIPO followed by atezolizumab, a humanized IgG monoclonal antibody against PD-L1. Following this, tumor resection was planned at the discretion of the investigator. This trial was withdrawn, but the trial registration notes that resubmission is expected. In an ongoing phase II trial (NCT02986178) 122 adults with recurrent malignant glioma will receive intratumoral infusion of PVSRIPO alone or in combination with lomustine.

*2.5. Reoviridae*

2.5.1. Pelareorep (REOLYSIN)

REOLYSIN is an unmodified wild-type serotype 3 reovirus (respiratory enteric orphan virus) that is nonpathogenic in humans. It was found to have potential for use as an oncolytic therapy due to increased replication in cells with upregulated Ras signaling, which is common in malignant cells[47,48,49].

In a phase I dose escalation trial, 12 patients with recurrent malignant glioma were given a single intratumoral stereotactic injection of REOLYSIN. One patient was noted to have stable disease, 10 had progressive disease, and one patient was not able to undergo further evaluation. Median overall survival was 21 weeks (range was 6-234 weeks), median time to progression was 4.3 weeks (range 2.6-39), and a maximum tolerated dose was not reached[50]. Of note, viral shedding was noted in the saliva of one patient and in the feces of two. One patient was also positive for reovirus at the start of the study, but became negative after treatment[50]. This warrants further investigation into the potential for maintenance of a viral reservoir and subsequent shedding when administering virotherapies. In another phase I dose escalation trial (NCT00528684), 15 adults with recurrent malignant gliomas were given REOLYSIN via CED in a single intratumoral injection over 72 hours. Following this, 10 patients had stable disease, one had partial response, and four had progressive disease. Median overall survival was 140 days and median time to progression was 61 days[51]. This trial was the first in which an oncolytic virus was administered via CED in the US.

There is currently one active phase I trial in which 6 pediatric patients with recurrent glioma will be enrolled. Patients will receive sargramostim (GM-CSF- a bone marrow stimulant), on days 1 and 2, which is to be followed by a 60 minute IV infusion of REOLYSIN on days 3-5. This treatment will be repeated every 28 days for 12 cycles.

*2.6. Paramyxoviridae*

2.6.1. MV-CEA

MV-CEA is an oncolytic measles virus derived from the Edmonston vaccine lineage and has been shown to have antitumor effect against malignant glioma[52]. In this case, the measles virus is

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engineered to include human carcinoembryogenic antigen (CEA), which is a peptide marker that can be used to detect viral gene expression[53]. A toxicology study was completed to demonstrate safety in non-human primates to support a phase I/II clinical trial in recurrent glioma[53].

### *2.7. Parvoviridae*

#### 2.7.1. Parvovirus H-1 (H-1PV, Parv-Oryx)

Parv-Oryx is an oncolytic single-stranded DNA virus whose natural host is the rodent. Parv-Oryx retains the ability to infect and replicate inside of human cells, but is not associated with pathology in non-neoplastic tissue[54,55,56,57]. Of note, its oncolytic mechanism of action is thought to work through the cathepsin-mediated cell death pathway, so it may be an effective therapeutic approach by which to target glioma cells with defective apoptotic pathways[58]. Parv-Oryx is also unique in that it does readily cross the blood-brain barrier, which is a clinically valuable feature as this has potential for IV administration and may circumvent the need for surgical catheter placement or intracranial injection if administered prior to tumor resection[59,60].

A phase I/IIa study of Parv-Oryx was completed in 2015 in patients with progressive primary or recurrent glioblastoma in Germany (NCT01301430)[59]. 18 patients were enrolled at 9 into each of two study arms. In the first group, patients received ParvOryx in a treat-resect-treat study design. The virus was initially administered via intratumoral injection followed by resection and another round of intracranial injection into the tumor bed 9 days later. In the second study arm, the first treatment was given intravenously. Following this, treatment was similar to the first study arm (resection and intracranial injection 9 days later). Notably, clinical response was not found to be dependent on either the dose or route of entry, indicating that oncolytic parvovirus is able to cross the blood-brain barrier. This was further evidenced by signs of immunogenicity in CD4+ and CD8+ infiltrated tumors. Median overall survival was 464 days and median progression-free survival was 111 days[60].

### *2.8. Summary of Clinical Experiences using OVs in High-Grade Glioma*

As discussed, a variety of OVs have been used in clinical trials in patients with HGG. Completed clinical trials are summarized in Table 1. Trials that are in progress at the time of submission are summarized in Table 2.

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1 **Table 1.** Completed clinical trials using oncolytic virotherapy in high-grade glioma.

| Agent | NCT | Study phase | Published results | n | Study population | Outcomes |
|---|---|---|---|---|---|---|
| G207 | NCT00157703 | Phase I | Markert et al 2014 | 9 | Recurrent malignant glioma | Safety demonstrated (AEs) Median survival from inoculation=7.5 months<br>mPFS= 2.5 months |
| | NCT00028158 | Phase Ib/II | Markert et al 2000 | 21 | Recurrent malignant glioma | Safety demonstrated (AEs)<br>mTTP= 3.5 months<br>mOS= 15.9 (glioblastoma) and 40.5 (anaplastic astrocytoma) |
| HSV1716 | (UK) | Phase I | Rampling et al 2000 | 9 | Recurrent malignant glioma | Safety demonstrated (AEs) |
| | (UK) | Phase I | Papanastassiou et al 2002 | 12 | Malignant glioma | Safety demonstrated (AEs) |
| | (UK) | Phase I | Harrow et al 2004 | 12 | Recurrent or newly diagnosed high grade glioma | Safety demonstrated (AEs) |
| AdV-tk | NCT00589875 | Phase II | Wheeler et al 2016 | 48 | Newly diagnosed glioblastoma | Safety demonstrated (AEs, DLTs) mOS=17.1 months<br>mPFS= 8.1 months<br>OS at 1, 2, 3 years= 90%, 53%, 32% |
| DNX-2401 | NCT00805376 | Phase I | Lang et al 2018 | 37 | Recurrent malignant glioma | Safety demonstrated (AEs, DLTs)<br>Study arm A (single injection)-<br>Tumor reduction in 72% of patients<br>mOS= 9.5 months<br>Study arm B (infusion & resection)<br>mOS= 13 months |
| | NCT02197169 (TARGET-1) | Phase Ib | Lang et al 2017 | 27 | Recurrent glioblastoma | Tolerability of DNX-2401 as monotherapy (as compared to combination with IFN-gamma) demonstrated (AEs)<br>OS-12 (33%)<br>OS-18 (22%) |

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| | | | | | | |
|---|---|---|---|---|---|---|
| | NCT01956734 (Spain) | Phase I | Alonso et al 2017 | 31 | Glioblastoma at first recurrence | Safety demonstrated when combined with TMZ (AEs), efficacy endpoints not yet reported |
| | NCT02798406 (CAPTIVE/KEY NOTE-192) | Phase II | Zadeh et al 2020 | 49 | Recurrent glioblastoma | Safety demonstrated when combined with pembrolizumab (AEs)<br>mOS=12.5 months<br>OS12= 54.5%, OS18= 20.8% |
| ONYX-015 | - | Phase I | Chiocca et al 2004 | 24 | Recurrent malignant glioma | Safety demonstrated (AEs, DLTs)<br>Median survival= 6.2 months (4.9 months for glioblastoma patients, 11.4 in AA/AO) |
| Toca511 + TocaFC | NCT01470794 | Phase I | Cloughesy et al 2016 | 43 | Recurrent high grade glioma | Safety (AEs, DLTs)<br>OS (HGG)= 13.6 months<br>OS (glioblastoma)= 11.6 months For all evaluable patients: OS6 (87.9%), OS9 (72.4%), OS12 (52.5%), OS24 (29.1%)<br>PFS= 3.2 months, PFS6= 16.3% |
| | NCT01156584 | Phase I | - | 54 | Recurrent high grade glioma | - |
| | NCT01985256 | Phase I | - | 17 | Recurrent or progressive high grade glioma | - |
| | NCT02414165 (Toca 5) | Phase II/III | Cloughesy et al 2020 | 201 | Recurrent glioblastoma/ anaplastic astrocytoma | Safety (AEs)<br>mOS= 11 months<br>Efficacy was not demonstrated over control arm |
| PVSRIPO | NCT01491893 | Phase I | Desjardins et al 2018 | 61 | Recurrent glioblastoma | mOS=12.5 months<br>OS 24M and 36M= 21% |
| REOLYSIN | NCT00528684 | Phase I/II | Forsyth et al 2008 | 12 | Recurrent malignant glioma | mOS=21 weeks (range 6-234)<br>mTTP 4.3 weeks (range 2.6-39)<br>MTD not reached |
| | NCT00528684 | Phase I/II | Kickielinski et al 2014 | 15 | Recurrent malignant glioma | mOS= 140 days<br>mTTP=61 days |

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Abbreviations: AE= adverse events; DLT= dose limiting toxicity; OS= overall survival; mOS= median overall survival; mTTP= median time to progression; mPFS= median progression free survival; PFS6= progression free survival at 6 months; AA= anaplastic astrocytoma; AO= anaplastic oligodendroglioma; TMZ= temozolomide.

**Table 2.** Clinical trials in progress or results not yet reported using oncolytic viruses in high-grade glioma.

| Agent | NCT | Study phase | n | Trial design/population | Outcomes (safety, efficacy) |
|---|---|---|---|---|---|
| G207 | NCT02457845 | Phase I | 12 | Pediatric progressive or recurrent supratentorial tumors | Safety, tolerability (AEs)<br>PFS, OS |
| | NCT03911388 | Phase I | 15 | Pediatric recurrent or refractory cerebellar tumors | Safety, tolerability (AEs)<br>PFS, OS |
| HSV1716 | NCT02031965 | Phase I<br>Terminated by sponsor | 2 | Pediatric refractory/recurrent high grade glioma | MTD, PFS and OS up to 15 years |
| rQNestin | NCT03152318 | Phase I | 108 | Malignant glioma | MTD |
| M032 | NCT02062827 | Phase I | 36 | Recurrent malignant glioma | MTD<br>TTP and survival up to 12 months |
| C134 | NCT03657576 | Phase I | 24 | Recurrent glioblastoma | Safety, tolerability (AEs)<br>PFS- 3d, 28d, 3M, 6M, 12M, OS up to 12M |
| DNX-2401 | NCT03896568 | Phase I | 36 | Recurrent high-grade glioma | MTD, AEs<br>Tumor response, TTP for 1 year |
| CRAd-S-pk7 | NCT03072134 | Phase I | 12 | Newly diagnosed malignant glioma | Neurological side effects, MRIs for progression |
| Toca 511+ Toca FC | NCT02598011 (Toca 7) | Phase Ib<br>Terminated by sponsor | 18 | Newly diagnosed high grade glioma | DLTs |
| PVSRIPO | NCT03043391 | Phase Ib | 12 | Pediatric recurrent malignant glioma | Toxicity , 24 month OS |
| PVSRIPO + atezolizumab | NCT03973879 | Phase Ib/2<br>Withdrawn, resubmission expected | _ | Recurrent malignant glioma | Safety (AEs), survival at 24M |
| PVSRIPO +lomustine | NCT02986178 | Phase II | 122 | Recurrent malignant glioma | Objective response (iRANO) at 24 and 36 M, duration of ORR, OS at 24 and 36M, safety (AEs) |
| REOLYSIN + GM-CSF | NCT02444546 | Phase I | 6 | Pediatric relapsed/ refractory brain tumors | MTD (DLT), AE, mOS, OR, TTP |

Abbreviations: AEs= adverse events; PFS= progression free survival; OS= overall survival; mOS= median overall survival; AEs= adverse events; MTD= maximum tolerated dose; DLTs= dose limiting toxicity; ORR= objective response rate; iRANO= immunotherapy response assessment in neuro-oncology; TTP= time to progression.

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## 3. Summary of Preclinical Experiences with Combinatory Virotherapy in Glioblastoma

Considerable effort is being made in the preclinical setting to investigate the utility of virotherapy. Several combinatory strategies have been explored to render virotherapy more efficacious with little to no neurological adverse effects in GBM mouse models.

Perhaps, the most attractive combinatory approach thus far has been the combination of oncolytic virotherapy with checkpoint inhibition. This has recently and extensively been reviewed[61–63]. The most prominent checkpoint molecules are PD-1 and CTLA-4. These are constitutively expressed on the surface of regulatory T cells and are upregulated on the surface of cytotoxic T cells during immune response. They serve to reduce apoptosis in regulatory T cells, and conversely serve to dampen cytotoxic T lymphocyte-mediated immune response. PD-1 and CTLA-4 have been successfully targeted in other indications without much success in the glioma setting. Compounding evidence from a stream of recent publications attributed this phenomenon to the scheduling of checkpoint inhibition around surgery and possibly the molecular profile of patients[64–66]. Cloughesy et. al. showed that administering neoadjuvant/adjuvant anti-PD-1 before and after surgical resection showed clinical benefit. This regimen was also shown to induce immune cell infiltration and augmented T cell receptor clonal diversity among tumor-infiltrating T lymphocytes[66].

Given these first successful instances of targeting PD-1 in glioma patients, the synergistic implications of checkpoint inhibition and augmented virotherapy-mediated immune response have become far-reaching. This is especially apparent since oncolytic virus combinations with checkpoint inhibitors have proven potent in the preclinical setting. Hardcastle et. al. showed that oncolytic measles virus infection in vitro induced secretion of DAMPs and upregulated PD-L1 expression[67]. This synergistic potential was further corroborated in vivo, where oncolytic measles virus combination with PD-L1 blockade was shown to significantly improve survival in a syngeneic GBM model[67]. Similarly, Errington-Maiset. al. recently demonstrated that viruses could prime the glioma microenvironment for ensuing checkpoint blockade[68]. Intravenously delivered reovirus upregulated tumor PD-L1 expression, thereby further opsonizing the tumor for subsequent anti-PD-L1 action. This combination ultimately led to improved survival in a preclinical mouse model of glioma[68]. The utility of virotherapy in combination with checkpoint blockade has also been proven to spur a potent secondary adaptive response. An anti-PD-1 expressing oHSV, NG34scFvPD-1, was shown to improve survival in syngeneic immunocompetent GBM mouse models[69]. Spectacularly, a second challenge with glioblastoma cells in mice already treated with the anti-PD-1 expressing oHSV proved futile, hence suggestive of a vaccinal effect[69]. It has been well-characterized that PD-1 blockade leads to the consequent upregulation of its counterpart checkpoint molecule, CTLA-4[66]. Saha et. Al., combined an IL-12-expressing oHSV with anti-CTLA-4 and anti-PD-1 in a mouse glioma model[70]. This triple combination extended survival, increased T effector to T regulatory cell ratios, and led to subsequent rejection of GBM re-challenge in the immunocompetent mouse model[70].

The extracellular matrix (ECM) has also been implicated in the propagation of phenotypes associated with the several hallmarks of cancer such as migration, immunosuppression and therapeutic resistance[71]. Particularly, the desmoplastic state characteristic of most solid tumors is largely due to the increased aggregation and dysregulated organization of ECM proteins[71,72]. Moreover, the previously discussed oncolytic HSV variant, HSV1716, has been associated with altering high-grade glioma cytoskeletal dynamics, hence proving replication is ECM-dependent[73]. ECM proteins have, therefore, also become attractive targets for enhancing viral replication. Particularly, integrins have been shown to be upregulated in the glioma microenvironment. The integrin ligand-containing adenovirus, DNX-2401 has proven potent through glioma cell lysis and subsequent release of DAMPs to elicit Th1 immune response in the immunocompetent glioma mouse model[74]. Integrin-mediated entry has also been utilized to improve both viral tropism and replication[75,76]. Remarkably, Lee et. al. showed ⊚1 integrin blockade improved replication of an HSV variant and promoted anti-tumor efficacy in patient derived primary GBM-bearing mice[77].

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Given the obligate parasitic nature of viruses, there has also been precedent to target replication-incompetency at the transcriptome level. Chemoradiation, which is part of the standard of care for high grade glioma (HGG) patients, exerts its effect by targeting the excessively replicative and expressive characteristic of glioma DNA. Chemoradiation and virotherapy, have thus been explored preclinically as combinatory options. The standard HGG chemotherapy, temozolomide (TMZ) is an alkylating agent which delivers a methyl group to purine bases of DNA, consequently spurring DNA damage. GuhaSarkar et. al. showed that TMZ administered post adenovirus + interferon-beta therapy resulted in a significant survival benefit compared to both modalities alone[78]. Since TMZ is typically given 4 weeks after surgery in the clinical setting, virotherapy could serve as an option to sensitize infiltrative glioma cells to the effects of chemoradiation.

**4. Discussion**

Inflammation poses a considerable challenge when designing clinical trials in the neuro-oncology space. Surgical intervention is associated with some degree of inflammation, and this is further compounded by the latent inflammatory effects seen after administration of virotherapy. Data from virotherapy clinical trials in patients with HGG is not always consistent. This is partially due to the fact that this patient population tends to be immunosuppressed to varying degrees, so responses will depend on each patient's baseline immunological status. Furthermore, HGG patients routinely receive concomitant steroid therapy to control tumor- associated cerebral edema. If the patient has undergone recent tumor resection, it is likely that their steroid doses were further increased. As noted in a clinical trial of HSV-1716, a patient who was on high dose dexamethasone at the time of viral administration had no resulting immune response[16]. Standard of care therapies such as chemoradiation can further dampen immune response. The confluence of these factors may require higher doses of viral inoculation in order to elicit the desired immune response.

This challenge may be addressed by modifying the delivery schedule. Administration of virotherapy at the time of resection, when there is additional inflammation at the site of disease, may poise the virus to be quickly neutralized by the immune system and therefore less efficacious. Harrow et. al. 2004 noted that HSV-1716 may have failed to produce lytic infection in the setting of surgery associated inflammation and proposed conducting viral inoculation at time distinct from surgical resection[16]. Although this necessitates an additional interventional procedure, it may ultimately increase the efficacy of virotherapy and yield a more significant response. Temporally separating surgery from viral inoculation may also elucidate the etiology of adverse events**.** Chiocca et. al. 2005 noted that after ONYX-15 was injected locally, 10 out of 24 patients had one or more AEs that (based on clinician judgement) were related to the trauma associated with surgery rather than to the therapeutic agent[33]. To further minimize swelling, it may be prudent to collect pre-infusion biopsies greater than 48 hours prior to delivery of virotherapy so that the peak of swelling will have already elapsed at the time of viral inoculation. Another solution may be to administer virotherapy through implantable CED catheters, which can be left in place post-resection until surgery-associated inflammation has subsided, then used to infuse virotherapy into the resection cavity. Furthermore, some intravenously delivered virotherapies have shown potent migratory features in the preclinical setting, highlighting their utility for delivery that is both spatially and temporally separated from tumor resection[68,79]. Under the above proposed schedules, pre-treatment tissue samples would still be available for pharmacodynamic analysis. Finally, it is prudent to consider that an increase in the number of cells that are turning over during surgery may result in loss of selectivity, as the virus may infect non-malignant cells that would have otherwise been spared from infection[11].

The method of delivery used may also contribute to the amount of inflammation induced at the time of viral inoculation. In some trials, up to 40 separate injections were proposed, which involves placement and positional adjustment of multiple needles. When delivering the therapeutic agent by more sophisticated methods such as CED or intraarterial infusion, fewer catheters must be placed in

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comparison to intracranial injection, which can significantly reduce the amount of procedure-related inflammation associated with viral delivery. Furthermore, CED through a reflux-free catheter may result in delivery of a greater number of viral particles and more complete tumor coverage in comparison to delivery by intracranial injection, which relies on viral transduction efficacy to achieve maximal tumor coverage after locoregional delivery. The use of CED through the lowest number of catheters possible may ultimately minimize surgery- associated inflammation and improve the efficacy of virotherapy.

It is crucial to recognize that some degree of inflammation after the delivery procedure is to be expected when administering a virotherapy and may be indicative of the desired immune response. Chiocca et. al. 2004 noted that on histological exam of recurrent tumors in two patients, lymphocytic and plasmocytic infiltrates were noted following ONYX-15 administration, potentially a consequence of viral infection of the tumor tissue[33]. Hu et. al. 2006 found that following OncoVex administration, four patients with multiple tumors showed inflammation at distal, uninjected tumors in addition to inflammation at the inoculation site[10]. Papanasstiou et. al. 2002 found that after inoculation, 4 out of 10 samples of paired distal sites were positive for HSV DNA. Since lytic virotherapy stimulates an immunogenic response that is associated with latent inflammation, responses may be inappropriately characterized as pseudoprogression by the RANO or Macdonald criteria[9]. For this reason, it may be necessary to develop new assessment tool by which to compare virotherapy induced inflammation to an expected baseline.

Another challenge faced in virotherapy clinical trials is adverse event monitoring. Clinical sequelae following virotherapy administration tend to be nonspecific, so it can be challenging to determine the etiology of symptoms- particularly in a population that is significantly immunocompromised at baseline. Hu et. al. 2006 found that after OncoVex administration, AEs included local inflammation and erythema as well as constitutional symptoms such as febrile illness[10]. Administration of a virus directly into the brain requires vigilance during adverse event monitoring due to the possibility of developing viral encephalitis. Although multiple clinical trials have demonstrated the safety of local administration of both engineered and one wild type virus (reovirus), it is important to consider modifications that can be made to engineered viruses to reduce neurovirulence in non-malignant cells. In the event that viral encephalitis does develop, lack of antiviral choice poses a significant challenge. The current standard of care in the case of HSV encephalitis includes IV administration of acyclovir. Should encephalitis occur when administering a virus from another family, available treatment would be supportive, not curative. Another risk associated with virotherapy is the potential for environmental shedding of the virus and subsequent infection of others. Forsyth et. al. 2008 found that after administering REOLYSIN, one patient was found to shed the virus in saliva and 2 were found to shed it in stool. It may be advisable to collect both stool and saliva to assess for environmental shedding when designing future virotherapy clinical trials. When assessing the scope of viral replication, lack of assay specificity can also pose a monitoring challenge. Existing assays may not be able to differentiate between the wild-type virus and the engineered (therapeutic) virus. Harrow et. al. 2004 noted this as a study limitation, as PCR could not distinguish between wild-type HSV from engineered HSV-1716[16]. This issue can be circumvented by insertion of a distinct reporter gene when engineering the virus. For example, G207 has LacZ insertion that can be used to distinguish it from wild-type herpes viruses during post-inoculation analysis. With regard to engineering modifications, although certain gene deletions may reduce neurovirulence, they can subsequently compromise the efficacy of the therapeutic. For this reason, G207, which was engineered with an additional modification to improve the safety profile, exhibits lower transduction efficacy than HSV-1716[15]. Such a compromise may allow for the virus to be more readily neutralized by the immune system, which could warrant multiple doses to achieve the desired effect.

There are some aspects of virotherapy in neuro-oncology that are incompletely understood and warrant further study. Perhaps the most perplexing data across multiple virotherapy trials is the

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clinical outcome of patients in the context of their baseline serology. There are conflicting results, as Markert et. al. 2014 found the most significant responses to be in seronegative patients, but noted that the previous G207 trial found the best responses to be in seropositive patients[12]. Hu et. al. 2006 found that seronegative patients who were given OncoVex GM-CSF had more pronounced constitutional symptoms, which limited the MTD for this[10]. Patients were also found to seroconvert after viral administration, however, the data is again inconsistent. Chiocca et. al. (2004) found that two out of 24 patients seroconverted from negative to positive[33]. Markert et. al. 2000 found that only one patient from the highest dose level seroconverted from negative to positive[11]. It may be beneficial to seroconvert patients prior to initiating virotherapy. In a phase I trial of PVSRIPO (NCT01491893), patients were given the poliovirus booster 2 weeks prior to infusion. This may prime the immune system for virotherapy and dampen AEs, allowing for a higher MTD. This warrants further investigation, as the mechanism of immune system involvement has not been completely elucidated and conclusive results are not seen across studies that have collected serological results. Of note, Hu et. al. proposed a multi-dosing scheme in which patients are initially given a low dose of the virus to allow for seroconversion prior to administration of the intended dose, which may modulate side effects without having a negative effect on tumor necrosis level[10]. As noted by Markert et. al. 2014, to fully understand this phenomenon, a larger prospective study should be conducted to determine whether or not pretreatment exposure is a biomarker for response to virotherapy treatment[12]. This could aid in identifying certain populations of patients with HGG who may be better candidates for virotherapy and stand to gain greater clinical benefit from these therapies.

Both preclinical and clinical studies have shown convincing potential for the use of viruses in treatment of HGG. As clinical trials advance to later phases, strategic combinations can augment the efficacy of single therapies. Markert et. al. 2014 notably gave a subtherapeutic 5 Gy dose of concomitant radiation because it was thought to enhance viral replication[12]. Takahashi et. al. showed sensitization of previously radioresistant glioma cells after inoculation with RRV in both in vitro and in vivo preclinical models[39]. Radiotherapy following treatment with Toca 511 and Toca FC is thought to exert a local effect on cells containing 5-FU, thereby sparing surrounding non-malignant tissue[39]. Although radiotherapy may increase viral transduction efficacy, it may come at a cost of immunocompetency, so further studies are warranted to discern the implications of giving concomitant radiation. Despite the immunosuppressive nature of chemotherapeutics, clinical trials are underway investigating the safety and efficacy of virotherapies in combination with standard of care chemotherapy**.** Immunomodulating agents such as CPA or sargramostim have been given prior to virotherapy in an effort to stimulate the immune system and augment efficacy of virotherapy (NCT03152318, NCT02444546). Combination of virotherapies with checkpoint inhibitors can also facilitate a robust immune response following inoculation, resulting in more pronounced anti-tumor effect. With emerging studies definitively characterizing a therapeutic window for checkpoint inhibition in the glioma setting, virotherapy and checkpoint blockade combinations are becoming increasingly attractive[64–66]. Combination with immunotherapies (such as pembrolizumab, ipilumab, and nivolumab) are being investigated and show promise in allowing the immune system to work in concert with viruses to exert anti-tumor effect. Investigating virotherapeutic combinations in late phase clinical trials shows promise in improving the prognosis of HGG.

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