

IMPERIAL

**Bacteriophage-mediated systemic gene
therapy of Diffuse Intrinsic Pontine Glioma
through the blood-brain barrier**

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A doctoral thesis submitted for the degree of
Doctor of Philosophy

Imperial College London
Faculty of Medicine
Division of Brain Sciences

Statement of originality

I hereby declare that the work presented in this thesis was conducted entirely by myself under the supervision of Dr. Amin Hajitou at the Department of Medicine, Imperial College London, during the year 2017-2024. I confirm that the work reported herein is my own, and the work of others has been appropriately credited and referenced within this thesis.

The contribution of other researchers' work is indicated below:

The work presented in Figure 12 (Chapter 2), Figure 32 (Chapter 3) was discussed with and performed by Dr Ángel Montero Carcaboso, a researcher at the Sant Joan de Déu Research Institute in Spain.

The work presented in Figure 13 (Chapter 3) and Figure 14 (Chapter 3) was performed with the assistance of Mr Justin Tsui, a previous BSc student at the Phage Therapy Group, Department of Medicine, Imperial College London.

Wenqing Yan (2024)

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Abstract

Diffuse intrinsic pontine glioma (DIPG) is the most aggressive childhood brain tumour with a poor survival rate of only 6-10% beyond 2 years with no effective therapies. Surgical resection is not viable due to tumour location and existing treatments such as chemotherapy and radiotherapy show poor therapeutic response. We have applied a targeted cytokine gene therapy by using an engineered hybrid phagemid adeno-associated virus (PAAV) to selectively target the tumour through cancer-selective integrin binding ligand. This vector is derived from recombinant adeno-associated virus (AAV) DNA and M13-filamentous phage capsid and genetically engineered to display the RGD4C peptide which targets $\alpha v\beta 3$ and $\alpha v\beta 5$ integrin receptors that are over-expressed on tumour cells and tumour vasculature. The tumour necrosis factor-related apoptosis inducing ligand (TRAIL), which has preferential toxicity in tumour cells, was selected as the cytokine therapeutic gene to be delivered via this targeted vector (RGD4C/PAAV). We characterised 6 primary human DIPG cells obtained from biopsies grown under different culture conditions (adherent and spheroid) and evaluated the efficacy of PAAV-mediated TRAIL gene delivery in DIPG cells. We demonstrated that all 6 DIPG cell cultures express the $\alpha v\beta 3$ and $\alpha v\beta 5$ integrin receptors and TRAIL death receptors which are crucial for the success of our therapy. Next, we showed that our targeted RGD4C/PAAV vector was able to deliver the reporter gene Lucia (secreted luciferase) with high transduction efficacy. Next, we showed the transduction of HSJD-DIPG-07 DIPG cells with our prototype (RGD4C/PAAV-TRAIL) was able to induce significant DIPG cell death. Finally, the efficacy of our prototype was tested for the treatment of DIPG *in vivo* through intravenous administration. RGD4C/PAAV-TRAIL showed selective tumour homing and prolonged animal survival. In this thesis, we demonstrate that RGD4C/PAAV provides targeting of TRAIL expression selectively to the tumour environment, ensuring systemic safety and therapeutic efficacy.

Acknowledgements

I would like to express my heartfelt gratitude to all those who have guided me one way or another in this work. This would not have been possible without the support of many.

To my supervisor Dr Amin Hajitou, for offering me the opportunity to work in his Phage Laboratory. He has been incredibly supportive throughout, even in challenging times such as during the pandemic and when I faced personal difficulties.

To my co-supervisor Dr Keittisak Suwan, who has provided me with guidance in day-to-day work in the laboratory and was always available to provide a listening ear.

To the Phage Therapy Group, Dr Mike Asavarut, Dr Mariam Al-Bahrani, Dr Sajee Waramit for their kindness and friendship along the way.

To the faculty education office, occupational health team, personal tutor and senior tutors for coordinating my deadline extension and supporting me.

To A*STAR, for awarding me the scholarship to attend medical school and funding my PhD.

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List of Abbreviations

DIPG	Diffuse Intrinsic Pontine Glioma
PAAV	Phagemid Adeno-Associated Virus
AAV	Adeno-Associated Virus
TRAIL	Tumour Necrosis Factor-Related Apoptosis Inducing Ligand
CNS	Central Nervous System
MRI	Magnetic Resonance Imaging
XRT	Radiotherapy
MS	Median Survival
OS	Overall Survival
WHO	World Health Organization
TMZ	Temozolomide
PCR2	Polycomb Repressive Complex 2
MGMT	O6- Methylguanine DNA Methyltransferase
BBB	Blood Brain Barrier
ECs	Endothelial Cells
CMT	Carrier-Mediated Transport
CED	Convection-Enhanced Delivery
PTEN	Phosphatase And Tensin Homolog
HSV-tk	Herpes Simplex Virus Thymidine Kinase
CAR	Chimeric Antigen Receptor
T-VEC	Talimogene Laherparepvec
GM-CSF	Granulocyte-Macrophage Colony-Stimulating Factor
GBM	Glioblastoma
LGG	Low-Grade Glioma
RES	Reticuloendothelial System
DNA	Deoxyribonucleic Acid
RNA	Ribonucleic Acid
siRNA	Small Interfering Ribonucleic Acid
Ad	Adenovirus
AAV	Adeno-Associated Virus
ss	Single-Stranded
ALL	Acute Lymphoblastic Leukaemia
X-SCID	X-Linked Severe Combined Immunodeficiency
Ad	Adenovirus
ds	Double-Stranded
kb	Kilobases
ITRs	Inverted Terminal Repeats
CRAds	Conditionally Replicating Adenoviruses
SARS-CoV-2	Severe Acute Respiratory Syndrome Coronavirus 2
HSV	Herpes Simplex Virus
IFN	Interferon
aHUS	Atypical Haemolytic Uremic Syndrome
TU	Transducing Units
AAVP	Adeno-Associated Virus/Phage
CMV	Cytomegalovirus

E. coli)	Escherichia Coli
TNF	Tumour Necrosis Factor
FasL	Fas Ligand
CD95L	Cd95 Ligand
TNF α	Tumour Necrosis Factor Alpha
TRAIL	Tnf-Related Apoptosis-Inducing Ligand
NK	Natural Killer
DD	Death Domain
FADD	Fas-Associated Death Domain
DISC	Death-Inducing Signalling Complex
c-FLIP	Cellular Flice-Like Inhibitory Protein
PARP-1	Poly(Adenosine Diphosphate Ribose) Polymerase-1
FBS	Fetal Bovine Serum
3D	3-Dimensional
BTSCs	Brain Tumour Stem Cells
TSM	Tumour Stem Medium
Trypsin-EDTA	Trypsin-Ethylenediaminetetraacetic Acid
PFA	Formaldehyde
PBS	Phosphate-Buffered Saline
BSA	Bovine Serum Albumin
FACS	Flow Cytometric Analysis
PCR	Polymerase Chain Reaction
rSAP	Recombinant Shrimp Alkaline Phosphatase
2XYT	2x Yeast Extract Tryptone
IPTG	Isopropyl-Beta-D-Thiogalactopyranoside
PEG	Polyethylene-Glycol
NaCl	Sodium Chloride
LB	Luria–Bertani
SRB	Sulphorhodamine B
TCA	Trichloroacetic Acid
qRT-PCR	Quantitative Real-Time Pcr
O.C.T	Optimal Cutting Temperature
RLU	Relative Luminescence Units
lucia	Lucia-Luciferase Reporter
mRNA	Messenger RNA
GFP	Green Fluorescent Protein
tmTRAIL	Transmembrane TRAIL
sTRAIL	Soluble TRAIL
H&E)	Haematoxylin And Eosin
HNA	Human Nuclear Antigen
DAPI	4',6-Diamidino-2-Phenylindole

1. Introduction

1.1 Diffuse intrinsic pontine glioma: the current landscape

Paediatric brain tumours are the leading cause of death from disease in children and are the second most common cancer in children accounting for 29% of childhood cancers in the UK. (National Registry of Childhood Tumours/Childhood Cancer Research Group [Online]).

Diffuse intrinsic pontine glioma (DIPG) are tumours of childhood with universally poor prognoses; despite only constituting for only 15-20% of all paediatric central nervous system (CNS) malignancies, it is the main cause of death due to brain cancer in children (1, 2). DIPG can occur in all age groups but is commonly diagnosed in children between the age of 5 and 10 years (3), more rarely occurring in adolescents and adults. It is an extremely aggressive brain tumour that arises in the ventral pons and is an extensively invasive malignancy with a diffuse infiltrative nature and presents with high-grade features.

Diagnosis is usually based on a combination of radiographic findings and clinical signs. On magnetic resonance imaging (MRI), DIPG has a pathognomonic histological appearance that makes diagnosis reliable. It is classified as an expansile pontine lesion involving at least half of the pons, hypo- to iso-intense on T1-weighted sequences, usually hyperintense on T2-weighted sequences, with minimal contrast enhancement and often encasing the basilar artery (4-6). While clinical signs and symptoms can vary with respect to tumour location and nature of tumour growth, patients typically present with a classic triad of worsening pontine symptoms - cerebellar signs (e.g., ataxia or dysmetria), long-tract signs (e.g., spastic myelopathy, hyperreflexia) and isolated or multiple cranial nerve palsies (unilateral or bilateral) (7). However, only about a third of patients have this triad at presentations, with most developing it later during disease progression (5, 6, 8)

As previously mentioned, DIPG is almost uniformly fatal and more than 90% of children die within 2 years of diagnosis with a median survival of 9 months (1, 2, 9). This is because cytoreductive surgery, a crucial positive predictor in the majority of primary CNS neoplasms, is not possible in DIPG due to its anatomical position in the pontine segment of the brain stem and its highly infiltrative nature (6, 10, 11). Palliative radiotherapy (XRT), usually at

54Gy over 6 weeks, remains the sole standard therapy offered to patients to improve survival and provide temporary relief of neurological symptoms, albeit only for a limited time as recurrence is rapid and unremitting, usually measured in weeks (6, 12). Median survival (MS) following XRT is 11 months, overall survival (OS) at 1 year is 30%, 10% at 2 years, with only occasional reports of survival 5 years following diagnosis (13, 14).

Histologically, DIPGs exhibit similarities to World Health Organization (WHO) grade III and IV astrocytomas found in other brain regions or in adults (15). As such, there is a persistent assumption that this paediatric cancer would respond similarly to treatments validated in their adult counterparts (16). Disappointedly, repurposing chemotherapy regimens that have had some modest success in adult brain cancers for children with DIPG has by the large showed no meaningful improvement in prognosis (Table 1). Over 250 clinical trials were conducted over the past decade, using conventional chemotherapy, concurrent chemoradiotherapy and several targeted therapies (15), however, these have consistently failed to demonstrate clinical benefit meaningfully superior to radiotherapy, with the MS ranging from 6.25 to 15 months (6, 13-15, 17-22). For example, temozolomide (TMZ), given both concurrently and after XRT, failed at achieving any survival benefit and was instead associated with significant comorbidities (21, 22). Other conventional chemotherapeutic agents produced a similar outcome: not only did they fail at prolonging survival, but often resulted in the development of significant haematological side effects (23-27).

This suggests that conclusions drawn from studies on adult brain cancers cannot be directly extrapolated to paediatric patients, emphasising the necessity for specific research focused on DIPG patients (16).

First author	Year published	Therapy	MS (months)	OS at 1 year (%)
Bailey	2013	TMZ (long regimen)	9.5	35
Bradley	2013	Motexafin-gadolinium, XRT	11.4	53
Chassot	2012	TMZ	11.7	50
Kim	2009	TMZ, thalidomide	12.7	58.3
Korones	2008	Vincristine, VP-16	9	27
Massimino	2008	Etoposide, cytarabine, ifosfamide, cisplatin, dactinomycin	12	45
Massimino	2008	Cisplatin/etoposide, cyclophosphamide/vincristine/met hotrexate	13	70
Massimino	2008	Cisplatin/etoposide, isotretinoin	9	29
Massimino	2008	Vinorelbine	9	43
Sirachainan	2008	TMZ, cis-retinoic acid	13.5	58
Broniscer	2013	Dasatinib/PDGFR, vandetanib/VEGFR	15.0	52
Michalski	2010	Tamoxifen/Estrogen receptor	6.3	16.1
Narayana	2010	BEV/VEGF, Irinotecan/topoisomerase	6.25	20.0

Table 1. Summary of relevant conventional and targeted therapy-based clinical trials in the treatment of DIPG. Adapted from (28)

The role of MRI in diagnosis, coupled with concerns about increased morbidity and surgical risks resulting from tissue biopsy, has, until recently, limited the availability of samples for preclinical research, impeding progress in biologically driven therapeutic advancements (29-31). Since the 2000s, non-profit foundations have actively supported DIPG research, leading to the establishment of the DIPG Collaborative and its associated DIPG Registry. This registry serves as a comprehensive database containing clinical, radiological, pathological, and molecular data. (10, 32). New stereotactic biopsy and autopsy protocols have also increased the availability of these primary tumour samples, allowing us to be able to describe the genomic landscape underpinning DIPG tumorigenesis using novel genetic and epigenetic

analysis. With this new data, scientific interest in this deadly disease has surged, with over 560 papers published between 1989-2020 (33). These efforts have contributed significantly to advancing the understanding of key oncogenic pathways and identifying high-frequency mutations that represent actionable targets (34-36). The first breakthrough in the understanding of DIPG's biology came in 2012 with the discovery of histone mutations that were present in up to 80% of tumours (2, 20, 37-39). This mutation differentiated DIPG from other paediatric and adult gliomas and play a pivotal role in pathogenesis (35, 40). The H3K27M histone mutation is a result of a conversion of lysine to methionine at residue 27, impairing polycomb repressive complex 2 (PCR2), leading to global hypomethylation (13). In 2016, following the updated WHO classification of brain tumours, the identification of the H3K27M mutation alone was deemed adequate to categorise tumours containing it as grade IV (41).

Additional somatic histone mutations were eventually identified and these often occurred in a mutually exclusive manner, allowing for a novel classification of paediatric gliomas based on epigenetic factors (42). The H3K27M mutation is thought to arise first and are almost always associated with specific, high-fidelity obligate partner partners throughout disease initiation and its spread, highlighting its importance as a potential oncogenic driver and mutual need for tumorigenesis (39, 43). These ubiquitously associated mutations either involve aberrations in the TP53 cell-cycle (TP53/PPM1D) or specific growth factor pathways (ACVR1/PIK3R1) (20, 35, 39, 44-47).

The significance of the unique genetic makeup of DIPG is highlighted when conventional therapeutic drugs that have been historically successful in other brain cancers are trialled. An example is TMZ, used in adult GBM patients to prolong survival, failing to yield any therapeutic advantage in DIPG through multiple clinical trials (15). TMZ acts primarily through the inactivation of DNA repair protein O6-methylguanine DNA methyltransferase (MGMT). The inefficacy of TMZ in DIPG patients may be attributed to the limited presence of MGMT methylation, found in only 3% of tissue samples with K27M expressing tumour cells (48). This discrepancy underscores the importance of a biological rationale in guiding clinical decision-making (49).

This improved understanding of the tumour biology of DIPG subsequently facilitated the development of various murine models, with the ability of replicating distinct aspects of

DIPG's biological characteristics (13, 17, 50, 51). Furthermore, a multitude of patient-derived cell lines have become available; mouse models, generated through cancer vectors or xenografts, allow for the study of novel therapeutics, and tumour cell labelling has also enabled a direct approach to assessing response (6).

1.2 New therapeutic approaches

A significant challenge in developing effective treatments for gliomas lies in ensuring that therapeutic agents achieve sufficient concentrations at the tumour site for effective therapy (52).

This is due to the presence of the blood brain barrier (BBB), a complex and highly selective physiological interface that separates the circulating blood from the CNS and serves as a critical regulator of molecular exchange between the blood and the brain parenchyma (53). It has a specific architecture where specialised endothelial cells (ECs) share a common basement membrane with pericytes, and the overall cerebral capillaries are covered by astrocyte endfeet (16, 54, 55). This is further reinforced by a complex of tight junction proteins that seal intercellular spaces, thus prevents blood cells, plasma components, and pathogens from entering the brain (56). Therefore, substances entering the brain parenchyma typically need to traverse both the luminal and abluminal plasma membranes of endothelial cells (56-58). There are mechanisms to traverse the BBB, which include carrier-mediated transport (CMT), paracellular transport, receptor-mediated transport and absorption-mediated transport (19) (Figure 1). Most small lipophilic substances are also able to pass through the BBB by transcellular transport, but this process is restricted by metabolic barrier properties, which include efflux transporters (59, 60) and drug-metabolising enzymes such as detoxification enzymes (e.g., monoamine oxidase, cytochrome P450) (61), commonly found in various organs and also present at the BBB (16). These selective properties function as a protective barrier for brain cells against neurotoxic compounds while simultaneously presenting a formidable obstacle for the effective delivery of therapeutic drugs to the brain parenchyma (19, 54, 59, 62-64).

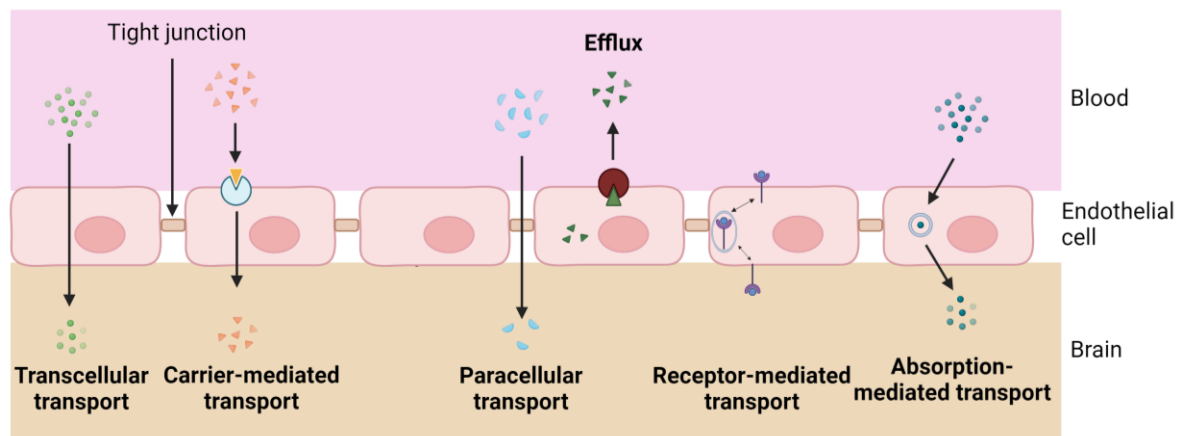


Figure 1. The blood brain barrier (BBB). The BBB regulates entry of nutrients to the brain and prevents entry of cells and large molecules via tight junctions. There are several mechanisms for transporting molecules across the BBB, including carrier-mediated transport, paracellular transport, receptor-mediated transport, and absorption-mediated transport. Created with BioRender.com

While the BBB is often disrupted in the context of advanced brain tumours (65), as indicated by contrast enhancement, it is frequently intact in DIPG, potentially explaining its lack of contrast enhancement and drug inaccessibility via systemic routes (5, 19, 31, 66, 67). Traditionally, to overcome this issue, drug dosage has been increased to potentiate greater amounts of tumour penetration but the necessary dose escalations for intra-tumoural efficacy often result in systemic side effects that impede therapeutic efforts (6, 18, 19).

Various innovative strategies have been developed to bypass or permeate the BBB, each tailored to overcome specific limitations associated with traditional drug delivery methods. Transient BBB disruption can be achieved through chemical (e.g., mannitol (68, 69)) or mechanical means (e.g., focused ultrasound (70, 71)), but the window of increased permeability is relatively short-lived and inconsistent (70, 71). Intra-arterial delivery involves injecting therapeutic agents directly into the arteries feeding the brain, often in conjunction with BBB-disrupting methods, relies on the extraction of agents from the circulation by the tumour during the first pass but requires sophisticated techniques to target specific areas without causing tissue damage (68, 72). Local drug delivery, utilising convection-enhanced delivery (CED), employs intracranial catheters to continuously administer a drug-containing infusate into the interstitial space, propelled by sustained, low hydraulic pressure (28). While

shown to be capable of delivering high drug concentrations and minimizing systemic exposure, thereby reducing toxicity (73), it remains an invasive surgical procedure limited by the accessibility of target brain regions and is typically performed in a single session. The duration of therapeutic drug concentrations after a single CED session remains unknown, raising concerns about their potential effectiveness before clearance from the target region (74). CMT utilises nanoparticles, liposomes, and similar carriers to traverse the BBB to deliver a broad range of therapeutic agents, including small molecule drugs, peptides, proteins, nucleic acids, and nanoparticles. It does so by mimicking endogenous transport mechanisms, evading efflux pumps, and engaging in receptor-mediated transcytosis (75). However, a major limitation of current CMT approaches is their lack of tumour-targeting specificity, which can lead to off-target effects and significant CNS toxicities (73).

1.3 Use of gene therapy for paediatric brain tumours

Due to the inefficient drug delivery across the BBB and development of drug resistance, gene therapy was envisioned as a promising strategy to overcome limitations of conventional therapies. Initially conceived for the treatment of congenital disorders, it is now applied in a variety of diseases including cancer (76). There are a few strategies that have been researched which are discussed below.

One approach is the delivery of functional copies of key tumour suppressor genes, such as p53 and phosphatase and tensin homolog (PTEN) which are often inactivated in cancer, through vector-mediated introduction into cancerous cells to reinstate their tumour control mechanisms (77, 78). However, ensuring sustained therapeutic gene expression and targeting infiltrative tumour cells remains challenging. Another method is via suicide gene therapy, which introduces genes encoding enzymes that convert non-toxic prodrugs into active cytotoxic agents within tumour cells, resulting in selective killing of cancer cells while sparing normal tissues. An example of this is the herpes simplex virus thymidine kinase (HSV-tk) gene used in conjunction with the prodrug ganciclovir (79-82). By delivering the HSV-tk gene into cancer cells via vectors, it can convert the subsequently administered ganciclovir into a toxic compound which enables selective tumour cell killing. However, this necessitates efficient gene delivery and careful management of potential bystander effects (83). Immunotherapy via genetically modified chimeric antigen receptor (CAR) T-cells uses the patient's own T-cells to express receptors targeting brain tumour-specific antigens (84). It

offers potent and specific anti-tumour potential but faces obstacles like off-target toxicity, overcoming immunosuppressive tumour environments, and ensuring CAR T-cell persistence (85). Lastly, oncolytic virotherapy uses genetically modified viruses like modified herpes simplex virus or adenovirus, to selectively infect and replicate within brain tumour cells to cause direct cell lysis and trigger an anti-tumour immune response. An example is talimogene laherparepvec (T-VEC), an oncolytic herpes simplex virus approved for the treatment of advanced melanoma that cannot be surgically removed (86). Through direct intra-tumoral injection, T-VEC preferentially infects and replicates within malignant melanoma cells, leading to cell lysis and the release of tumour-derived antigens. Concurrently, T-VEC has been designed to express granulocyte-macrophage colony-stimulating factor (GM-CSF), a cytokine that enhances the antigen-presenting capabilities of dendritic cells, thereby amplifying the host immune response against the tumour. T-VEC's effectiveness is limited to accessible tumours due to the need for direct intra-tumoral injections, and its impact on distant metastases and considerations for viral spread and pre-existing immunity are key research areas to enhance its efficacy (87-89). Additionally, these strategies can be used to improve efficiencies of co-administered treatments such as chemotherapy and radiotherapy, and directly induce death in tumour cells (90).

1.4 Non-viral vectors

A key challenge in gene therapy is the efficient delivery of genes to target cells and tissues, which is achieved using specialised carriers called vectors. Careful vector selection is critical for successful gene therapy, requiring consideration of important factors such as transgene expression, distribution within the tumour microenvironment, immunogenicity, and safety profiles (91-93). Since their initial development in the 1990s, the utilisation of both viral and non-viral vectors for gene transfer has exhibited promising efficacy within pre-clinical research settings (94), in brain cancers such as glioblastoma (GBM) and low-grade glioma (LGG) (95). However, their clinical translation remains complicated by many challenges (96).

To exert therapeutic effects within the brain, nucleic acid-based treatments must surmount multiple barriers, including degradation by nucleases, systemic clearance, reticuloendothelial system (RES) uptake before successfully traversing the BBB (97). Following sufficient diffusion within the brain and tumour penetration, the therapeutic gene must then undergo

cellular endocytosis and escape from endosomal degradation before reaching its intracellular target (98).

Non-viral vectors, comprised of synthetic or naturally derived materials, encapsulate or complex with genetic material for cellular delivery via a wide range of physical and chemical techniques in a method known as transfection. Physical methods involve transient cell membrane disruption using mechanical forces, electrical pulses, ultrasound (sonoporation), hydrodynamic pressure, or laser-based energy to facilitate entry of genetic material into target cells.

More commonly, genetic material is delivered via chemical systems, where negatively charged nucleic acids are transported by carriers such as lipid-based vectors (liposomes, cationic lipids), polymeric vectors (polyethylenimine, dendrimers), or inorganic nanoparticles (gold, calcium phosphate, silica-based). These complexes primarily enter cells through endocytosis (99). Hybrid systems combining these approaches are also under development. Non-viral vectors often exhibit lower immunogenicity, simplified manufacturing, and greater versatility for modification compared to viral vectors (97). However, limitations such as reduced transfection efficiency, instability in biological environments, and challenges in cell-specific targeting persist.

1.5 Mammalian viruses (lentivirus, adenovirus, adeno-associated virus)

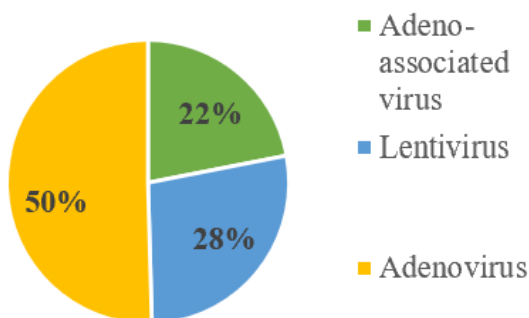
In contrast, viral vectors have a natural affinity for mammalian cells and thus can infect cells with great efficiency. After modification and removal of vital sequences of their genomes to hinder replication, vectors can be made safer for use in tumour cell targeting and oncolytic therapy (97). Most research has focused on the use of eukaryotic viruses as vectors as they provide superior transgene delivery (100-102). However, despite their potential, clinical approval for viral vectors faces challenges. Briefly, these include manufacturing complexities, high costs, immunogenicity, inflammatory risks, the potential for oncogenic mutations, and limited cargo capacity (103-106). Their wide tropism for mammalian cell-membrane receptors (107) can lead to undesired uptake by the liver, reticuloendothelial system and unwanted tissues, as well as marked immunogenicity that can cause induction of the inflammatory system leading to degeneration of transduced tissues (102, 106, 108).

Overcoming these limitations is crucial for unlocking the full therapeutic potential of viral vectors in brain tumour treatment.

A viral vector is typically defined by three core components: the capsid and/or envelope, which is the protein structure that encapsidates the genetic payload and determines the vector's tissue or cell tropism and antigen recognition;; the transgene, which is the therapeutic gene of interest designed to induce a desired effect when expressed within the target cells; and the regulatory cassette, which comprises genetic elements (enhancer, promoter, etc.) that control the transgene expression, determining whether it remains episomal (non-integrated) or integrates into the host cell's genome, as well as the duration of expression (109).

Design strategies for these components vary across different viral vector platforms. The three most widely used viral vectors are lentivirus, adenovirus (Ad), and adeno-associated virus (AAV) (109) (Figure 2), each with unique considerations, advantages, and limitations. Understanding their distinct characteristics is crucial for selecting the optimal vector for specific therapeutic applications.

a Number of clinical trials



b

Vectors	Number of clinical trials
Adeno-associated virus	250
Lentivirus	315
Adenovirus	575
Total	1140

Figure 2. Summary of the most used viral vectors in clinical trials.

a Pie chart showing the percentage of adenovirus, adeno-associated virus, or lentivirus vectors in use in clinical trials. **b** Table showing the number of clinical trials currently employing different viral vector platforms. Adapted from (109).

1.5.1 Retroviruses and lentiviruses

Lentiviruses, a genus within the Retroviridae family, are enveloped, single-stranded (ss) Ribonucleic acid (RNA) viruses approximately 100nm in diameter (110). Their viral particle encapsulates two sense-strand RNAs, along with key proteins: nucleocapsid, reverse transcriptase, integrase, and protease (109). The principal characteristic of retroviruses, including lentiviruses, is their ability to transduce mitotic cells due to their passage through nuclear pores (111). Notably, lentiviral vectors can also transduce post-mitotic and quiescent cells, whereas other retrovirus-based platforms, such as gammaretroviral vectors, require active cell division for successful infection (109, 112).

The entry of infectious lentiviral particles into host cells is mediated by interactions between envelope glycoproteins and specific cell surface receptors. Upon successful binding, the viral lipid bilayer fuses with the cell membrane, releasing the viral genetic cargo into the cytoplasm (113, 114). Their genome then undergoes reverse transcription into Deoxyribonucleic acid (DNA), which is then efficiently integrated into the target cell genome. This integration is in a non-random manner favouring transcriptionally active sites and enables long-term transgene expression, making them ideal for applications requiring permanent gene transfer (119-121).

The lentiviral genome allows for relatively easy manipulation into replication-defective vectors. Their structure, with minimal overlap between *cis*-acting regulatory sequences and *trans*-acting protein-coding regions, facilitates the removal of viral protein-coding genes without significantly impacting the essential regulatory sequences needed for the therapeutic gene's function. This creates approximately 8 kilobases (kb) of space for a transgene expression cassette (99, 115), potentially accommodating multiple genes within a single vector (116-118).

Lentiviral vectors have revolutionized cancer immunotherapy, particularly in the development of CAR-T cell therapies. Their ability to integrate into the genome allows for the stable expression of engineered CARs in T cells. This approach has achieved remarkable success in treating relapsed and refractory acute lymphoblastic leukaemia (ALL), with remission rates as high as 90% (119). Kymriah (tisagenlecleucel), a CAR-T therapy based on lentiviral vectors, became the first FDA-approved treatment for paediatric B-cell ALL (119).

Numerous clinical trials continue to explore the use of lentiviral vectors for various diseases, including metabolic, immune, and congenital disorders (109).

Despite their potential, retroviruses vectors carry significant limitations. Their integration into the host genome poses a risk of insertional mutagenesis, where activation of proto-oncogenes can lead to cancer development (111). An early clinical trial that used a gammaretroviral vector to treat X-linked severe combined immunodeficiency (X-SCID) led to serious adverse effects where four out of the nine patients who received the treatment showed uncontrolled clonal T cell proliferation following an undesired transcription of genes after integration of the retrovirus vector next to the LMO2 gene and went on to develop T-cell leukaemia (120, 121).

Lentiviral vectors are favoured for gene delivery due to their reduced risk of insertional mutagenesis compared to gammaretroviral vectors (122-124). While third-generation, self-inactivating lentiviral vectors further improve safety, there remains a risk that they could activate nearby genes, highlighting the ongoing need to refine vector design (125). Other limitations of retroviral vectors include their low efficiency *in vivo* and other immunogenic problems (99, 126).

1.5.2 Adenoviruses

Ad is a large and complex, non-enveloped, double-stranded (ds) DNA virus. It is 70 to 90 nm in size and possesses an icosahedral protein capsid that accommodates a 26–45 kb linear, dsDNA genome (127). Ad are ubiquitous: they have been isolated from a large number of different species and over 100 serologically different types of Ad exist, among which 49 types infect humans (128). Based on the specificity of the Ad, it may attach to different proteins on the cell surface to gain entry into the target cells (129). It is able to infect a broad range of cell and tissue types because its cell entry is primarily mediated by the interaction of the viral knob domain with the coxsackie and adenovirus receptor, expressed on most human cells (91, 129-131). Additionally, they can utilise endocytosis after interacting with cell surface integrins (91).

Ad was the first DNA virus to undergo comprehensive therapeutic development, favoured for its clearly understood biology, ease of genetic manipulation, considerable capacity for

transgenes (up to 36 kb), simplicity of mass production, and a beneficial safety profile (127). The effectiveness of Ad as tools for gene therapy has led to the approval of more than 450 clinical trial protocols (127). The most commonly used serotypes in gene therapy are the human Ad serotype 5 and serotype 2, which are common cold viruses that most adults have been exposed to – circulates in humans with a seropositivity rate of 40–60% (132). As such, they have low pathogenicity with few, and mild side symptoms associated with the common cold (97).

Ad vectors were initially produced by deletion of E1 and E3 regions, which makes them replication-defective and are replaced by the expression cassette, allowing them to deliver large DNA sequences (optimum size of therapeutic genes is around 7-8 kb) (91, 133). Three generations of Ad vectors were developed, with increasing transgene capacity - the final generation is referred to as the helper-dependent or gutless Ad, and has all of their viral sequences deleted, except for the inverted terminal repeats (ITRs) and the packaging signal (127). These third-generation Ad vectors exhibit significantly reduced *in vivo* immune response compared to earlier-generations while maintaining high transduction efficiency and tropism (134).

Ad vectors have an episomal genome, which minimises the risk of insertional mutagenesis (126). However, this also means their gene expression is transient and limits Ad gene therapy to treatment strategies in which only temporary protein expression is needed (135). For chronic conditions, treatments would have to be repeated at specific intervals. Efficient gene transfer is also difficult upon a second administration of the virus due to production of neutralising antibodies. In addition, the natural tropism of Ad makes it difficult to control the systemic delivery of vectors *in vivo*. Natural and acute immunologic responses against Ad have made their clinical application limited to a few tissues, such as the liver, lung, or localised cancer gene therapy (99, 136). One example is Oncorine (H101), an oncolytic Ad which received approval in China for nasopharyngeal carcinoma in combination with chemotherapy (137). However, of particular concern, there was a case where an Ad serotype 5 vector administered during a gene therapy trial triggered lethal systemic inflammation in the patient, meaning that this innate and pre-existing immunity to Ad vectors continues to pose a significant challenge (138).

For applications requiring transient gene expression, such as stimulating an anti-cancer immune response or triggering apoptosis, non-integrating delivery vehicles offer a suitable solution. Conditionally replicating Ads (CRAds) are developed for this purpose - they provide a potent dual mechanism for enhanced tumour penetration and localised amplification of anti-tumour effects. First, they directly induce oncolysis through their replication and lytic cycle, reducing tumour mass. Secondly, CRAds can act as delivery vehicles for therapeutic genes, augmenting tumour cell destruction and stimulating robust anti-viral and anti-tumour immune responses (127).

These days, Ad have had more success in the development of vaccines, in fact, four types of Ad vaccines for the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) have been approved since the global pandemic (139).

1.5.3 Adeno-associated viruses

AAV was initially discovered as a contaminant in adenovirus preparations (140). It is a small non-enveloped ssDNA virus from the non-pathogenic parvovirus family (92). It has an icosahedral capsid composed of three types of structural proteins, VP1-3, and has a ssDNA of approximately 4.8kb in length (141). AAVs are replication-deficient and traditionally require co-infection with a helper virus, such as Ad or Herpes simplex virus (HSV), for efficient infection inside the host cell (142, 143). On their own, AAVs are thought to be non-pathogenic and are yet to be concretely associated with any disease in humans.

AAV vectors are favoured for *in vivo* gene therapy due to several advantages. They can transduce both dividing and quiescent cells and achieve robust transduction efficiency *in vivo*. After entering the cell, the virus's genome forms episomal concatemers within the host cell nucleus, ensuring stable transgene expression over extended periods, particularly in non-dividing cells like neurons (144). There are at least 12 natural AAV serotypes, each exhibiting different tissue tropisms, which enhances targeted delivery options (127). Their relatively low immunogenicity, non-pathogenicity, and established clinical safety record further solidify their therapeutic value (127, 145).

AAV's small genome aids in rapid penetration into solid tumours like gliomas (146). Specifically, it was reported that a single intracranial injection of AAV encoding human

interferon (IFN)- β in human and murine GBM models increases tumour cell death and promotes long-term survival (147). Many researchers have developed high-efficiency AAV for GBM cells, by selection in culture of a chimeric AAV capsid library generated by DNA shuffling of different *cap* genes, with several different AAV serotypes (148, 149). AAV vectors have shown potential in treating a variety of neurological disorders, including genetic diseases, neurodegenerative disorders, and brain tumours. Some approved gene therapies on the market include Luxturna (voretigene neparvovec-rzyl) for the treatment of Leber congenital amaurosis and Hemgenix (etranacogene dezaparvovec) for the treatment of Haemophilia B (99). The ability of AAV vectors to efficiently transduce neurons and other cells in the CNS, combined with their safety profile and capacity for sustained gene expression, makes them an attractive option for CNS therapies (68).

One key limitation of AAV vectors is their limited transgene capacity. To address this, up to 96% of the AAV genome can be removed for therapeutic gene insertion (150). However, their production remains complex and often results in low viral yields and high costs (151). While primarily episomal, AAV integration into chromosome 19 can occur at a 1-3% frequency at high viral doses or in the presence of adenoviral replicase which can result in disruption of gene regulatory regions or unintended activation of nearby genes (152). Cell gene expression can also induce toxicity or unwanted immune responses, particularly in off-target tissues. While the capsid proteins of AAV vectors offer some tissue specificity, systemic diseases often necessitate high doses for clinical trials to achieve sufficient transgene expression in the target cell populations (153). These high doses increase the risk of severe adverse effects like hepatotoxicity (154), neurotoxicity (155), atypical haemolytic uremic syndrome (aHUS) (156), and even death in critical cases (157-160). Increased target specificity of AAVs would reduce the necessary viral dose and minimise off-target adverse effects. Despite these limitations, AAV vectors nonetheless hold great potential to revolutionize the clinical management of human diseases.

Table 2 provides a comparative overview of lentivirus, Ad and AAV, highlighting their distinct properties, strengths, and limitations as gene therapy vectors.

Vector	Genome	Cloning capacity	Advantages	Disadvantages
Lentivirus (a type of retrovirus)	Enveloped, single-stranded RNA genome, reverse-transcribed into DNA upon cell entry	~8kb	Long-term expression	Insertional mutagenesis
			Broad tropism	Complex and costly production
				Immune reactions
Adenovirus	Non-enveloped, double-stranded DNA	Up to 8kb (early generation), up to 36kb possible with modifications	High transduction efficiency and robust transgene expression.	Highly immunogenic, limiting repeat administrations
			Broad cell tropism.	
			Diverse applications, can be designed as replicative or oncolytic vectors	Transient gene expression
			Easy of manipulation and high-titer production	Pre-existing immunity can lead to vector clearance or adverse reactions
Adeno-associated virus	Non-enveloped, single-stranded DNA genome	~4.7kb	Low immunogenicity	Limited packaging capacity
			Diverse natural serotypes with varying tissue tropisms.	Risk of integrate into the host genome at a low rate
			Relatively safe profile.	Difficult and expensive to manufacture.

			Small size allows penetration into solid tumours	Pre-existing immunity can lead to vector clearance
			Long-term gene expression, particularly in non-dividing tissues	

Table 2. Summary of key characteristics for three commonly used viral vectors in gene therapy: lentivirus, Ad and AAV.

1.6 Bacteriophage vectors

While the most popular vehicles used in gene therapy are eukaryotic viruses, increasing interest surrounds the use of prokaryotic viruses, or bacteriophages. As obligate parasites of bacteria, bacteriophages are the most abundant biological entities on Earth (161). Studies demonstrate their potential as vectors for targeted delivery of genes and drugs to cancer cells after systemic administration (108). Examples include the successful delivery of tumour-suppressing non-coding RNAs to hepatocarcinoma cells and cytochrome P450 to cervical carcinoma cells which induced targeted tumour cell death (162-164). Furthermore, research into bacteriophage-based cancer vaccines offers a promising avenue for future immunotherapies (165).

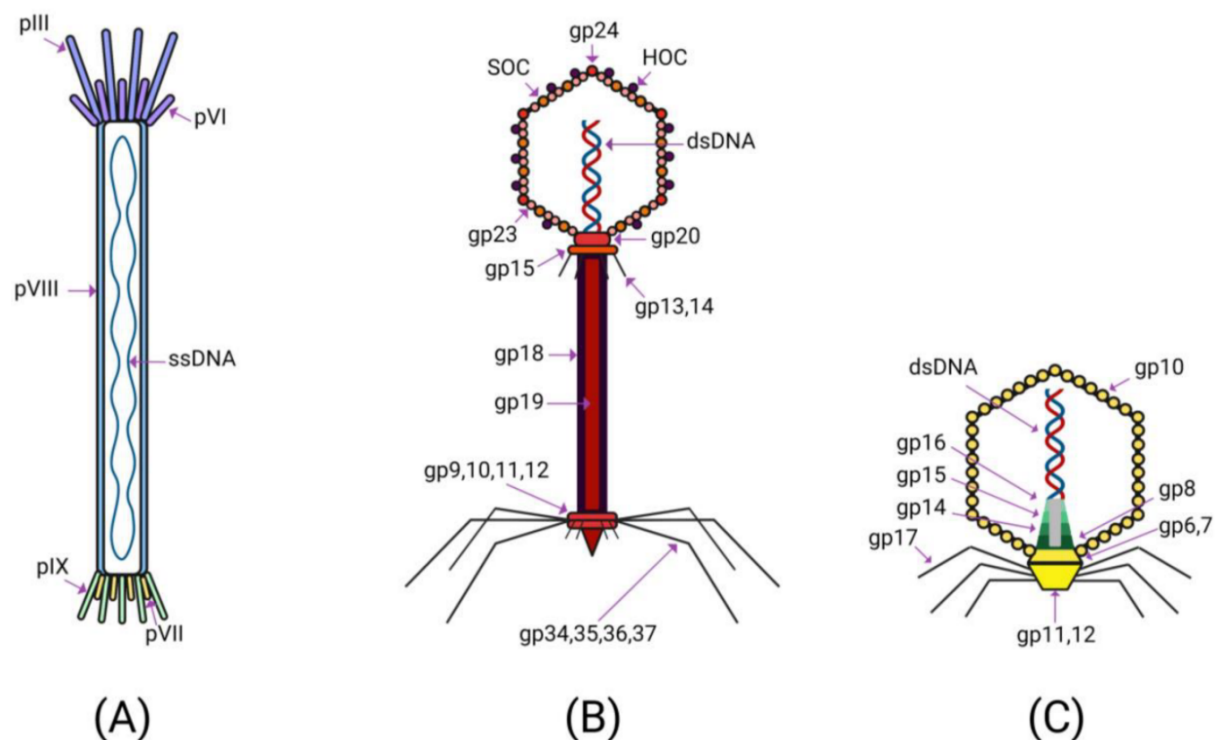


Figure 3. The structures of (A) M13 phage, (B) T4 phage and (C) T7 phage. Source from (166).

Bacteriophage particles possess well-defined three-dimensional structures, typically comprising a capsid head harbouring their genetic material (ss or ds, DNA or RNA) and a tail (Figure 3) (161, 167). A crucial structural element is the baseplate at the tail's terminus, bearing thin, elongated fibrils that facilitate initial attachment to the bacterium (168). The

baseplate contains receptor-binding proteins that recognize specific molecules on the bacterial surface. Phage-bacteria interactions exhibit high specificity, as the host must express the appropriate surface receptor to enable phage binding and internalisation (169).

Among commonly used bacteriophages, the filamentous M13 bacteriophage (referred to as phage) is exceptionally well-suited for phage display-based technology. This method involves inserting foreign genes into the phage genome, resulting in the display of encoded proteins on its surface. Researchers have leveraged this technique to identify receptors selectively expressed in tumour tissues and subsequently employed these phages to deliver therapeutic genes (100).

The potential of phage for DIPG research stems from their ability to cross the BBB (170-172). A landmark study in the 1940s demonstrated the therapeutic efficacy of intraperitoneally administered in treating bacterial infections, including meningitis, indicating that there was effect on the brain (170). While this finding is significant, it's important to note that the BBB may be compromised by inflammation during meningitis. This observed effect suggests the potential for BBB crossing but warrants further investigation into phage permeability under intact BBB conditions. More recently in the 2000s, a study exploring Alzheimer's disease treatment demonstrated the use of filamentous phage to deliver anti-beta amyloid antibody fragments intranasally (171). Subsequent brain section imaging revealed phage presence associated with plaques (Figure 4). Despite this evidence of brain targeting, the overall phage levels remained low (171).

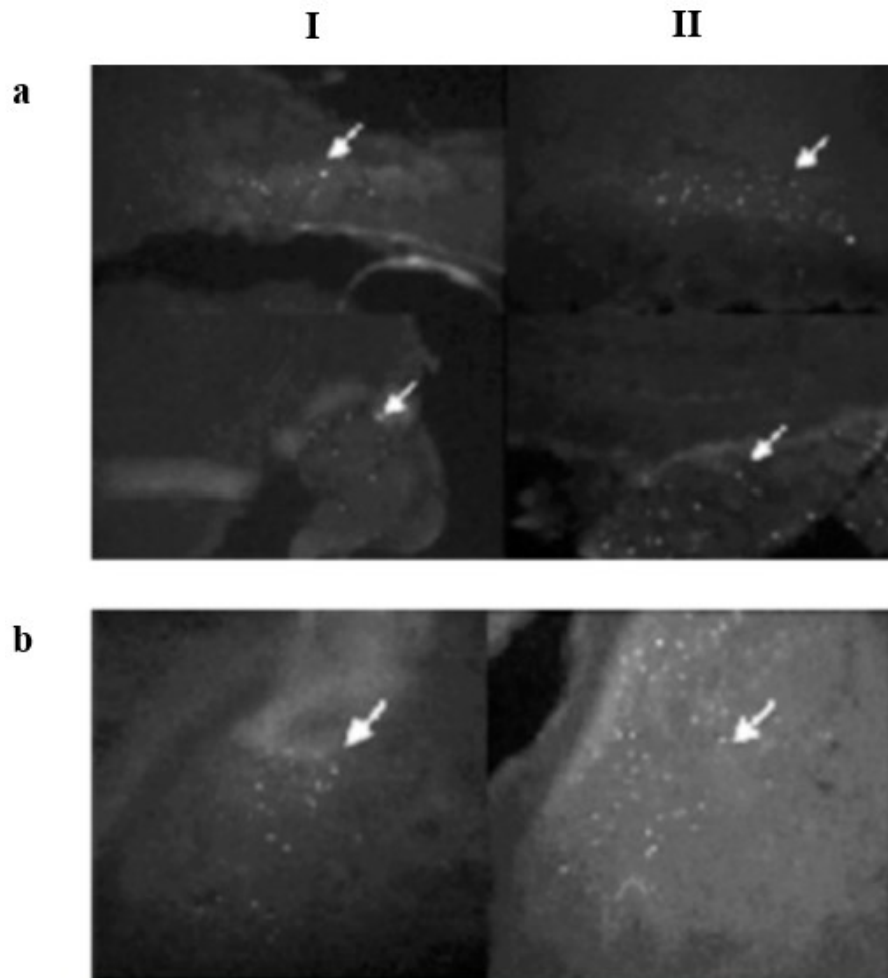


Figure 4. Immunofluorescence detection of filamentous bacteriophage in BALB/C brain sections. The presence of linear filamentous bacteriophage in mice olfactory bulb sections (a) and hippocampus region (b) were detected after one administration (I) and after three daily administrations (II) of the wild-type phage (indicated by arrows). The sections were observed on a fluorescence microscope at a magnification of x20. This shows that intranasal administration of filamentous bacteriophage in mice resulted in accumulation of phage in brain. Source from Frenkel and Solomon (171).

A 2019 study on human glioblastoma reaffirmed the potential for phage to cross the BBB. Researchers demonstrated that phage-targeted gene therapy, combined with systemic temozolomide, suppressed orthotopic GBM growth in mice more effectively than either treatment alone (173). In this study, the phage was genetically engineered to display a tumour-specific ligand for display (further discussed below), allowing it to target and hone to the brain tumour. Immunofluorescence staining confirmed that after systemic (tail vein)

administration, the phage accumulated specifically within the tumour tissue and not in surrounding healthy brain tissue (Figure 5). This finding strongly suggests the phage's ability to cross the BBB and selectively target the tumour.

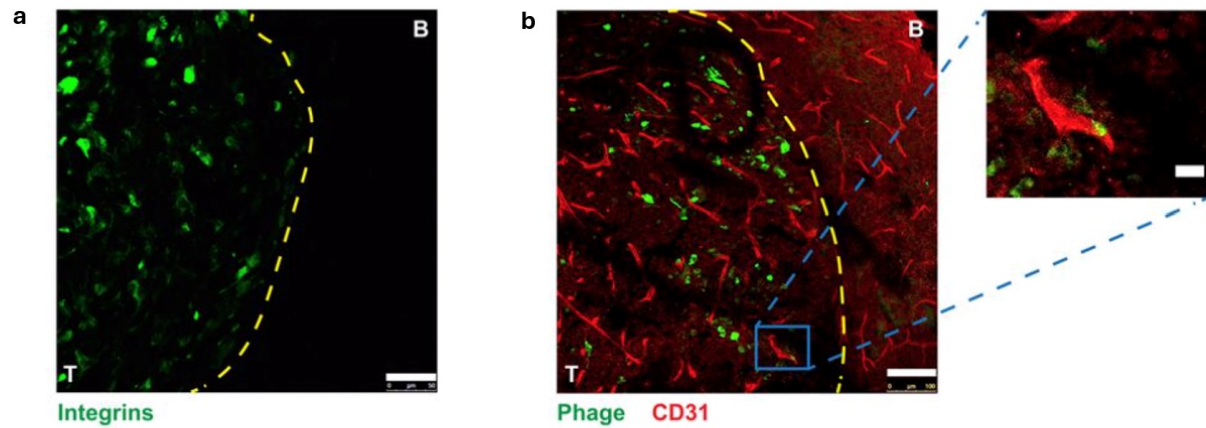


Figure 5. Administration of phage vector to an orthotopic model of human GBM. Brain tumours were established in immunodeficient nude mice by intracranial implantation of U87 glioblastoma cells. Subsequently, 5×10^{10} transducing units (TU) of phage was administered to mice via tail vein. At 18 h after vector administration, tumour-bearing nude mice were killed, and tumours and control organs were collected.

a. Immunofluorescence staining of α_v integrin (green) in frozen brain section including the intracranial tumour (T) but not in the surrounding healthy brain (B), demarcated by the yellow dashed line. Scale bar, 100 μm .

b. Co-staining of CD31 (red) and phage (green). CD31 is primarily located on the surface of endothelial cells and hence represent the presence of blood vessels. Phage particles are seen in the intracranial tumour (T) but not in the surrounding healthy brain (B), demarcated by the yellow dashed line. Scale bar, 100 μm

In addition, phage can be found distributed on blood vessels indicated on the higher magnification view. Scale bar, 25 μm

Source from Przystal et al (173)

1.7 Ligand-directed targeting

Phage possess advantages such as ease of modification, cost-effectiveness, and the ability to be produced in high titers. However, their lack of natural tropism toward mammalian cells presents a significant limitation for their direct use in mammalian cell transduction (100).

By combining the favourable biological attributes of eukaryotic and prokaryotic viruses, a chimeric virus vector was constructed comprising of recombinant AAV and M13-derived filamentous phage, named AAV/Phage (AAVP) (100). The genome of this vector was made by inserting the AAV transgene cassette into an intergenomic region of the phage, regulated by the cytomegalovirus (CMV) promoter flanked by AAV serotype 2 full-length ITRs. This vector is engineered to display selective ligand peptide motifs on its coat proteins to allow binding to targeted cells and subsequent internalisation of ligand-directed delivery vectors (174) (Figure 6). The pIII coat proteins of the phage was engineered to display the double-cyclic peptide CDCRGDCFC (RGD-4C), which binds to specific α_v integrin receptors ($\alpha_v\beta_3$ or $\alpha_v\beta_5$) that are over-expressed in both tumour and supporting angiogenic vasculature (100, 175). This allows for superior ligand-directed delivery and cellular transduction of therapeutic transgenes as a targeted platform and these functional attributes have been confirmed in preclinical models of several cancers including prostate, breast cancer (100) and soft-tissue sarcomas (176). One study used AAVP vectors to target human melanoma cells grafted to nude mice, which showed high specificity for tumour vasculature, induction of apoptosis in tumour vessels, significant inhibition of tumour growth but with no systemic toxicity to normal organs (177).

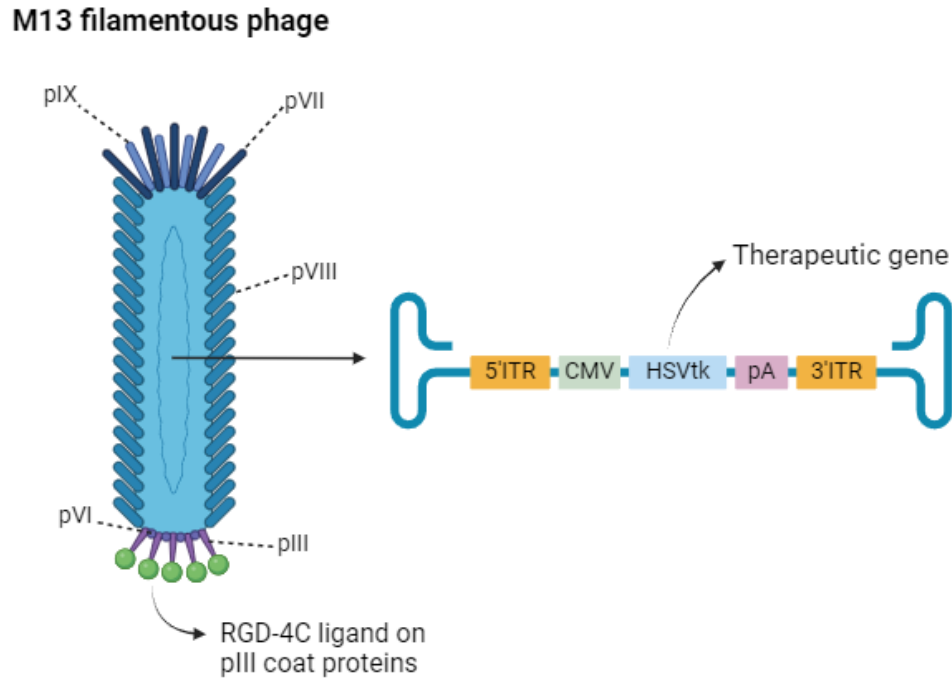


Figure 6. Structure of the hybrid vector AAV/phage (AAVP) developed by Hajitou et al (178). The particle contains a chimeric genome of a CMV-transgene cassette flanked by inverted terminal repeats, 3' ITR and 5' ITR, of AAV-2 and the genome of M13 filamentous bacteriophage. The vector also has $\alpha_v\beta_3$ or $\alpha_v\beta_5$ integrin-targeting RGD4C ligand on the PIII minor coat protein. Created with BioRender.com

This vector platform was further improved by using the phagemid system to produce the next-generation vector known as Phagemid-AAV (PAAV). PAAV vector is a chimeric virus – a M13 filamentous phage containing a hybrid genome constructed using DNA sequences from the AAV serotype 2. The gene of interest is regulated by the constitutively active cytomegalovirus, CMV promoter and flanked by full-length ITRs (Figure 7). The phagemid contains an f1 origin of replication, which is used for single stranded replication and packaging into phage particles; as well as an origin of replication for double stranded replication once it enters the target cell. It can be selected with ampicillin during cloning and production of virus vector. In this hybrid vector model, most of the phage genome is removed which allows for longer DNA sequences to be accommodated but necessitates the use of helper phage viruses to provide the capsid and other phage components. RGD-4C peptide motif is first displayed on the pIII coat protein of the M13 bacteriophage to produce a targeted backbone helper virus. The helper virus is then used to transduce TG1 *Escherichia*

coli (*E. coli*) bacteria containing the engineered phagemid, and selection of the targeted vectors containing phagemid can be conducted using selection pressure with antibiotics. The new model was able to accommodate longer DNA sequences, had higher transduction efficiency and could be produced at higher titre over the original AAVP.

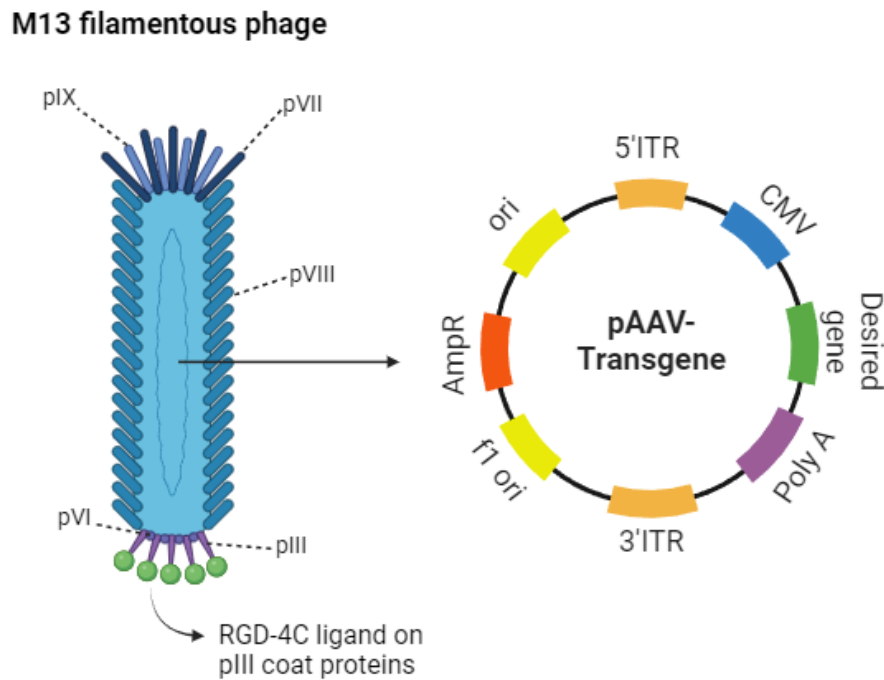


Figure 7. Structure of the hybrid vector pAAV. This new vector is modified from the previous AAVP model such that it contains no structural genes in its genome, significantly reducing the size of the construct. The small construct allows for larger gene inserts to be accommodated and can improve efficacy in gene transduction. However, a helper phage is necessary to provide this hybrid vector with capsid and other components for assembly. Created with BioRender.com

Another strategy to enhance efficiency is by displaying the histidine rich H5W ligand (GLFHAIAHFHGGWHGLIHGWYG), an endosomal escape peptide on the recombinant pVIII coat proteins (Figure 8). Intracellular barriers such as endosomes can limit the rate of gene expression even if the efficiency of internalisation is high, by trapping vectors and preventing it from exerting its therapeutic effects (179, 180). The histidine side chain can form zwitterions, allowing it to act as a proton sponge and buffer the low pH in the endosome following ligand-directed endocytosis of the phage vector. When protons enter the endosome,

water is drawn into the endosome, causing pores to form on the endosome and thereby releasing the phage vector.

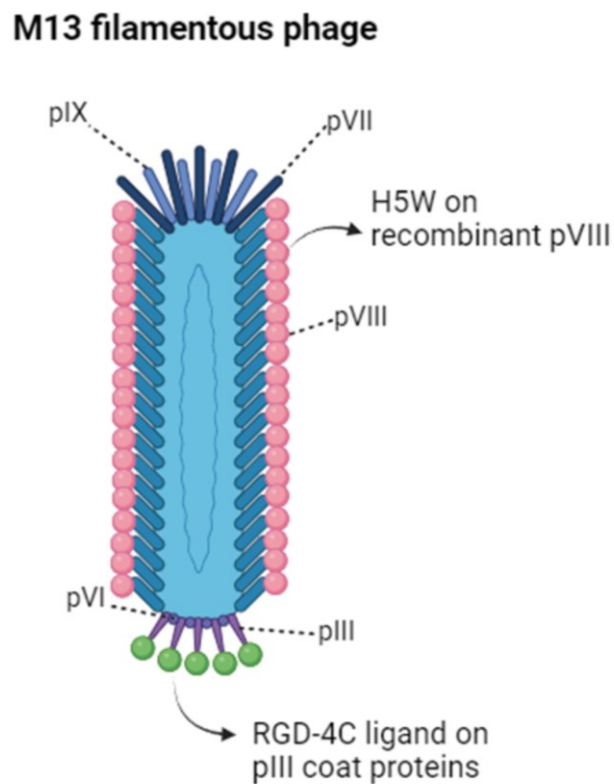


Figure 8. Schematic showing Phage with H5W endosomal escape ligand expressed on recombinant pVIII receptor. Created with BioRender.com

1.8 Tumour necrosis factor superfamily as therapeutic genes in cancer

Among the known hallmarks of cancer, induction of apoptosis is a promising strategic approach to eradicating cancer cells (181). This cellular self-destruct sequence can be initiated via intrinsic pathways, often contingent on p53 activity in response to intracellular stressors like DNA damage, or through extrinsic pathways, triggered by the binding of pro-apoptotic ligands to specific receptors on the cell surface (182).

The Tumour Necrosis Factor (TNF) superfamily, encompassing ligands such as Fas ligand (FasL), CD95 ligand (CD95L), and tumour necrosis factor alpha (TNF α), plays a significant role in the extrinsic apoptosis pathway and have been recognised for their potent anti-tumour efficacy (90, 183). However, the clinical application of these molecules has been constrained

by their potential to induce considerable toxicity and inflammation, limiting their application to the clinic (90, 184-187).

In this context, TNF-related apoptosis-inducing ligand (TRAIL), also known as Apo2L and TNFSF10, represents a safer approach. Discovered in 1996, TRAIL distinguishes itself by preferentially inducing apoptosis in a variety of neoplastic cells while sparing normal cells, thereby mitigating the risk of systemic toxicity (184, 188, 189). Preclinical and early clinical trials employing recombinant TRAIL and antibodies targeting TRAIL receptors have shown that there is selective toxicity towards tumour cells with minimal adverse effects on healthy tissue (184, 190-192). This selective cytotoxicity positions TRAIL as a favourable candidate for clinical research and therapeutic application, offering a more targeted method for cancer treatment.

TRAIL is a ~35 kDa protein that is expressed in various tissues including spleen, thymus, prostate and lung, and also by cells of the immune system such as natural killer (NK) cells, T cells, macrophages and dendritic cells (90, 183). It is synthesised as a Type II transmembrane protein which, similarly to other members of the TNF superfamily, apart from being present in its membrane-bound version, can also be proteolytically cleaved from the cell surface by a cysteine protease to form a soluble fragment of ~24 kDa (183, 193, 194). Under physiological condition soluble TRAIL exists in the blood plasma at around 100 pg/ml, a concentration that does not lead to apoptosis in cell lines in vitro (195). The exact role of TRAIL as a soluble form or as a membrane-bound version in different contexts is currently uncertain (196), though both forms have been found to enhance the functionality of activated immune cells and are secreted in exosomes to regulate immunity and induce apoptosis in cancer cells (197, 198). The membrane-bound conformation appears to be more potent, as TRAIL is biologically active as a homotrimer and this specific conformation presumably facilitates cross-linking of ligand-receptor complexes, thereby increasing signaling strength (90). The soluble form is less potent, but effects can be enhanced by engineering an extracellular domain fused to motifs such as a leucine zipper, which helps with stabilization and formation of homotrimers (90, 184). The effectiveness of the membrane-bound form of TRAIL in vivo is constrained by its need for direct cell-to-cell contact, which can be hindered by the size of the tumour mass, suggesting a need for approaches to enhance TRAIL's bioavailability for better anti-cancer effects.(188)

1.9 Anti-tumour activity of TRAIL

Like other members of the TNF superfamily, TRAIL triggers apoptosis by interacting with and activating specific receptors on the surfaces of target cells (90, 192) (Figure 9). Five receptors have been identified for TRAIL: TRAIL R-1 (DR4) and TRAIL R-2 (DR5) are death receptors featuring a cytoplasmic death domain (DD) that facilitates protein-protein interactions and recruits TNF receptor-associated death domain proteins to initiate apoptosis. In contrast, TRAIL R-3 (DcR1) and TRAIL R-4 (DcR2) act as decoy receptors, lacking the ability to trigger apoptotic signalling and thus inhibiting TRAIL's apoptotic effects. Additionally, osteoprotegerin, a soluble receptor, can bind to TRAIL, potentially acting as another decoy or inhibitor by sequestering TRAIL extracellularly (90, 184, 192). Both forms of TRAIL are capable of binding these target receptors (199).

TRAIL initiates apoptosis through the extrinsic pathway by engaging with death receptors TRAIL-R1 and TRAIL-R2 on the cellular membrane. When homotrimer TRAIL binds to death receptors, TRAIL-R1 or TRAIL-R2 form a trimer and recruit adaptor protein Fas-associated death domain (FADD). This leads to the formation of the death-inducing signalling complex (DISC), within which initiator caspase 8 is auto-activated via proteolysis. Subsequently, the activated caspase 8 cleaves effector caspase 3, triggering a cascade that results in the cleavage of cellular components critical for apoptosis (90). This pathway may be inhibited by the cellular FLICE-like inhibitory protein (c-FLIP), which, despite sharing some structural similarities with caspase-8, lacks its proteolytic function, allowing it to bind to FADD without triggering apoptosis. If TRAIL binds to the decoy receptors instead, there is no recruitment of FADD and subsequent apoptosis is prevented.

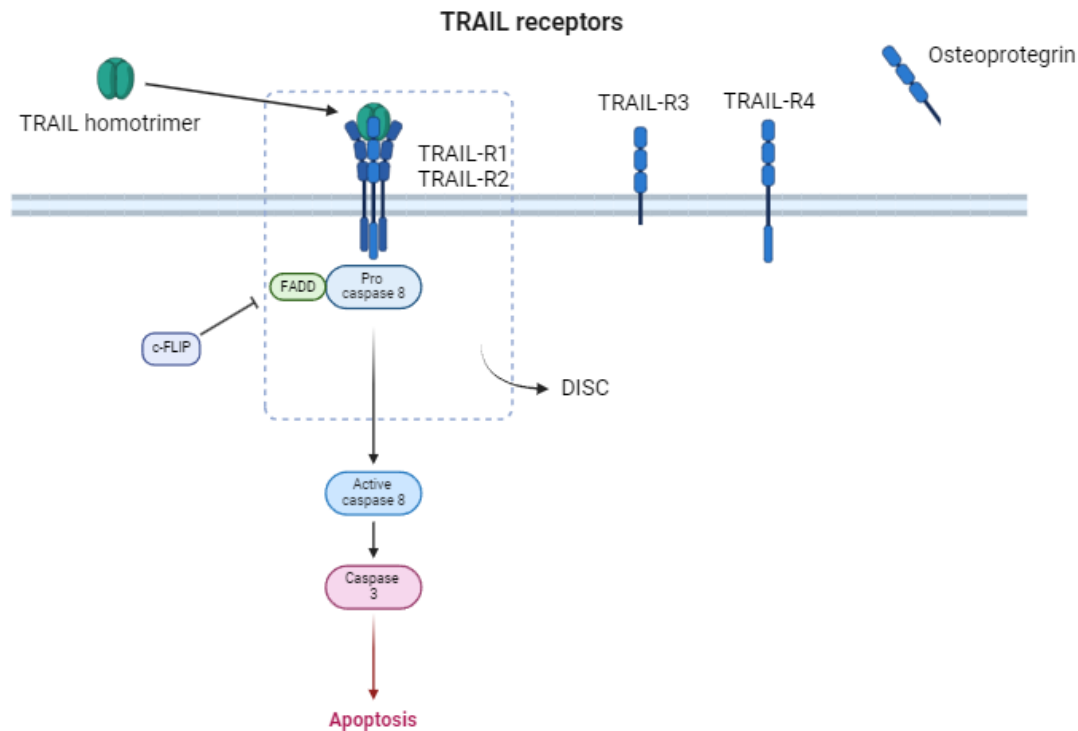


Figure 9: TRAIL and its receptors. In the extrinsic pathway of apoptosis, TRAIL forms homotrimers that recognise and bind to death receptors (TRAIL-R1 or TRAIL-R2), which in turns recruits adapter FADD. FADD recruits procaspase 8, which is cleaved into active caspase 8, which in turn cleaves caspase 3, eventually triggering apoptosis. TRAIL-R3 and TRAIL R-4 are decoy receptors incapable of activating apoptosis. Osteoprotegrin is a soluble receptor, which also acts as a decoy receptor. Created with BioRender.com

TRAIL receptors have been identified in both glioblastoma and grade II astrocytomas (200, 201), furthermore, their expression is an independent factor affecting survival rates in glioblastoma patients (200). In DIPG, a recent study demonstrated that the induction of TRAIL-R2 plays a key role in the effectiveness of imipridone-based combination therapies, especially in H3K27M-mutant DIPG cell lines, suggesting that TRAIL receptors are present in DIPG and are involved in mediating therapeutic response (202).

TRAIL can also trigger necroptosis in cells that are resistant to apoptosis, demonstrating diversity in its mechanisms in inducing cell death (203, 204). Necroptosis, a caspase-independent programmed cell death pathway, has a unique signalling pathway that is typically triggered by external toxic stimuli. TRAIL's role in necroptosis involves activating poly(adenosine diphosphate ribose) polymerase-1 (PARP-1), essential for engaging the

necrosome components RIPK1/RIPK3 and initiating cell death (204). The exploration of necroptosis for therapeutic applications, especially in glioma treatment, is an area of active research, emphasizing its potential to enhance cancer therapy strategies (205). Furthermore, the TRAIL-receptor system plays a pivotal role in eliminating tumour-supportive immune cells, with its expression on NK cells representing a crucial immune strategy for eradicating cancer cells (190).

Studies have shown that TRAIL is consistently expressed in low-grade astrocytomas, anaplastic astrocytomas, glioblastomas, and cell lines derived thereof, and that malignant glioma cell lines are susceptible to TRAIL-induced apoptosis (206). In a study examining efficacy of TRAIL-producing human adipose tissue-derived mesenchymal stem cells against experimental brainstem glioma, TRAIL led to a significant reduction of tumour volume and caused 3-fold survival gain (207). In hepatocellular carcinoma, TRAIL (50ng/mL) sensitises chemotherapeutic drugs such as mitomycin and 5-fluorouracil, decreasing their threshold by 50-100 folds (208). These findings highlight the potential of TRAIL as a versatile therapeutic agent, demonstrating its effectiveness across a range of tumour types, by enhancing susceptibility to apoptosis and sensitizing tumours to conventional chemotherapy.

1.10 Selection of models to test gene therapy in DIPG

When developing cancer therapies, selecting a representative cell culture model is crucial for successfully translating *in vitro* results to *in vivo* applications. Traditionally, glioma cells were cultured as adherent monolayers in the presence of fetal bovine serum (FBS). However, 3-dimensional (3D) serum-free spheroid cultures have gained popularity for *in vitro* drug screening (43).

While adherent cultures offer ease of maintenance and cost-effectiveness, their flat, single-layer formation can misrepresent the tumour environment. This can influence cell activity, morphology, gene expression, and crucially, responses to stimuli and drugs (209). In contrast, spheroids, grown on non-adherent plates, form 3D structures that better mimic the original tumour's architecture (210). This allows them to maintain their morphology (211) and receive environmental stimuli as they would *in vivo* (212). Furthermore, spheroids are often theorised to be enriched in brain tumour stem cells (BTSCs) (213), potentially making them more representative of the original tumour (214-216), though this notion is debated (217).

In DIPG research, the best model for predicting *in vivo* results remains unclear. Different culture conditions can activate distinct signalling pathways and significantly alter gene expression profiles (43). Research groups have used both adherent monolayer (67, 218-221) and spheroid cultures (13, 222-225) for DIPG investigations. In this paper, both methods are employed, with emphasis on spheroids.

1.11 Hypothesis and aims

This paper hypothesises that applying PAAV as a delivery vector to express TRAIL could establish a targeted and efficient therapeutic strategy for DIPG

In this study, I aim to investigate if TRAIL is suitable as a potential therapeutic gene for the treatment of DIPG. This will be done using RGD4C-targeted PAAV as a gene delivery vehicle that can specifically target tumour cells and ensure gene transfer to DIPG cells, first *in vitro* and then, *in vivo* with an animal model.

To achieve this, this paper aims to specifically:

- 1) Validate the efficacy of phage-guided therapeutic gene delivery *in vitro* using human primary DIPG lines derived from patient biopsies,
- 2) Test and compare the efficacy of TRAIL transgenes in inducing tumour cell death *in vitro*
- 3) Investigate the use of this new phage vector *in vivo* using SCID mice bearing human DIPG as a proof of concept

2. Materials and methods

2.1 Materials

2.1.1 Cell culture lines

Name	Tissue Origin	Source
DIPG (HSJD-DIPG-07, HSJD-DIPG-11, HSJD-DIPG-12, HSJD-DIPG-017, HSJD-DIPG-019, HSJD-DIPG-021)	Biopsy from pontine gliomas	Kindly provided by Dr Carcaboso from St Juan de Deu Hospital Barcelona, Spain

Table 3. Cell lines used during the investigation.

2.1.2 Pharmacological and chemical reagents

Name	Solvent	Source
FuGENE ® 6 Transfection Reagent	Cell culture medium	Promega

Table 4. Pharmacological and chemical reagents used during the investigation.

2.1.3 Kits

Name	Source
Gel extraction kit	Qiagen
Plasmid midi kit	Qiagen

Table 5. Kits used during the investigation.

2.1.4 Antibodies

Primary antibodies	Host	Species reactivity	Application	Source
α v integrin ab55991	Rabbit	Human	FACS	Abcam
β 3 Ab34409	Mouse	Human	FACS	Abcam
β 5 Ab15459	Rabbit	Human	FACS	Abcam
α V β 3 MAB3050	Mouse	Human	FACS	R&D Systems
α V β 5 MAB2528	Mouse	Human	FACS	R&D Systems
TRAIL R1 Anti-human CD261	Mouse	Human	FACS	BioLegend
TRAIL R2 Anti-human CD262	Mouse	Human	FACS	BioLegend
TRAIL R3 Anti-human CD263	Mouse	Human	FACS	Miltenyi Biotec
TRAIL R4 Anti-human CD264	Mouse	Human	FACS	Molecular Probes

Secondary antibodies	Host	Species reactivity	Application	Source
Anti-rabbit Alexa Fluor 594	Goat	Rabbit	FACS	Abcam
Anti-mouse Alexa Fluor 488	Goat	Mouse	FACS	Abcam

Table 6. Antibodies used during the investigation.

2.1.5 Plasmids

Plasmid	Genome	Source
pUNO1-hTNFA	Contain the human TNFa gene	Invivogen
pUNO1-hTNFSF10a	Contains the human TRAIL gene	Invivogen

Table 7. Plasmids used during the investigation.

2.2 Methods

2.2.1 Maintenance of cell culture

DIPG cells were cultured with Tumour Stem Medium (TSM) (47.5% Neurobasal-A Medium, 47.5% D-MEM/F12, 1% HEPES Buffer Solution, 1% Sodium Pyruvate MEM, 1% MEM Non-Essential Amino Acids Solution, 1% GlutaMAX-I Supplement, 1% Antibiotic-Antimycotic). 2D DIPG cells were maintained with TSM supplemented with 10% FBS, while 3D DIPG cells were maintained with TSM supplemented with growth factors (2% B27, 20ng/ml Human EGF, 20ng/ml Human FGF, 10ng/ml PDGF-AA, 10ng/ml PDGF-BB, 2ug/ml Heparin solution). The cells were cultured in T-75 flasks and maintained in a 5% CO₂ humidified cell culture incubator at 37°C. 2D cells were passage once they reached 80-90% confluence. The medium was aspirated, and cells were washed twice with 1X sterile PBS. Cells were detached using 5ml of trypsin-ethylenediaminetetraacetic acid (Trypsin-EDTA) and incubated at 37°C for 5 minutes. Trypsin was deactivated by adding 10ml of fresh medium to the cells. This is then transferred to a sterile falcon tube and centrifuged at 1000pm for 5 minutes to pellet the cells. The cell pellet is then resuspended in 10ml of culture medium and 1ml aliquot was transferred into a new flask containing 14ml of fresh medium. 3D cells were transferred directly to a new flask containing fresh medium once the medium starts to turn colour.

Reagents	Vendor	Final Concentration
Neurobasal-A Medium	Life Technologies	47.5%
D-MEM/F12	Life Technologies	47.5%
HEPES Buffer solution	Life Technologies	1%
Sodium Pyruvate MEM	Life Technologies	1%
MEM Non-Essential Amino Acids Solution	Life Technologies	1%
GlutaMAX-I Supplement	Life Technologies	1%
Antibiotic-Antimycotic	Life Technologies	1%

Table 8. Reagents that makeup the TSM Base.

Reagents	Vendor	Final Concentration
B27	Life Technologies	2%
Human EGF	Peprtech	20ng/ml
Human FGF	Peprtech	20ng/ml
PDGF-AA	Peprtech	10ng/ml
PDGF-BB	Peprtech	10ng/ml
Heparin solution	Sigma	2µg/ml

Table 9. Growth Factors that are added to the TSM Base to form the Working 3D TSM.

2.2.2 Integrin Immunofluorescence Staining

Coverslips were first autoclaved, flamed, and exposed to UV light for 30 minutes. They were then placed in 24-well plates and coated with 50mg/ml poly-D-lysine. DIPG cells were seeded on the coverslips and grown in the incubator until 70% confluence. The cells were then washed 2X with Phosphate-buffered saline (PBS) and then fixed with 4% formaldehyde (PFA) for 10 minutes at room temperature. After fixation, the cells were washed 3X with PBS and treated with 50mM ammonium chloride for 5 minutes to reduce autofluorescence. The cells were washed again for 3X with PBS and blocked with 2% Bovine serum albumin (BSA) (in PBS) for 30 mins at room temperature. After blocking, primary antibodies (1:100 mouse anti- α V, 1:50 mouse anti- β 3 and 1:100 rabbit β 5 integrins) diluted in 1% BSA-PBS blocking reagent was added to the wells and the plate was kept in a moist chamber at 4°C overnight. The next day, the cells were washed 3X with PBS and incubated in the dark with 1:750 Alexa-Fluor conjugated antibodies and 1:2000 4',6-diamidino-2-phenylindole (DAPI) for one hour at room temperature. The cells were washed 3X with PBS and the coverslips were rinsed with sterile water and mounted with Prolong gold antifade reagent, sealed in nail polish and allowed to dry in the dark. Images were taken using a fluorescence microscope. Results for the staining of Integrin α V, β 3, β 5 were compared to control samples (without any antibodies, mouse secondary antibody only and rabbit secondary antibody only)

2.2.3 Flow Cytometric Analysis (FACS)

All the FACS results were acquired using the Flow cytometer (BD FACSCaliburTM) and analysed using the FlowJo software (FlowJo, LLC).

Cells were washed with ice-cold PBS and dissociated using cell dissociation buffer (Gibco Cell Dissociation Buffer, enzyme-free, PBS). The cells are then centrifuged at 2500rpm for 5 minutes and pelleted.

2.2.3.1 Integrin expression

DIPG cell were resuspended in PBS/10%FBS/1%NaN₃ and stained with primary antibodies (α V, β 3, β 5, α V β 3, α V β 5 integrins) diluted in 3% BSA/PBS for 30 mins. Next the cells were washed 3X in PBS/10%FBS/1%NaN₃ and incubated with secondary antibodies diluted in 3% BSA/PBS for 15 mins. The cells were then washed for 2 more times in PBS/10%FBS/1%NaN₃ before sorting by flow cytometer. Cells that were stained with secondary antibodies (mouse, rabbit secondary) alone were used as controls.

2.2.3.2 TRAIL receptors

DIPG cells were stained for TRAIL-R1, TRAIL-R2, TRAIL-R3 and TRAIL-R4 in Falcon tubes. Primary antibodies conjugated with fluorescent dyes for the 4 different receptors were diluted 1:100 were directly added to the DIPG cells. They were left alone for 30 mins at room temperature. Finally, the cells were washed 3 times in PBS with 1% BSA.

2.2.4 DNA cloning

Polymerase Chain Reaction (PCR) was done using Q5 High-Fidelity DNA Polymerase (New England Biolabs) and the reaction mixture was prepared.

Reagents	Volume
5X Q5 reaction buffer	5 μ l
10mM dNTPs	0.5 μ l
10uM forward and reverse primers	1.25 μ l
Template DNA plasmid	100-200ng
Q5 High-Fidelity DNA polymerase	0.25 μ l
5X Q5 High GC Enhancer	5 μ l
Nuclease-free water	Up to total volume of 25 μ l

Table 10. Reagents that make up the mixture for DNA cloning.

The PCR cycle was set as follows:

1. First stage (1X) of initial denaturation at 95°C for 1minute.
2. Second stage (35X): 95°C for 15 seconds
3. 60°C for 20 seconds (Vary according to the melting temperature 55°C)
4. 72°C for 30 seconds
5. Third stage (1X) of 72°C for 1minute followed by 4°C on hold.

PCR products were then analysed using gel electrophoresis.

2.2.4.1 Restriction Enzyme digestion

Plasmid digestion was performed according to the manufacturer's instructions (New England Biolabs, NEB). The temperature, incubation time and heat inactivation time were dependent on the enzymes used. The digested DNA was then analysed on 1% agarose gel.

Reagents	Volume
Restriction enzymes	3 units
DNA	1µg
1X reaction buffer	As appropriate
1% Bovine Serum Albumin	As appropriate
appropriate volume of H_2O	Up to total appropriate volume

Table 11. Reagents that make up the mixture for restriction enzyme digestion.

2.2.4.2 Dephosphorylation of DNA fragments

The 5' and 3' ends of the digested DNA from restriction enzyme digestion were dephosphorylated using recombinant shrimp alkaline phosphatase (rSAP, NEB). Two units of rSAP were added to the digestion reaction and digested for 2hrs at 37°C, followed by heat deactivation at 65°C for 20 minutes.

2.2.4.3 Agarose gel electrophoresis

Agarose gel was prepared (1% agarose) in 1X TAE buffer and heated in the microwave until the agarose was completely dissolved. The heated solution was then allowed to cool and 1X SYBR safe DNA stain (Invitrogen™ S33102) was added. This solution was then poured into a casting tray containing a set of combs and allowed to solidify at room temperature. After solidification, the gel was placed in a gel box filled with 1X TAE buffer and the gel comb is removed to reveal wells. DNA samples for analysis were prepared by adding 1X

loading dye (Fisher scientific). The DNA ladder (Fisher scientific) was loaded at the starting lane along with the samples to determine the DNA fragment size. Electrophoresis was set at 80-100V for about 60-90 minutes, depending on size of DNA samples. DNA bands were then visualised under the UV using the UVP Bio Doc-It imaging system. If purification and extraction of the DNA fragment was needed, the bands were visualised using Pearl Blue Light Transilluminator (Pearl biotech) and the DNA bands of the required molecular weight were cut out with a scalpel. The DNA was then extracted using QIAquick gel extraction kit (QIAGEN).

2.2.4.4 DNA ligation

Ligation was performed with T4 ligase (New England Biolabs). The reaction mixture was first prepared, then incubated at 16°C overnight or at room temperature for 2 hr. Then, the reaction was deactivated at 65°C for 10 minutes and chilled on ice.

Reagents	Volume
10X T4 ligase buffer	2µl
Vector DNA	50ng
Insert DNA	37.5ng
T4 DNA ligase	1µl
Nuclease-free water	Up to total volume of 20µl

Table 12. Reagents that make up the mixture for DNA ligation.

2.2.4.5 Transformation of Competent *E.coli* cells

The transformation process was performed using NEB 5-alpha competent *E.coli* cells.

1. Thawed competent bacteria (100µl) on ice for 30 minutes.
2. Add the ligation reaction mixture or plasmid DNA (1pg-100ng) to *E.coli* cells and incubate on ice for 30 minutes.
3. Place in a heated water bath set to 42°C for 30 seconds for heat shock and return the mixture to ice for 5 minutes.
4. Add 300µl of SOC medium at room temperature and place in shaker at 37°C for 45 minutes at 180rpm.
5. Spin the mixture gently to produce a bacteria cell pellet and decant some of the excess supernatant.

6. Using the cell pellet and some leftover supernatant, spread on 2xYT agar plate containing appropriate antibiotics (kanamycin or ampicillin) and incubate overnight at 37°C.

2.2.4.6 Selection of plasmids and sequencing

Bacteria cell colonies are picked the following day and sent for sequencing to select for the correct sequences.

2.2.5 Production, purification and titration of PAAV (Figure 10)

2.2.5.1 PAAV preparation

The plasmid was transformed into TG1 (mix and go) as per manufacturer's instruction and allowed to grow overnight in 2x Yeast Extract Tryptone (2XYT) medium containing 100ug/ml ampicillin at 37°C with shaking at 250rpm. The next day, 1ml of this culture was added to a flask containing 100ml 2XYT with ampicillin and allowed to grow until its optical density reaches mid-log phase (OD_{600} is between 0.4-0.8). The culture was then split into two flasks and 15µl of helper phage was added to each flask and then incubated for 30 minutes with shaking at 150rpm. The culture is then added to a flask with 450ml of 2XYT medium containing ampicillin (50mg/ml), kanamycin (50 mg/ml) and 1mM Isopropyl-beta-D-thiogalactopyranoside (IPTG) and incubated for 18 hours at 37°C shaking at 250rpm.

2.2.5.2 PAAV purification

The overnight cultures were transferred to 250 ml centrifuge tubes and centrifuged at 6000g for 15 minutes at 4°C. The supernatant was collected in a new sterile bottle and the bacteria pellet discarded. 20-30% of the supernatant volume of Polyethylene-glycol/Sodium chloride (PEG/NaCl/Triton-X: 168g of PEG MW8000 with 196.46g NaCl and Triton-X dissolved in 800mL ddH₂O) was added and allowed to precipitate in the cold room overnight. The mixture was centrifuged the next day at 10000g for 30 minutes at 4°C. The supernatant was discarded, and the pellet resuspended in 15ml of PBS and 20-30% of total volume of PEG/NaCl to precipitate the virus in the cold room overnight. The next day, the mixture was centrifuged again at 10000g for 30 minutes at 4°C and the supernatant was discarded. The pellet is resuspended in 1ml of PBS and left overnight at 4°C to dissolve completely. Finally, the virus is spun using a bench top centrifuge at 13000rpm for 10 minutes and the supernatant

PAAV solution was filtered through a 0.45µm low protein binding PVDF filter membrane and kept at 4°C.

2.2.5.3 PAAV titration

Naïve TG1 was cultured in 2XYT until a OD₆₀₀ between 0.4-0.8. Serial dilutions of the PAAV solution were made in 1X PBS from 10³ up to 10¹¹. 5µl of the PAAV dilution was added to infect 500µl of naïve TG1 and incubated at 37°C for 30 minutes. Then, 100µl of the infected TG1 was plated on 2XYT agar plates in duplicates (either containing 100µg/ml ampicillin or 50µg/ml kanamycin) to estimate the viral titre of the PAAV and helper phage. The agar plates were incubated at 37°C overnight and colonies were counted the following day. The PAAV titres were calculated from the ampicillin plates as bacterial TU per µl (TU/µl). The colonies on the kanamycin plates represent contaminated helper phage, which titre should be less than 10% of the PAAV titres on the ampicillin plates.

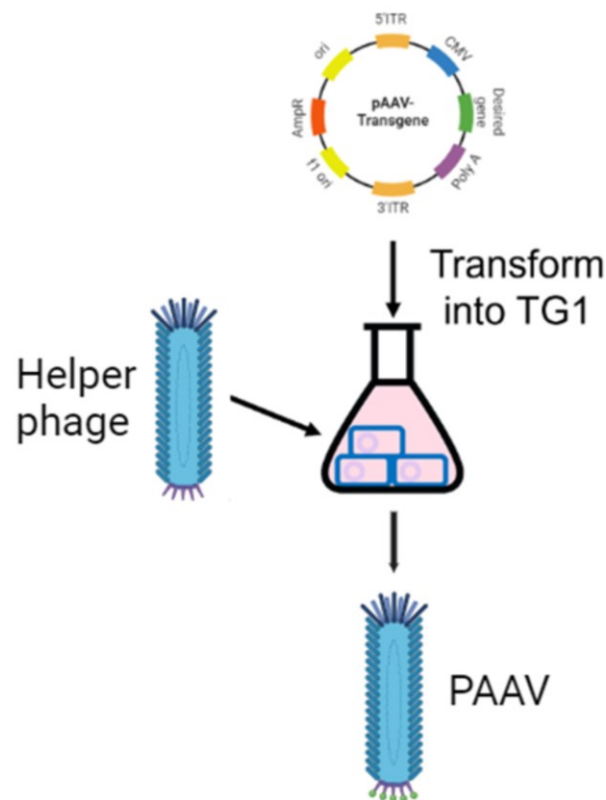


Figure 10. Schematic showing production of PAAV phage vector with helper gene.
Created with BioRender.com

2.2.6 Construction of plasmid with TRAIL using Snapgene (Figure 11)

We produced chimeric PAAV vectors carrying the transgene encoding TRAIL. To do so, we produced plasmids encoding human TRAIL. We cloned the gene cassettes with the transgenes into the phage construct. The phage vectors were amplified in TG1 host bacteria and then isolated and purified. Vectors were titrated by infection of host bacteria for colony counting on Luria–Bertani (LB) agar plates under antibiotic selection and expressed as bacterial TU. Non-targeted vectors, lacking RGD4C, were produced as negative controls for targeting.

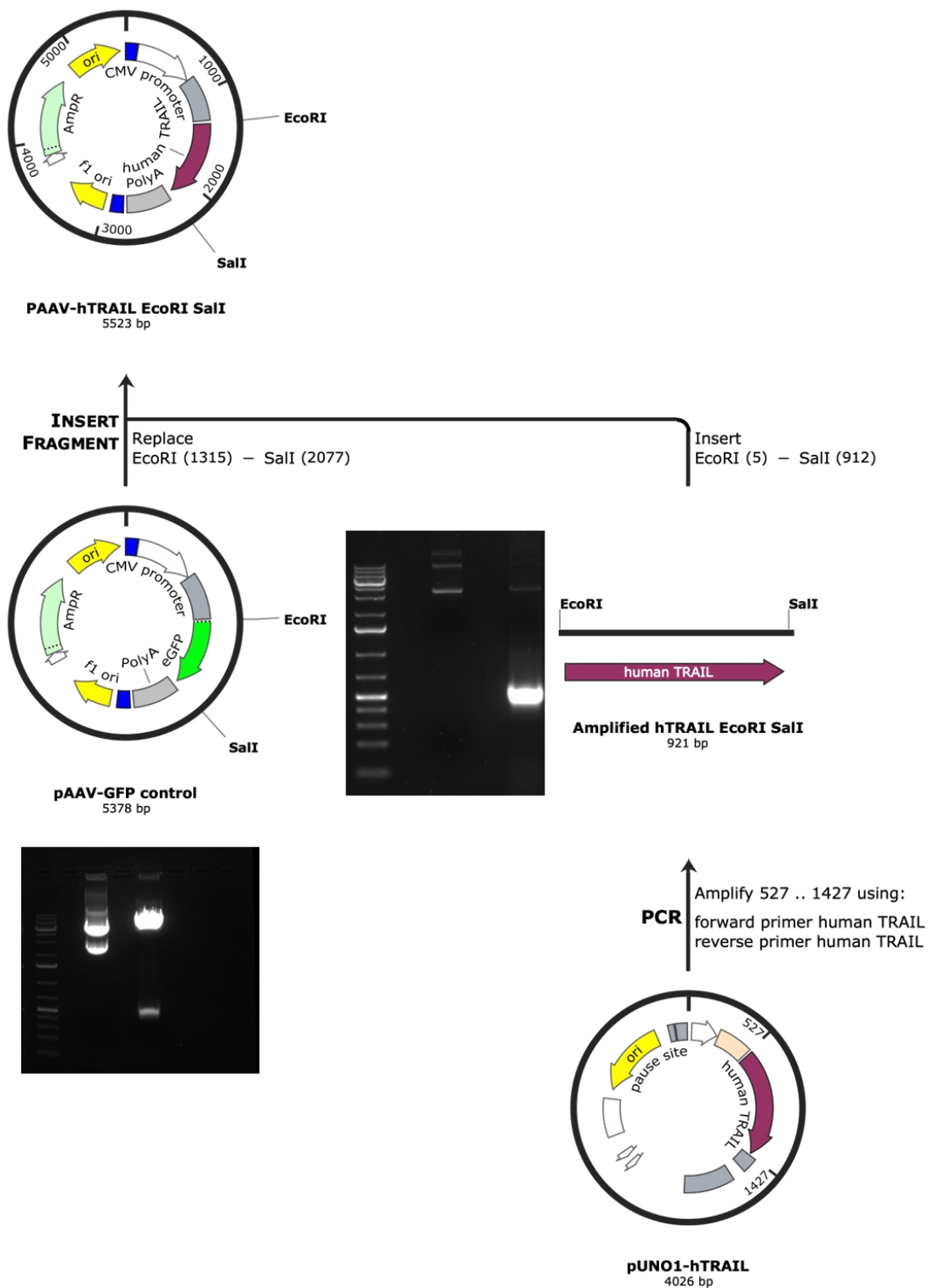


Figure 11: Snapgene construct of human TRAIL. From the commercially bought pUNO1-hTRAIL, the construct is modified via enzyme restriction digestion to form a new construct for the phage vector. Specifically, a previous construct of PAAV, expressing the transgene GFP, is used as the backbone that will ligate with the new TRAIL fragment. Images of gel electrophoresis are taken to verify the size of DNA segments.

2.2.7 DIPG transduction with PAAV vector

2.2.7.1 2D DIPG cell transduction

DIPG cell cultures were trypsinised to form single cells and counted using a haemocytometer. The cells were seeded in 96-well plates at a density of 1000 cells/well in 100µl of TSM supplemented with 10% FBS and incubated at 37°C in 5% CO₂ for 48 hours until the cells are 60% confluent. The transduction medium was prepared by adding different types of bacteriophage vector to TSM with 1% FBS. Growth medium was removed and replaced with the pre-prepared transduction medium. The cells were incubated with the vectors for 24 hours, then the medium was changed. The cells were observed over a period of 7 days.

2.2.7.2 3D DIPG cell transduction

The transduction medium was prepared by adding different types of bacteriophage vector to TSM with growth factors. DIPG cells were transduced with viral vectors containing non-targeted control vector with lucia, targeted vector with lucia and incubated for 7 days. 5µL of culture containing the secreted luciferase and 15µL of QUANTI-Luc (InvivoGen) was added to a 96-well white opaque microplate (BD, Falcon). The Microplate was read using Promega Glomax Microplate Reader. Readings were taken on Day 1, 2, 4 and 7.

2.2.8 QUANTI-Luc Luminescence assay

The cells were seeded in 96-well plates and transduced with PAAV-lucia vectors containing the Lucia-luciferase reporter (lucia) gene.

The secretion of lucia protein from transduced cells into the culture medium was monitored daily. 5µl of the sample supernatant containing the secreted luciferase was added to 15µl of QUANTI-Luc assay solution (InvivoGen) in a 96-well white opaque microplate (BD, Falcon). The luminescence was measured using Promega Glomax Microplate Reader.

2.2.9 Sulphorhodamine B (SRB) Assay

2D cells were washed 2X with 1X PBS and fixed overnight at 4°C with a final concentration of 10% trichloroacetic acid (TCA) in serum free medium. Plates are then washed with slow running tap water and left to dry at room temperature. Cells were stained with 100µl of 0.4% SBR for 30 minutes on a shaking platform. Unbound SRB was washed with 1% acetic acid

and the plates were left to dry at room temperature. Bound SRB was dissolved in 80µl of 10mM Tris pH 10.5 and left on a shaking platform to completely dissolve the SRB. Absorbance was read at 490nm.

2.2.10 Cell Titre Glo Cell Viability Assay

CellTiter-Glo® 3D Reagent was added to 3D cell cultures in a 1:1 ratio and transferred to a 96-well white opaque microplate. The Microplate was read using Promega Glomax Microplate Reader. This assay was used to determine the number of viable cells in 3D cell cultures.

2.2.11 Quantitative Real-Time PCR (qRT-PCR)

Cells were collected in TRIZOL reagent (Ambion life technologies, Ref. 15596026) and total RNA was isolated using the PureLink RNA mini kit (Ambion by life technologies, cat. No. 12183018A).

Reagent	Volume
RNA	2µg
DEPC-treated water	Up to a volume of 16µl
10X DNase I enzyme	2µl
10X DNase buffer	2µl

Table 13: Reagents that make up the qRT-PCR reaction.

After preparing the reaction mixture, incubate at room temperature for 12 minutes. Then, add 2µl EDTA and heat inactivate at 65°C for 10 minutes to stop the DNase activity. DNase treated RNA samples were then set as a template for reverse transcription and cDNA was synthesised using GeneAmp RNA PCR core kit (Applied Biosystems, Ref.N8080143).

The comparative C_t method (ΔC_t) was used for quantification of gene expression using GAPDH as a reference gene, and $\Delta\Delta C_t$ value was calculated against reference sample (RNA extracted from non-transduced cells/ transduced $-\Delta\Delta C_t$ with the non-targeted vector).

2.2.12 In vivo work

Orthotopic DIPG mouse models were generated by Dr Carcaboso, via injection of tumour spheres from human biopsies into the 4th ventricle of the brain via a syringe. Mice were

monitored by daily weighing to detect any weight loss resulting from the tumour growth. Mice bearing detectable tumours (69-84 days after tumour inoculation), were intravenously administered via the tail vein with targeted TRAIL vectors or control non-targeted vectors (5×10^{10} TU/mouse) (Figure 12).

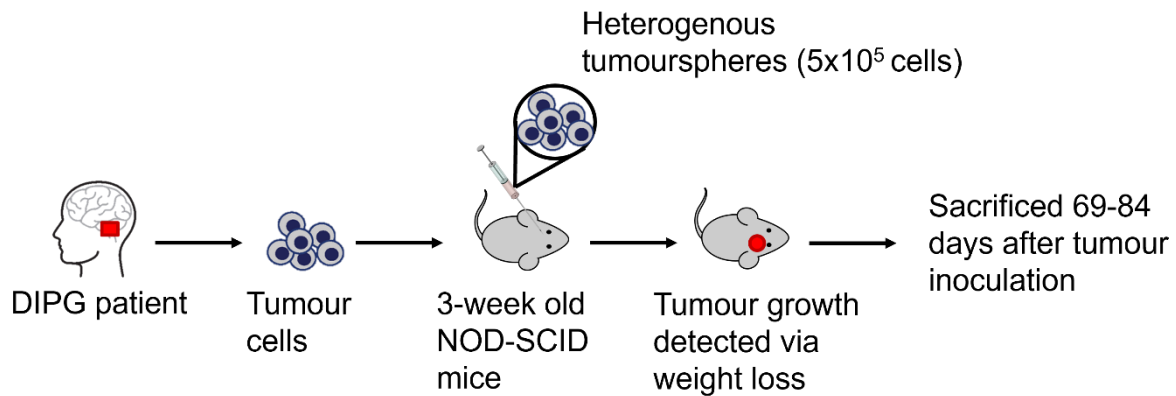


Figure 12. Timeline of in vivo studies.

2.2.12.1 Haematoxylin and Eosin (H&E) staining

Tumours and healthy tissues were collected from each mouse and mounted in optimal cutting temperature (O.C.T) compound, snap frozen in ethanol and dry ice and kept at -80°C . Frozen tissues were cut to $10\mu\text{m}$ and placed on slides to be kept at -20°C .

To process the slides:

1. The tissue sections were fixed in 95% ethanol for 10 minutes.
2. Rinse in tap water with 2-3 changes of water in between.
3. Stained with Haematoxylin for 5 minutes.
4. Rinse in tap water for 5 minutes.
5. Stained with eosin for 5 minutes.
6. Rinse in tap water.
7. Place in 70% ethanol, then in 90% ethanol and lastly 100% ethanol
8. Rinse in xylene two times.
9. Mounted in DPX mounting medium and leave dry in a fume hood for 24hrs.

2.2.12.2 Fluorescent staining of frozen sections

1. Frozen sections are generated as per previously mentioned.

2. Washed with 1xPBS and 0.3% Triton X-100 (PBS-T)
3. Blocked with 10% goat serum in 0.1% PBS-T containing 1% BSA for 60 min at RT.
4. Slides were incubated overnight at 4°C with rabbit α v integrin (1:750) diluted in 0.1% PBS-T with 1% goat serum.
5. Sections were then washed 3x in 0.1% PBS-T and stained for 1 hr at RT with the secondary antibodies AlexaFluor 594 goat anti-rabbit (1:3000) containing DAPI (1:8000).
6. Samples were washed 3x with 0.1% PBS-T, fixed for 30 sec with 4% PFA, washed 3x with PBS and then mounted.

2.2.13 Statistical analysis

Statistical analysis was performed using Graphpad Prism. P values were generated using one-way ANOVA. Data in graph are shown as mean $\pm$ standard error of mean. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$, **** = $p < 0.0001$; ns = non-significant.

3. Results

3.1 DIPG cells in different culture mediums exhibit differences in morphology

The initial focus of this study was investigating how different culture conditions influence the morphology of primary DIPG cells. Studying cell morphology is crucial because it offers insights into cellular behaviour, health, and responses to its environment. It also offers a visual clue on the tumour proliferation and potential responses to treatment. We used 6 primary DIPG cell lines derived from patient biopsies. Importantly, all cell lines share the H3K27M mutation, a prevalent characteristic of DIPG, ensuring the chosen cells broadly represent the tumour population. The presence of this mutation in these cells was verified through other studies : HSJD-DIPG-07 (226), HSJD-DIPG-11 (227), HSJD-DIPG-12 (226), HSJD-DIPG-17 (228), HSJD-DIPG-19 (229) and HSJD-DIPG-21 (228).

For analysis, cells were seeded under two distinct culture conditions: traditional adherent cultures where cells grow in a flat monolayer (Figure 13), and spheroid cultures where cells form 3D structures (Figure 14). After 7 days, we imaged and observed the differences in cell morphology of the same cell grown in the two different culture methods. Understanding how culture conditions affect morphology is vital for selecting the optimal method that best preserves tumour-like characteristics, ensuring more reliable drug testing and research outcomes.

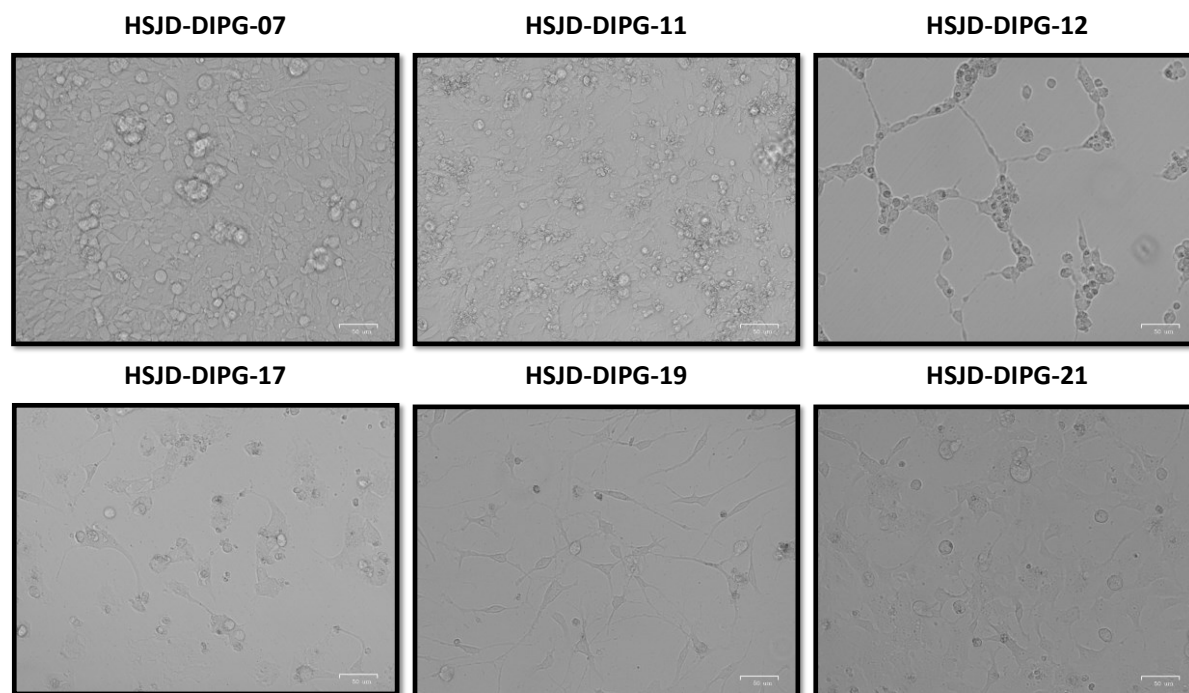


Figure 13. 6 DIPG patient-derived cells cultivated in adherent medium. Cells are seeded in equal number at the start, then observed after 7 days on light microscope. Scale bar, 50 µm.

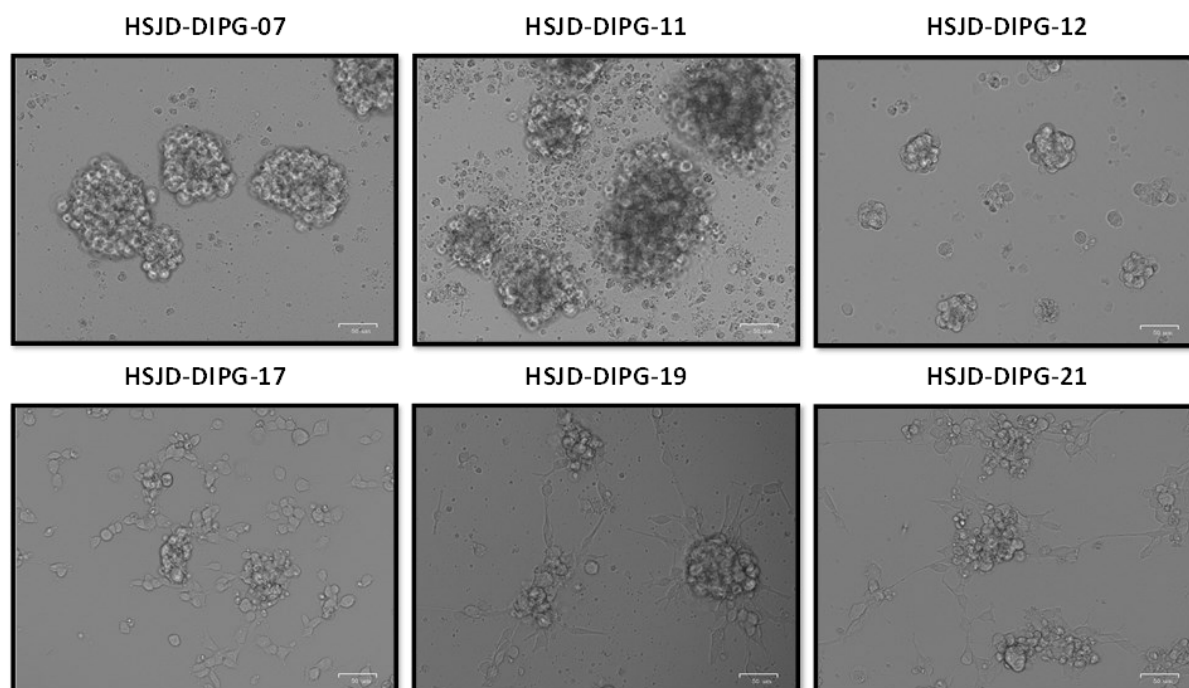


Figure 14. 6 DIPG patient-derived cells cultivated in adherent medium. Cells are seeded in equal number at the start, then observed after 7 days on light microscope. Scale bar, 50 µm.

Despite the use of these cells in DIPG research, there is not much published visual documentation regarding their morphological characteristics under different culture conditions. Our observations revealed distinct morphological appearances among these lines. In adherent cultures, HSJD-DIPG-17 exhibited a notably more fibroblast-like morphology with extended cellular processes, contrasting with the epithelial-like appearance of HSJD-DIPG-07, HSJD-DIPG-11, and HSJD-DIPG-12 cultures.

Furthermore, when grown as spheroids, HSJD-DIPG-07, HSJD-DIPG-11, and HSJD-DIPG-19 demonstrated a propensity for neurosphere formation, while HSJD-DIPG-12, HSJD-DIPG-17, and HSJD-DIPG-21 failed to form large aggregates. These observations highlight the heterogeneity in cellular behaviour and the importance of visual documentation to understand the influence of culture conditions on DIPG cell lines. Such visual records are valuable for providing comparative references for researchers and enhancing reproducibility within the field.

3.2 DIPG cells express integrin receptors which are targetable by RGD4C

The aim of this experiment was to validate the potential of the RGD4C-containing phage vector to efficiently target DIPG cells. Since integrins serve as the vector's primary binding and internalisation targets, establishing their abundance on the surface of these tumour cells is crucial. FACS analysis was employed to quantify the expression levels of $\alpha\text{v}\beta 3$ and $\alpha\text{v}\beta 5$ integrin heterodimers across the 6 DIPG cell lines. The observation of strong, positive staining across all variants, with particularly high expression in spheroid cultures, provides a compelling rationale for utilising this phage-based therapeutic approach (Figure 15). Furthermore, the decision to select the HSJD-DIPG-07 line for subsequent experiments is justified by these results, as it has shown robust integrin expression, making it an ideal model to investigate the vector's targeting efficiency and potential therapeutic efficacy.

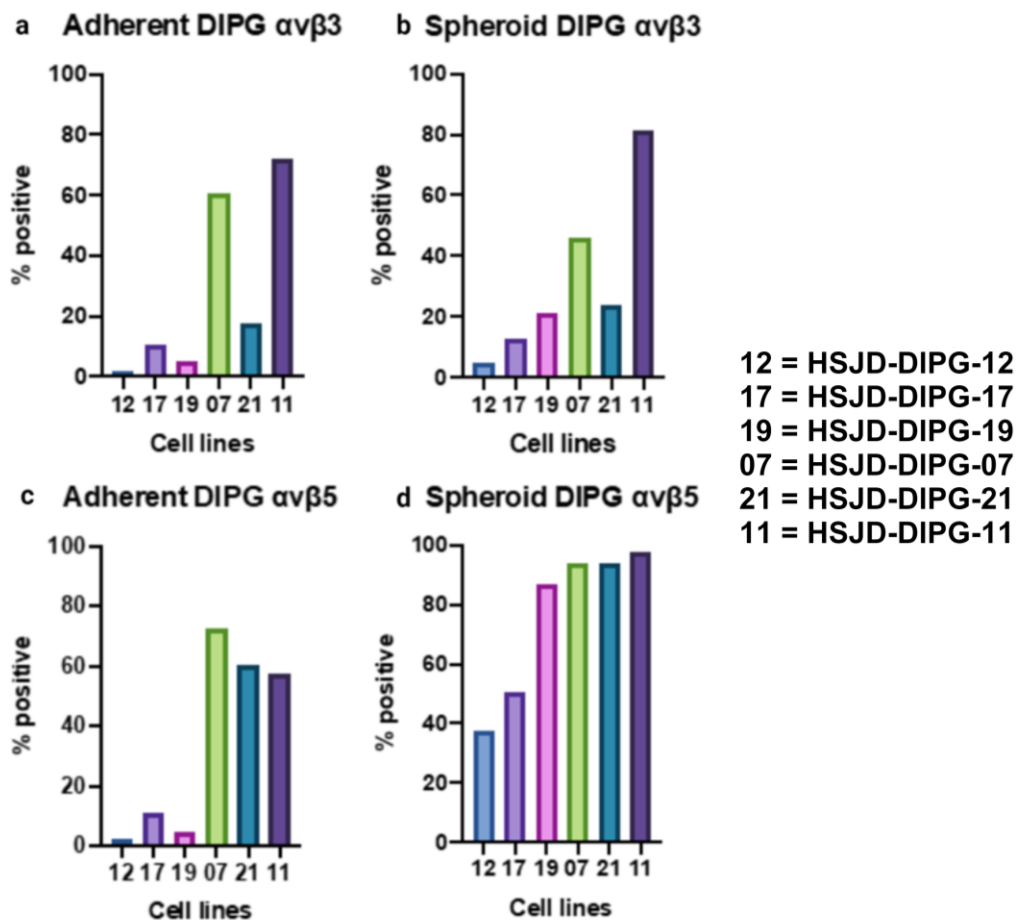


Figure 15. FACS analysis of 6 DIPG cell lines for $\alpha\text{v}\beta 3$ and $\alpha\text{v}\beta 5$ integrin expression. Cells were cultured in either adherent or spheroid medium, then stained for either $\alpha\text{v}\beta 3$, or $\alpha\text{v}\beta 5$ antibodies, then analysed by FACS. The bars show the % of cells that were positive for specified integrin. n=1

To further corroborate the FACS results, we utilised immunofluorescence staining, which provided direct visualisation of integrin expression on the DIPG cells (Figure 16). Cells stained strongly for the target integrin receptors, particularly αv integrin. The inclusion of the control groups, such as cells incubated with secondary antibodies only, ensures confidence in the specificity of the observed staining and minimises the potential for misinterpretation due to background fluorescence.

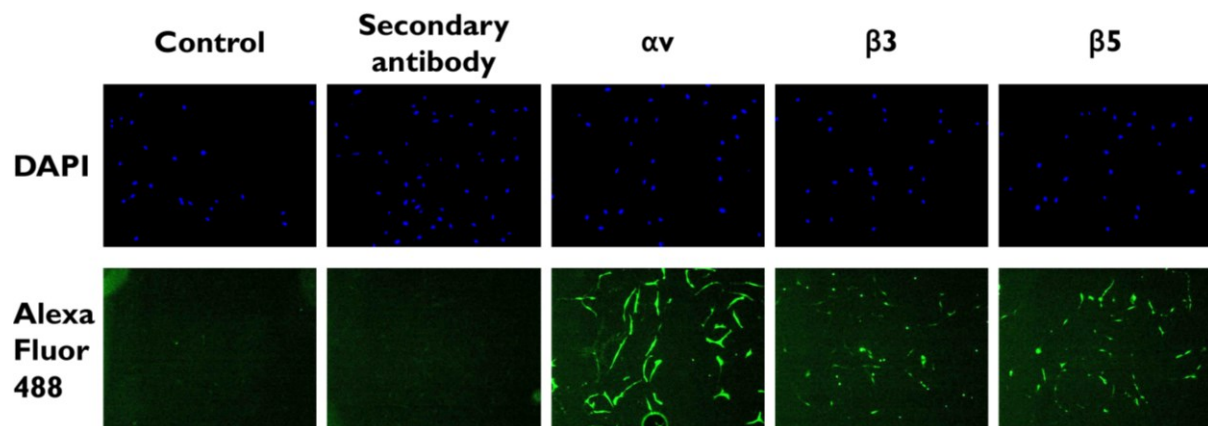


Figure 16. Fluorescent microscopic images of HSJD-DIPG-07. Cells were incubated with a primary anti-phage antibody and Alexa Fluor-488 labelled secondary antibody (green) to assess integrin ($\alpha v/\beta 3/\beta 5$) expression. Images of control cells with no antibodies or secondary antibodies only were taken to account for background fluorescence. Nuclei were stained with DAPI (blue).

3.3 Transgene expression is improved with display of histidine rich H5W ligand on phage coat protein, and is dose-dependent

While the initial results demonstrate the phage vector's ability to target DIPG cells via their integrin receptors, a key challenge remains in optimising intracellular delivery of the therapeutic payload. Phage vectors, like many other nanoparticles, can still face intracellular barriers like endosomal entrapment, hindering their therapeutic potential. To overcome this limitation, we incorporated the H5W endosomal escape peptide into the phage capsid protein to enhance transduction efficiency.

To verify this, we measured the relative luminescence units (RLU) produced by DIPG cells transduced with phage engineered to express Lucia. Four sample groups were used: 1)

control cells, 2) cells exposed to untargeted phage, 3) cells exposed to RGD4C-targeted phage, and 4) cells exposed to RGD4C-targeted phage expressing H5W. Higher RLU indicates greater transgene expression and thus, higher transduction efficiency.

As theorised, control samples and untargeted phage vectors showed minimal RLU, confirming the specificity of RGD4C-mediated targeting. Importantly, RGD4C-targeted vectors expressing H5W demonstrated a significant increase in Lucia expression compared to those without H5W (Figure 17). This highlights the effectiveness of H5W in promoting endosomal escape and improving phage vector delivery. These findings underscore the potential of endosomal escape ligands as a crucial strategy to maximise the therapeutic impact of phage-based therapies.

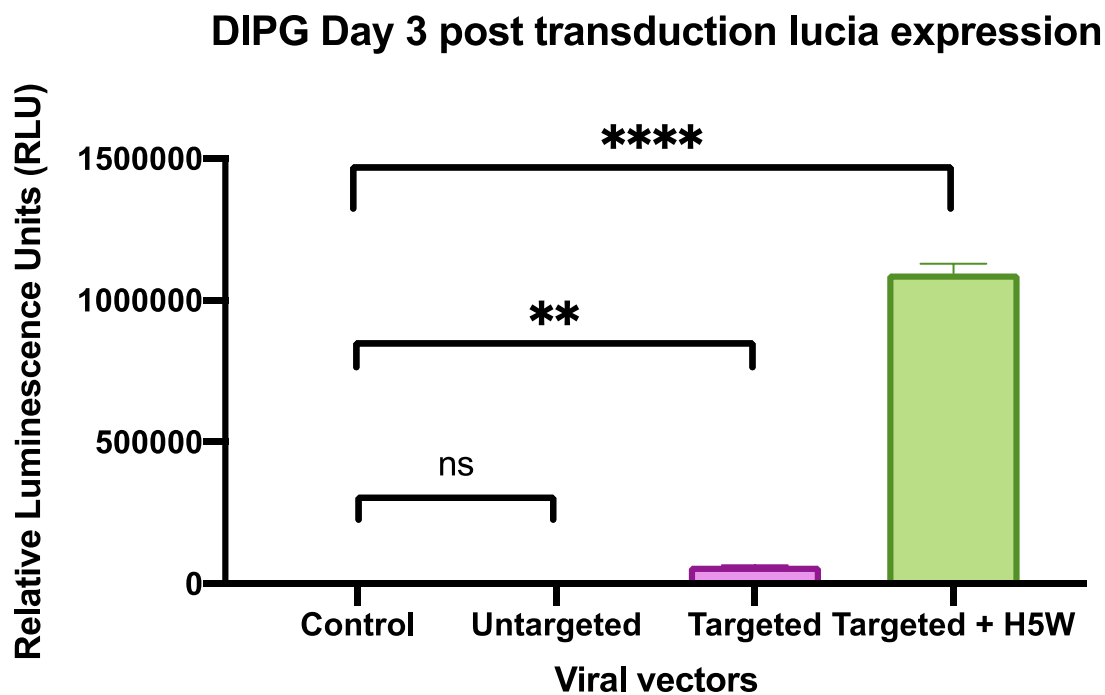


Figure 17. Transduction of HSJD-DIPG-07 cells with untargeted, targeted or targeted with H5W PAAV-lucia. DIPG cells were seeded in 96-well plate and grown to 70% confluence, then transduced at 1 million bacterial TU/cell. No virus was added to the control group. RLU was measured on day 3 by collecting 10µl of cell media and adding to 25µl of lucia substrate, then measuring using a luminometer. Data are represented as mean $\pm$ SEM of triplicate wells, experiment was repeated twice. ** = $p < 0.01$, **** = $P < 0.0001$, ns= non-significant.

In a follow-up experiment, we investigated the dose-dependent effects of PAAV on transgene expression. This is crucial for determining the optimal therapeutic dose and understanding how the number of phage vectors influences the efficiency of gene delivery. We used untargeted phage as a control and compared it to targeted phage and targeted phage expressing H5W at two different doses: 1 million (1m) and 2 million (2m) TU. Since we have not previously experimented with PAAV in DIPG, we based our dosage on a similar study which used this phage vector in human GBM (173). After 3 days, we collected the cell medium and measured relative luminescence units (RLU) as a proxy for transgene expression.

Consistent with previous results, the untargeted control group exhibited minimal RLU, while both targeted phage groups demonstrated a significant increase. Importantly, the targeted phage with H5W yielded the highest RLU, highlighting the role of endosomal escape in maximising transduction. In addition, within both the targeted and targeted with H5W groups, the 2m TU dose produced almost double the RLU compared to the 1m TU dose. This directly demonstrates a dose-dependent effect *in vitro*, where a higher number of phage vectors translates to increased transgene expression.

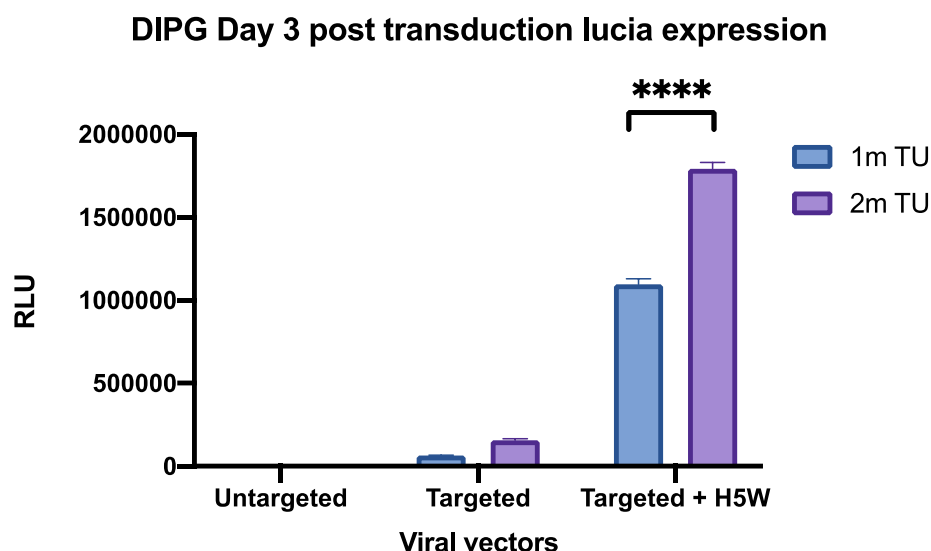


Figure 18. Transduction of HSJD-DIPG-07 cells with untargeted, targeted or targeted with H5W PAAV-lucia. DIPG cells were seeded in 96-well plate and grown to 70% confluence, then transduced at 1m or 2m bacterial TU/cell. No virus was added to the control group. RLU was measured on day 3 by collecting 10µl of cell media and adding to 25µl of lucia substrate, then measuring using a luminometer. Data are represented as mean ± SEM of triplicate wells, experiment was repeated twice. **** = $P < 0.0001$.

3.4 Transgene expression is dependent on integrin receptor expression in DIPG cells

Our previous work demonstrated that culturing DIPG cells in adherent compared to spheroid media influences the expression levels of $\alpha V\beta 3$ and $\alpha V\beta 5$ integrin heterodimers in all 6 primary DIPG cells (Figure 15). Since our phage vector specifically targets these integrins, we hypothesised that higher integrin expression would correlate with increased transgene expression upon cell transduction.

To investigate this, we cultured HSJD-DIPG-07 cells in both adherent and spheroid media and transduced them with targeted H5W phage vector expressing the Lucia transgene. We collected the cell medium over 3-5 days and measured RLU to assess gene expression. Importantly, DIPG cells cultured in spheroid medium consistently demonstrated higher Lucia expression compared to their adherent counterparts, with a particularly pronounced difference on Day 5 (Figure 19). These findings support our hypothesis, suggesting a direct correlation between integrin expression levels and transduction efficiency. This reaffirms the specificity of our phage vector in targeting DIPG cells.

Additionally, it highlights the significant impact that different culture conditions can have on cellular responses to the same therapy. Given our current understanding, it remains unclear whether adherent or spheroid cultures more accurately represent *in vivo* tumour behaviour. Therefore, employing both culture methods in preclinical drug testing offers a more comprehensive approach. This could potentially reveal how variations in the tumour environment influence treatment responses and guide the selection of the most promising therapies for clinical translation.

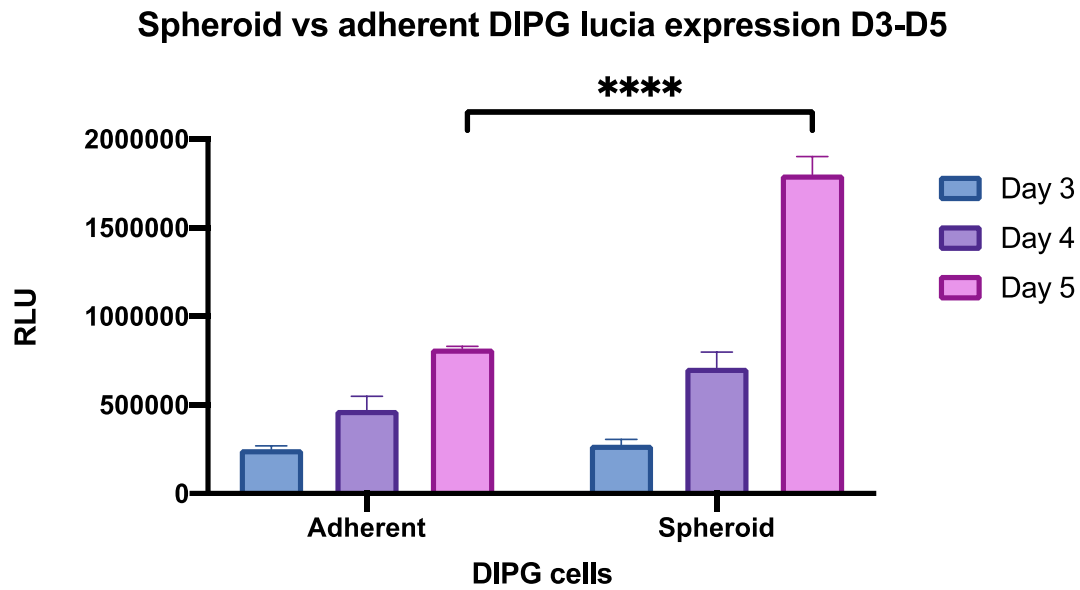


Figure 19. Transduction of adherent or spheroid DIPG cells with targeted PAAV-lucia. DIPG cells were seeded in 96-well plate, then transduced at 1m TU/cell after 2 days. RLU was measured from day 3 to 5 by collecting 10 μ l of cell media and adding to 25 μ l of lucia substrate, then measuring using a luminometer. Data are represented as mean $\pm$ SEM of triplicate wells, experiment was repeated twice. **** = $p < 0.0001$.

3.5 Astrocytes do not express integrin receptors and are hence not targeted by phage vector, confirming the specificity of vector and its sparing of normal brain tissue

While confirming the phage vector's ability to target DIPG cells based on integrin expression levels, we need to ensure its specificity towards tumour cells while minimising potential harm to healthy brain tissue. To address this, we used primary human astrocytes as representatives of normal brain cells. Using FACS, we compared integrin receptor expression between HSJD-DIPG-07 cells (cultured in both adherent and spheroid forms) and astrocytes. The results were reassuring, astrocytes demonstrated minimal expression of both $\alpha V\beta 3$ and $\alpha V\beta 5$, levels that were almost undetectable compared to the high expression observed in the DIPG cells (Figure 20). These findings provide preliminary evidence suggesting that the phage vector has a potential safety profile, favouring selective targeting of tumour cells with minimal impact on healthy astrocytes.

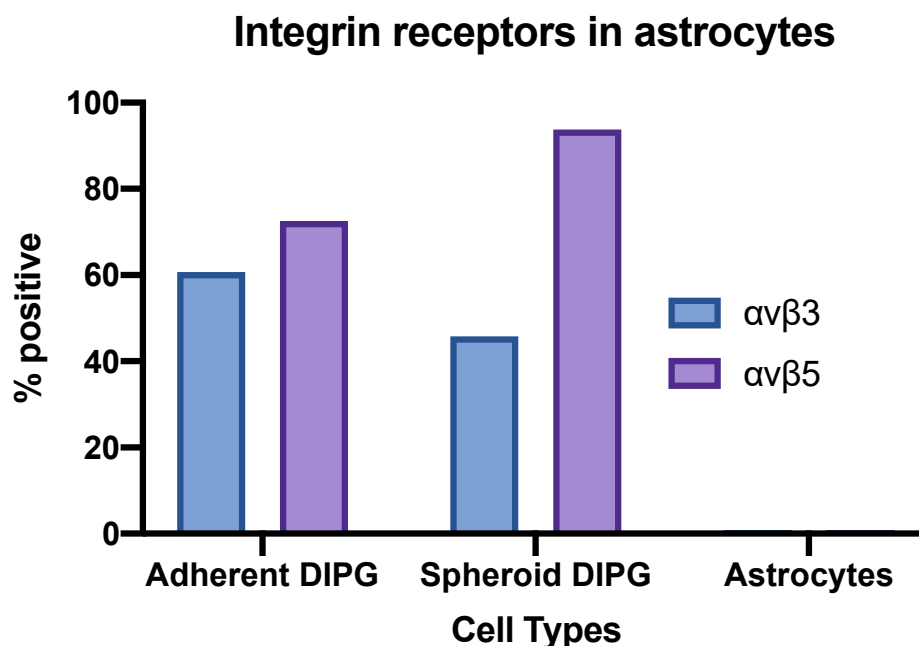


Figure 20. FACS analysis of HSJD-DIPG-07 cells for $\alpha V\beta 3$ and $\alpha V\beta 5$ integrin expression. DIPG cells cultured in adherent or spheroid medium, and astrocytes were analysed by FACS. The bars show the % of cells that were positive for specified integrin. Astrocyte integrin expression is minimal. n=1

Building upon the observation of negligible integrin expression in astrocytes, we sought to definitively confirm that the phage vector would not induce transgene expression in these astrocytes due to their lack of target integrin receptors.

We designed an experiment using three groups: 1) control cells, 2) untargeted phage, and 3) targeted phage. Lucia expression was monitored from day 3-5. Critically, we observed extremely low Lucia expression (approximately 5000 times less than in DIPG cells, refer to Figure 19), with no significant differences among the three groups (Figure 21). This compelling result strongly indicates that the phage vector does not target astrocytes and therefore does not induce transgene expression in these normal brain cells. This finding provides encouraging evidence for the potential safety and tumour specificity of our phage-based therapeutic approach.

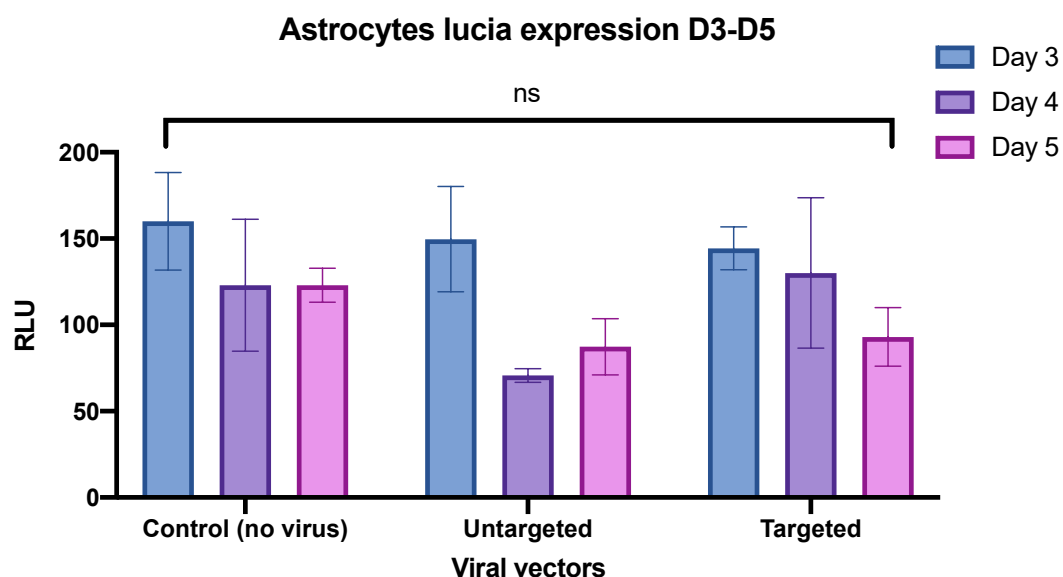


Figure 21. Transduction of human astrocytes with targeted or untargeted H5W PAAV-lucia. Astrocytes were seeded in 96-well plate and grown to 70% confluence, then transduced at 1m TU/cell. No virus was added to control group. RLU was measured from day 3 to 5 by collecting 10µl of cell media and adding to 25µl of lucia substrate, then measuring using a luminometer. Data are represented as mean $\pm$ SEM of triplicate wells, experiment was repeated twice. There was no significant difference between the 3 groups.

3.6 DIPG cells express TRAIL-R2, allowing for TRAIL targeting

Harnessing the potential of TRAIL as a therapeutic transgene rests upon the presence of its death receptors on the surface of DIPG cells. Since death receptor expression profiles can vary significantly across different cancer types, it is imperative to verify their presence in DIPG cells before employing a TRAIL-based therapeutic strategy.

To address this, we performed FACS staining of HSJD-DIPG-007 cells cultured in both adherent and spheroid media to assess the expression of all known TRAIL receptors. Encouragingly, DIPG cells demonstrated robust expression of TRAIL R-2 in both culture conditions, suggesting that TRAIL would effectively bind to and target these tumor cells (Figure 22). While we were unable to visualise staining for TRAIL R-1, R-3, and R-4, the observed TRAIL R-2 expression supports the potential of TRAIL as a promising therapeutic agent for DIPG.

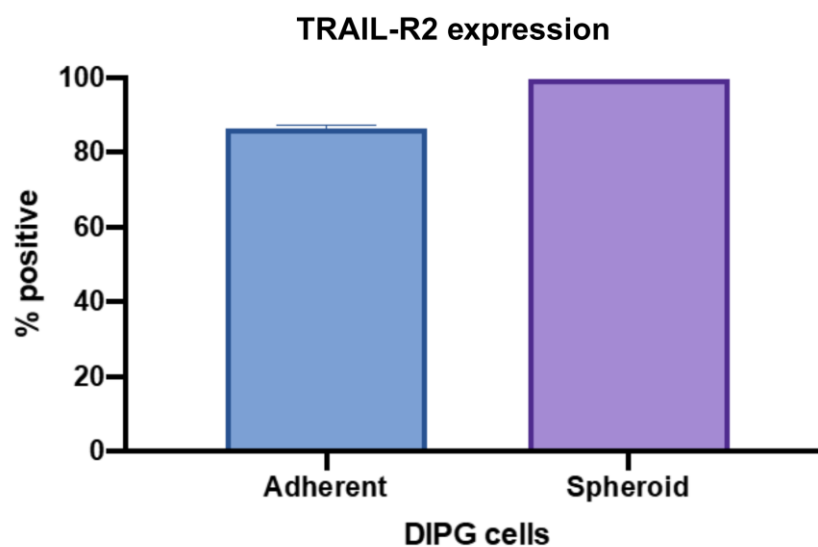


Figure 22. FACS analysis of HSJD-DIPG-007 cells for TRAIL-R2 expression. DIPG cells cultured in adherent or spheroid medium were stained with the primary anti-human CD262 (conjugated with fluorophores) antibodies for 30 minutes, then washed and analysed by FACS. Control cells were not stained. Data are represented as mean $\pm$ SEM of triplicates, experiment was repeated twice. There was no statistical significance.

3.7 Astrocytes express less TRAIL R-2 compared to DIPG cells

Building upon our findings, we extended our investigation to include TRAIL R-2 expression on human astrocytes, as a model for healthy brain tissue. Importantly, FACS analysis revealed that astrocytes express TRAIL R-2 to a lesser degree compared to DIPG cells in both adherent and spheroid cultures (Figure 23). This differential expression is reassuring from a safety standpoint. It suggests that a TRAIL-based therapeutic approach would preferentially target tumour cells with higher TRAIL R-2 expression, potentially minimising adverse effects on healthy astrocytes. These findings align with current literature indicating that TRAIL can induce apoptosis more selectively in tumour cells compared to many normal cell types (184, 190-192), further supporting the potential safety and tumour specificity of TRAIL.

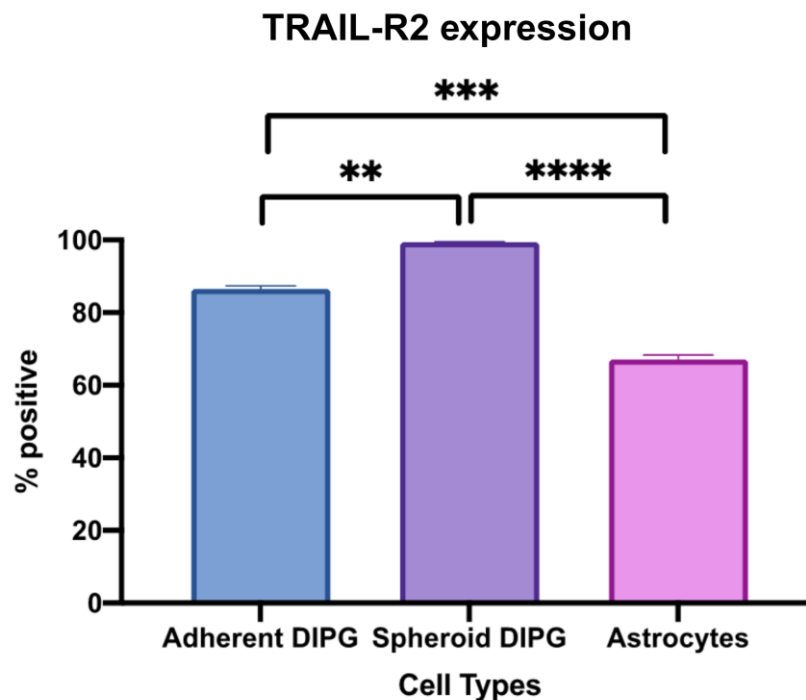


Figure 23. FACS analysis of HSJD-DIPG-007 cells and astrocytes for TRAIL R-2 expression. DIPG cells cultured in adherent or spheroid medium, and astrocytes were stained with the primary anti-human CD262 (conjugated with fluorophores) antibodies for 30 minutes, then washed and analysed by FACS. Control cells were not stained. Data are represented as mean $\pm$ SEM of triplicates, experiment was repeated twice. ** = $p < 0.01$, *** = $p < 0.01$ **** = $p < 0.0001$.

To further corroborate our FACS findings at the molecular level, we collected messenger RNA (mRNA) from these cells and performed qRT-PCR to compare their relative expression of TRAIL R-2. mRNA serves as an indicator if genes are actively being expressed in a cell or tissue. Consistently, our analysis showed that astrocytes had lower relative mRNA levels of TRAIL R-2 compared to DIPG cells, particularly those grown as spheroids (Figure 24).

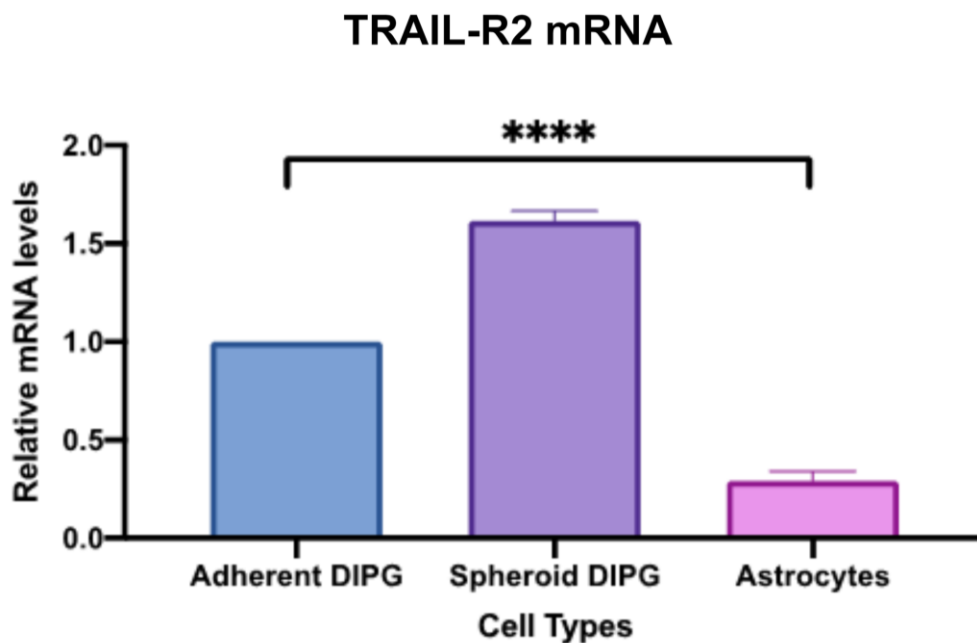


Figure 24. Relative TRAIL R-2 mRNA expression based on qRT-PCR. mRNA was collected from human astrocytes, adherent and spheroid DIPG cells and TRAIL R-2 expression levels were compared using their $2\Delta\Delta C_t$. Results were first normalised by GAPDH housekeeper gene, then to adherent DIPG TRAIL R-2 expression level. Data are represented as mean $\pm$ SEM of triplicates, experiment was repeated twice. **** = $p < 0.0001$.

3.8 TRAIL is able to induce killing of DIPG cells, both in direct gene transfection and in phage vector delivery

To directly assess the potential of TRAIL to kill DIPG cells and recognising the incomplete understanding surrounding transmembrane and soluble TRAIL, we initially employed both forms to compare their cytotoxic effects.

We transfected DIPG cells with either a control plasmid (expressing GFP), transmembrane TRAIL (tmTRAIL), or soluble TRAIL (sTRAIL). The reason for using a control plasmid is to account for any potential toxicity associated with the FuGene transfection reagent itself. After 24 hours, microscopic examination revealed a noticeable decrease in cell density in both the tmTRAIL and sTRAIL wells, with the sTRAIL well potentially exhibiting the most pronounced reduction (Figure 25).

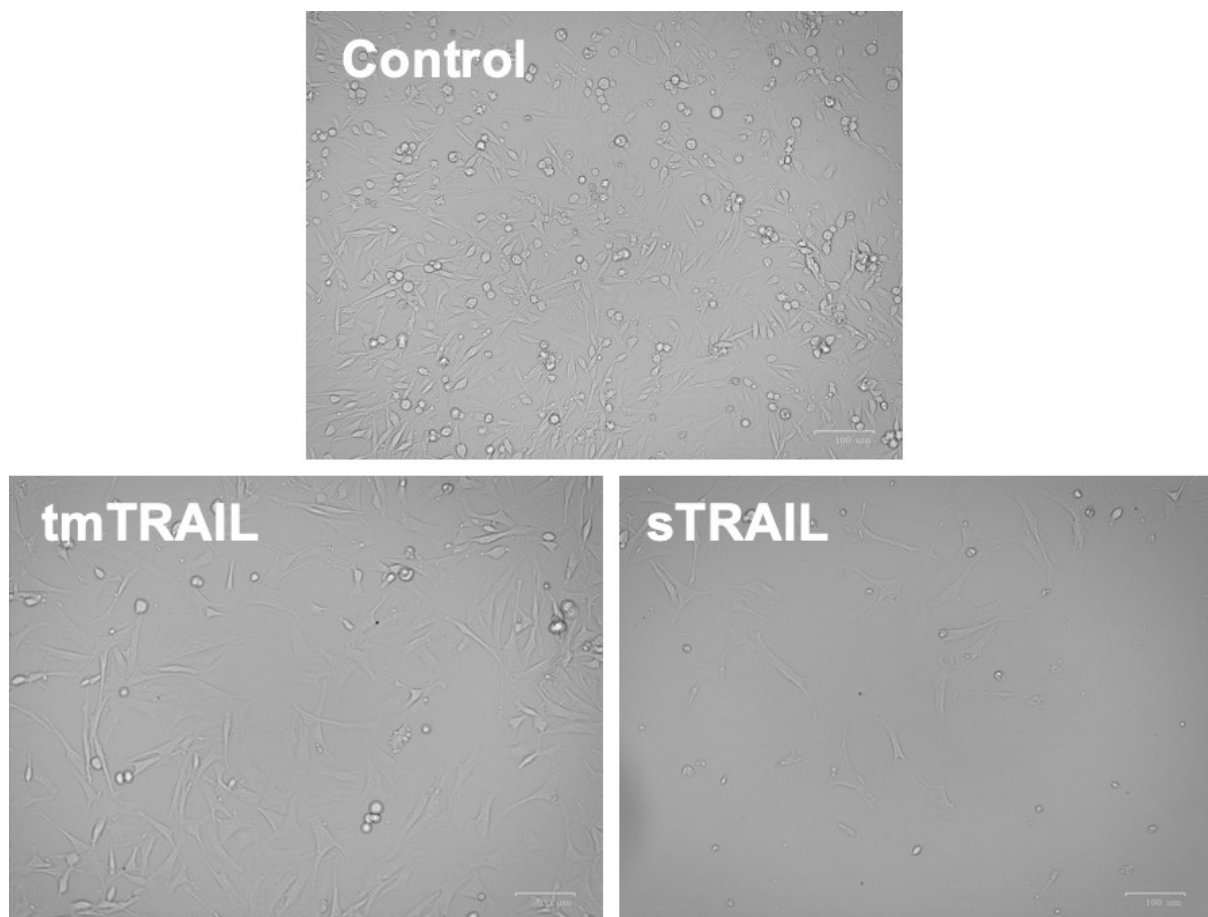


Figure 25. Transfection of adherent HSJD-DIPG-007 cells. DIPG cells were seeded in 96-well plate until 70% confluent, then transfected with 0.4ng of plasmid DNA for 6 hours (control/GFP, tmTRAIL or sTRAIL), after which the media was replaced. Cells were imaged on light microscope. Scale bar, 75 μ m.

To quantify this observation, we assessed cell viability with the SRB assay and results were normalised using non-transfected control cells (Figure 26). Importantly, both tmTRAIL and sTRAIL demonstrated significant cell killing in DIPG cultures, with comparable efficacy when delivered directly. While the transfection protocol was limited to adherent cells, this experiment establishes the potential of TRAIL to induce tumour cell death in DIPG.

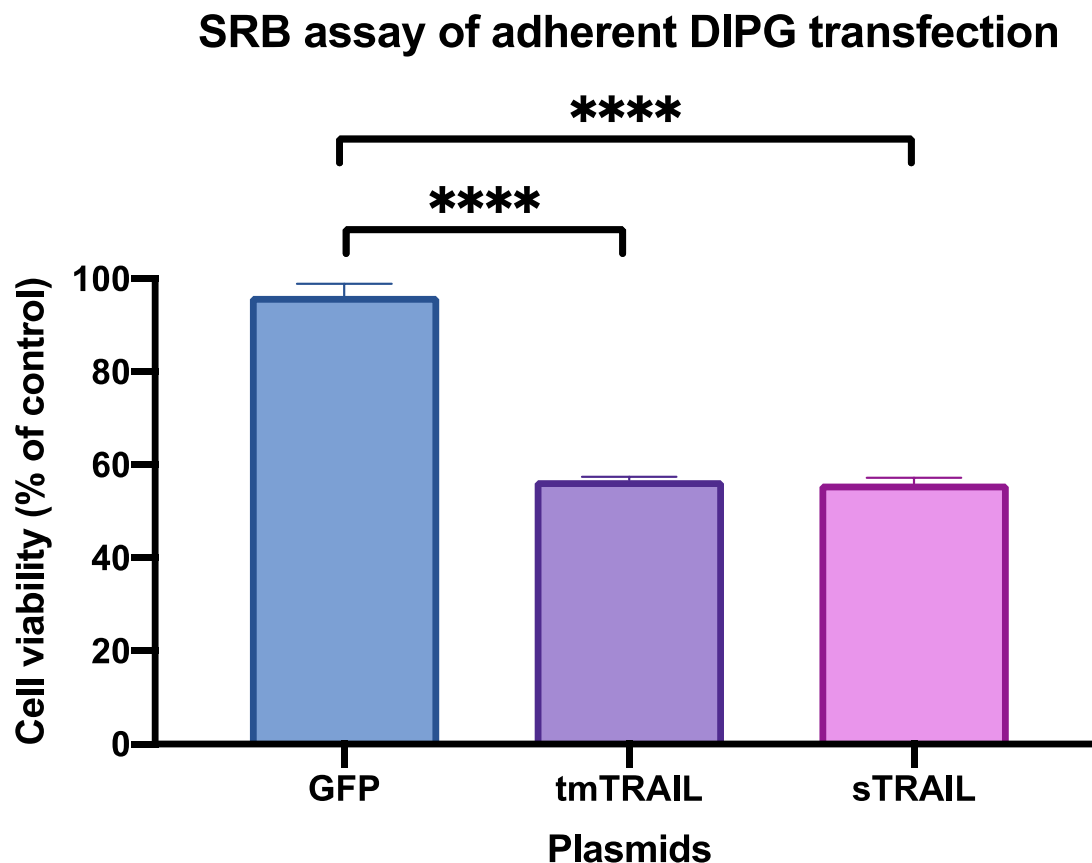


Figure 26. Cell viability after transfection of adherent HSJD-DIPG-007 cells. Cells were transfected with GFP, tmTRAIL or sTRAIL plasmids with FuGene. DIPG cells were seeded in 96-well plate until 70% confluent, then transfected with 0.4ng of plasmid DNA for 6 hours (GFP, tmTRAIL or sTRAIL), after which the media was replaced. Cells with only FuGene added was used as the control to normalise the results. Cell viability was determined after 24 hours with SRB assay. Results were normalised with cells without plasmid DNA added. Data are represented as mean $\pm$ SEM of triplicates, experiment was repeated twice. **** = $P < 0.0001$.

Building upon our transfection findings, we aimed to determine if transmembrane TRAIL (tmTRAIL) and soluble TRAIL (sTRAIL) had differential killing effects when delivered by our targeted phage vector system. We constructed PAAVs carrying both transgenes and transduced DIPG cells in spheroid culture. Four experimental groups were established: untargeted tmTRAIL, targeted tmTRAIL, untargeted sTRAIL, and targeted sTRAIL, alongside a control group with no virus added for normalization.

After 6 days, a visible decrease in cell density was observed under the microscope in the sTRAIL well (Figure 27).

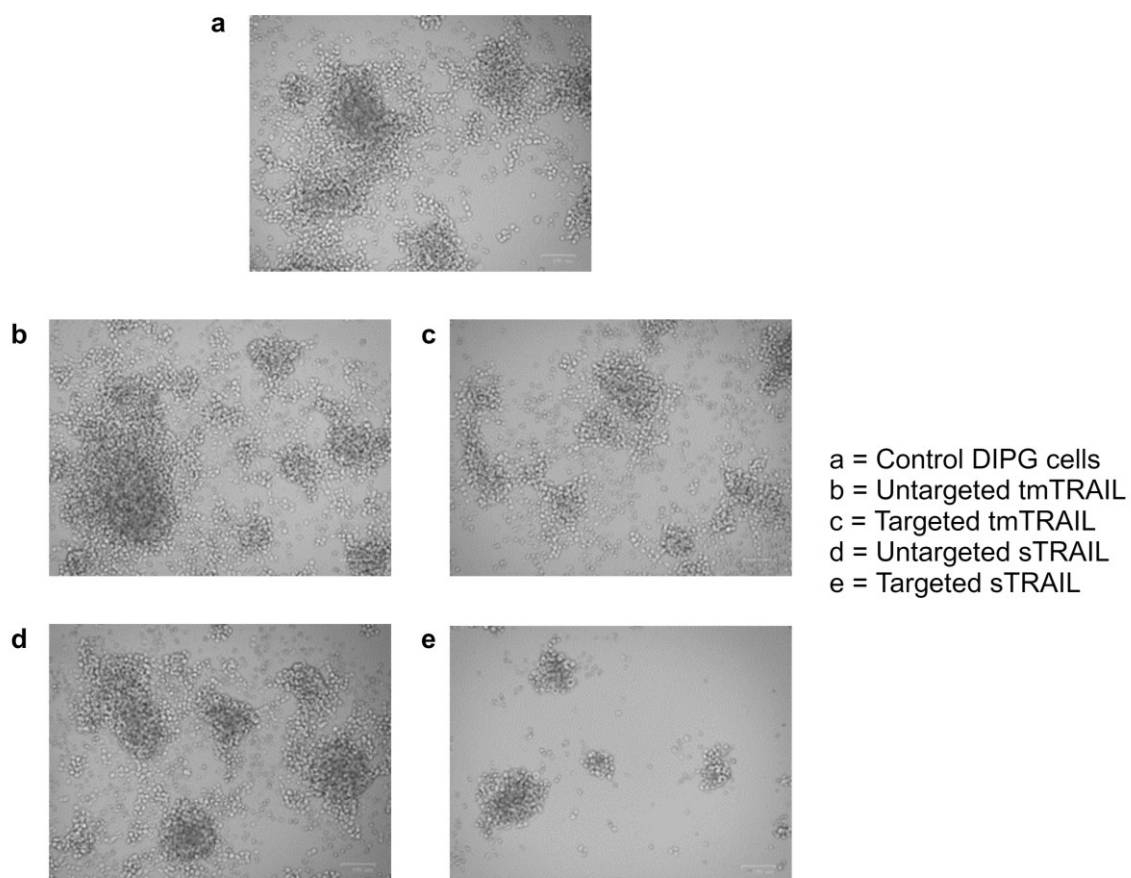


Figure 27. Transduction of HSJD-DIPG-007 cells. Spheroid DIPG cells were seeded in 96-well plate then transduced with untargeted or targeted tmTRAIL untargeted or targeted sTRAIL at 1.5m TU/cell after 2 days. Lucia was used as a control viral vector. Cells were imaged on light microscope. Scale bar, 100 μ m.

This observation was subsequently quantified using the Cell Titre Glo assay. Surprisingly, targeted tmTRAIL did not show a significant increase in cell death compared to its untargeted counterpart. Conversely, targeted sTRAIL induced a striking 70% reduction in cell viability compared to both the control and untargeted sTRAIL groups (Figure 28).

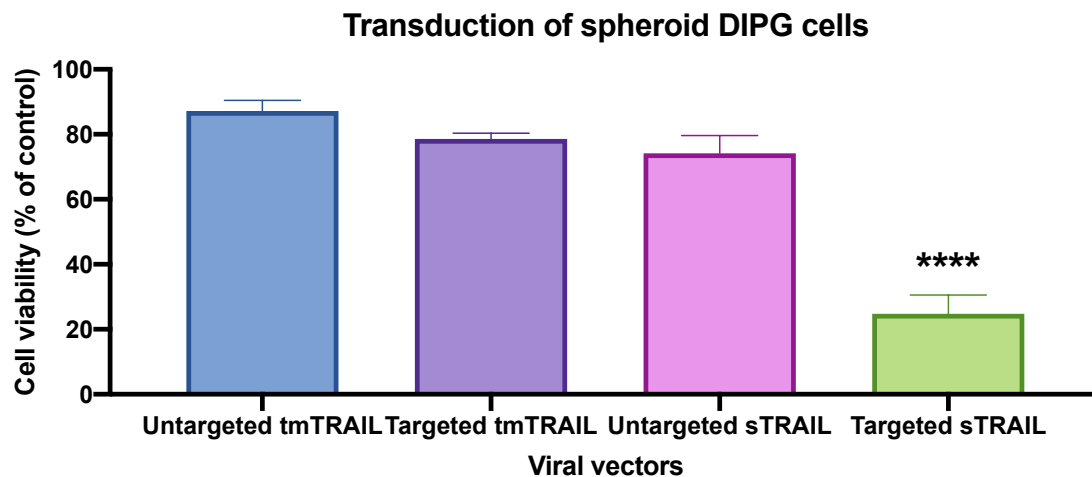


Figure 28. Cell viability of HSJD-DIPG-007 cells after transduction. Spheroid DIPG cells were seeded in 96-well plate then transduced with untargeted or targeted tmTRAIL untargeted or targeted sTRAIL at 1.5m TU/cell after 2 days. Lucia was used as a control viral vector. Cell viability was determined on Day 6 by adding Cell Titre Glo media reagent to the cell culture in 1:1 ratio and measuring using a luminometer. Results were normalised with non-transduced cells. Data are represented as mean $\pm$ SEM of triplicates, experiment was repeated three times. **** = $P < 0.0001$.

We wanted to corroborate that the observed cell killing was attributable to TRAIL expression within the DIPG cells after transduction. To achieve this, we collected mRNA from cells following transduction with both non-targeted and targeted vectors. By analysing these samples using qRT-PCR, we found a significantly higher sTRAIL mRNA expression level in the cells transduced with the targeted vectors (Figure 29). This finding strongly suggests that the enhanced cell death observed with targeted sTRAIL delivery is a direct consequence of increased TRAIL expression within the tumour cells.

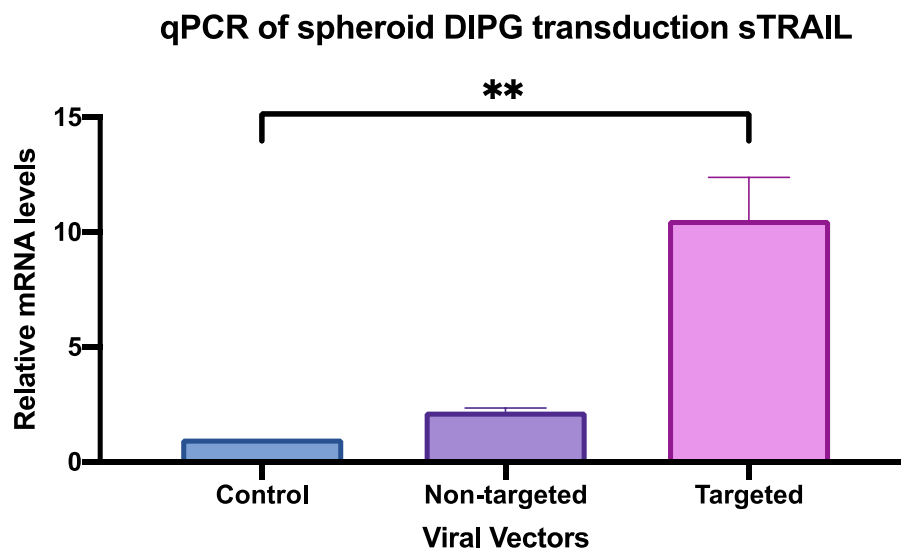


Figure 29. Relative TRAIL mRNA expression after transduction. mRNA was collected from control cells with no virus added, cells transduced with non-targeted or targeted vectors. TRAIL expression levels were compared using their $2\Delta\Delta C_t$. Results were first normalised by GAPDH housekeeper gene, then to spheroid DIPG TRAIL expression level. Data are represented as mean $\pm$ SEM of triplicates, experiment was repeated twice. ** $p=0.0014$

3.9 Phage vector is able to cross the BBB in immunodeficient mice bearing intracranial DIPG

A critical next step in our investigation was to evaluate the tumour specificity and biodistribution of RGD4C/PAAV following intravenous administration within a relevant *in vivo* model. To achieve this, we utilised immunodeficient mice bearing intracranial DIPG tumours. In vivo studies allows us to assess how the vector interacts with complex biological systems and to determine whether it can successfully reach and target tumour cells within the unique environment of the brain after systemic delivery.

Before administration, the phage vector was produced in large titers and purified using monolith ion-exchange columns (BIA-Separations GmbH) to remove endotoxins and ensure safety. Orthotopic DIPG mouse models were established by Dr. Carcaboso through the injection of human biopsy-derived tumorspheres (HSJD-DIPG-007) into the 4th ventricle of the brain. Mice were carefully monitored for tumour development, and intravenous tail-vein administration of either nontargeted vectors or targeted vectors (5×10^{10} TU/mouse) was performed once tumours were detectable tumours (69-84 days post-inoculation).

After 22 hours, mice were euthanised, and their brains were collected for immunohistochemical analysis. Paraffin-embedded brainstem and cerebellum sections were evaluated. H&E staining and immunohistochemistry with anti-human nuclear antigen (HNA) revealed diffusely infiltrating tumour cells throughout the brain (Figure 30), confirming successful tumour engraftment and providing the basis for assessing vector targeting and distribution.

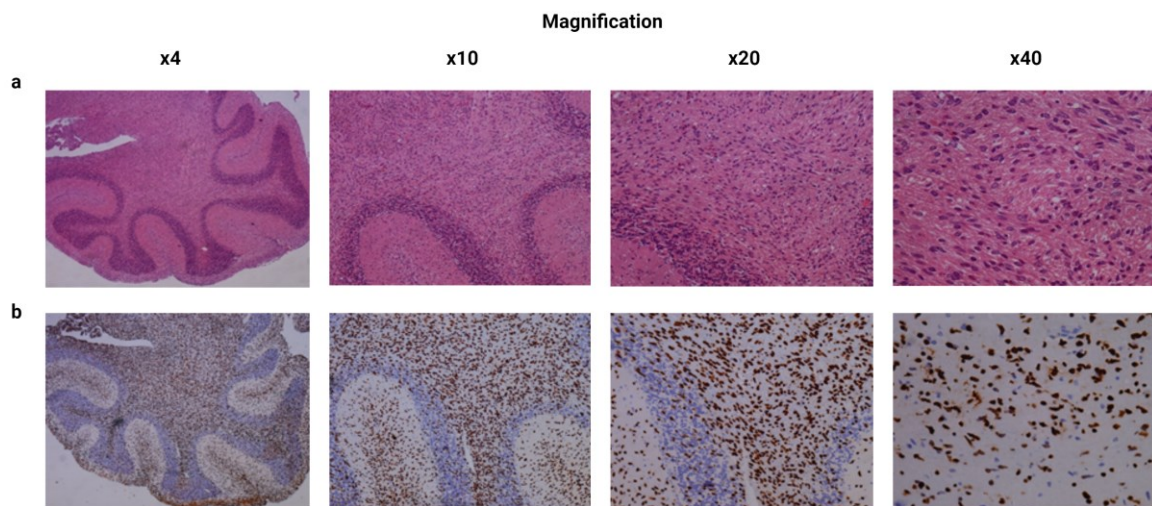


Figure 30. Histological analysis of DIPG tumour. Tumours were collected from each mouse and 10 μm sections were produced. The sections were observed on a fluorescence microscope at a magnification of x4, x10, x20 and x40.

- a. H&E staining
- b. Immunohistochemistry with anti-HNA. Brown nuclei are human cells while blue nuclei are mouse cells.

To investigate PAAV's ability to bypass the BBB and demonstrate tumor specificity in our mouse model, we prepared frozen sections and used immunofluorescence with an anti-phage antibody for detection of phage. Our results provided compelling evidence: we observed accumulation of phage particles within the tumour only in mice that have been treated with targeted sTRAIL (Figure 31), demonstrating the vector's ability to effectively cross the BBB.

This crucial finding has several significant implications. Firstly, it confirms that our targeted phage vector can successfully overcome the highly restrictive BBB following intravenous administration. Since the BBB poses a major challenge for treating DIPG by limiting the delivery of therapeutics, this finding carries immense importance. Secondly, the observed tumour accumulation highlights the vector's specificity, suggesting that the RGD4C ligand effectively guides the phage towards DIPG cells even after systemic tail-vein administration. Overall, these findings underscore the therapeutic potential of this phage-based delivery system for DIPG, opening avenues for targeted delivery of various therapeutic genes directly to the tumour site.

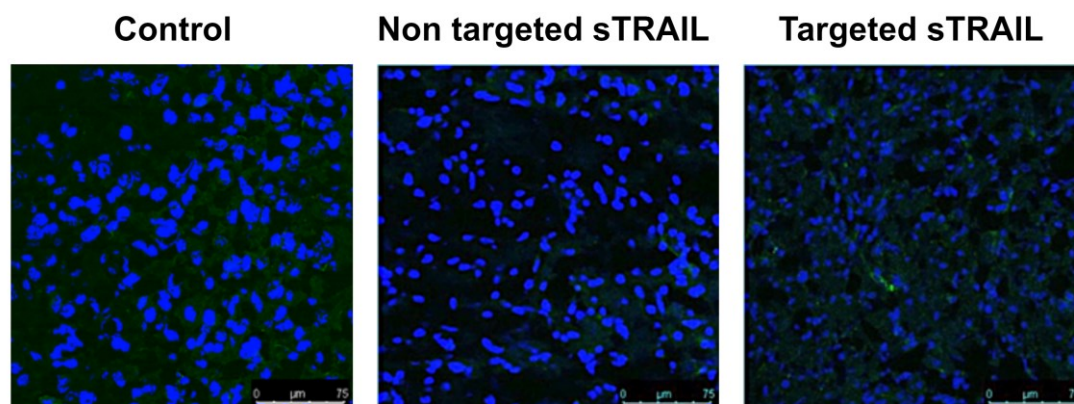


Figure 31. Immunofluorescence staining of DIPG tumour. Tumours were collected from mice that have received control (no virus), non-targeted sTRAIL vector, or targeted sTRAIL vector. 10 μm cryostat sections were produced and co-stained for both DAPI (blue) and phage (green) and viewed under fluorescence. Scale bar, 75μm.

3.10 Phage vector is able to improve survival of mice

Building upon our promising in vitro findings, we conducted a preliminary investigation into the anti-cancer activity of our phage vectors following intravenous administration in NOD.SCID mice bearing HSJD-DIPG-007 tumours. This study was conducted in collaboration with Dr. Carcaboso, who provided expertise in mouse model selection, treatment and management of animals, and survival data analysis.

Mice were divided into three groups:

- Group 1 (control): Received 200 µl of PBS intravenously (n = 7)
- Group 2 (non-targeted sTRAIL): Received 5×10^{10} TU of non-targeted sTRAIL vector in 200 µl PBS intravenously (n = 7)
- Group 3 (targeted sTRAIL): Received 5×10^{10} TU of targeted sTRAIL vector in 200 µl PBS, intravenously (n = 8)

Mice were carefully monitored for weight changes, with a 20% weight loss generally considered the survival endpoint.

Interestingly, both non-targeted and targeted vectors prolonged survival in tumour-bearing mice, although the non-targeted vector appeared to confer a slightly longer survival advantage (Figure 32). While formal toxicity studies were not conducted, the treatment appeared well-tolerated, with minimal weight loss observed in the treated mice.

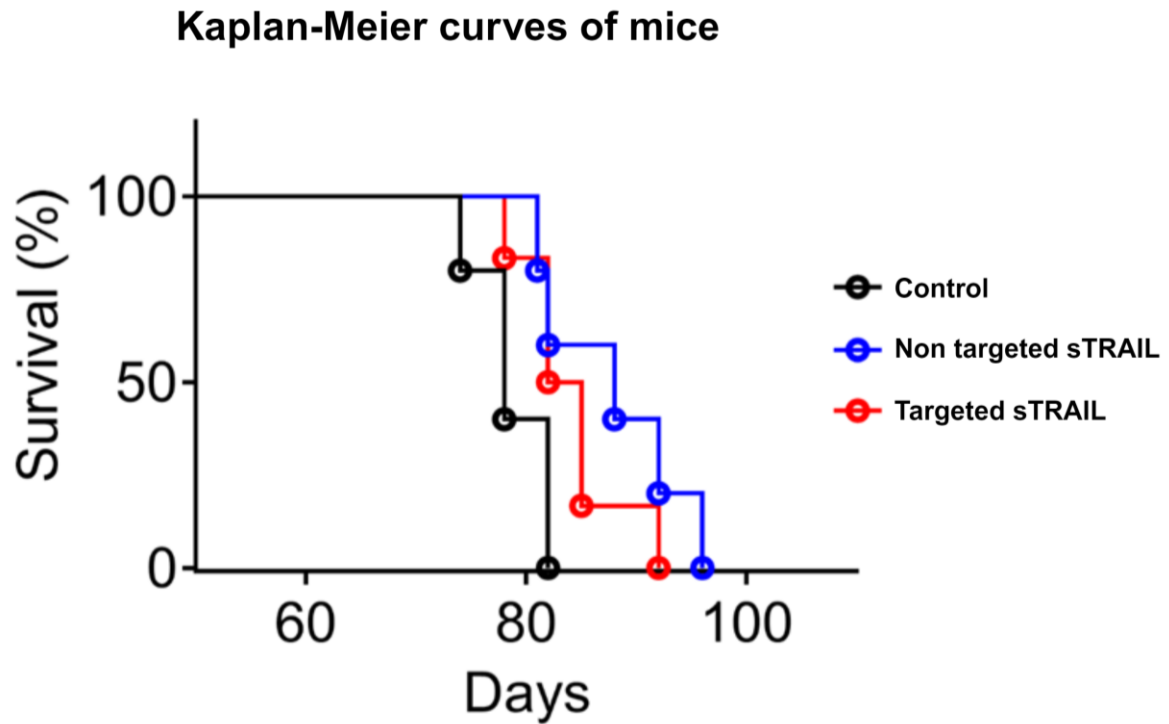


Figure 32. Survival of mice with HSJD-DIPG-007 tumours. The Median survival for control was 78 days, for non-targeted TRAIL was 88 days ($P = 0.0403$ vs control) and for targeted sTRAIL was 83.5 days ($P = 0.0458$ vs control). Median survivals were calculated using Kaplan-Meier curves. Statistic test is log rank test.

3.11 Overview of findings

The main findings of this thesis support the potential of a targeted phage-based approach for treating DIPG (Figure 33). Key findings include:

- **Morphological Heterogeneity:** DIPG cell lines exhibit distinct morphological differences when cultured under adherent or spheroid conditions. This highlights the importance of careful culture method selection for drug testing, as responses may vary depending on the model system.
- **Integrin-Mediated Targeting:** High $\alpha\text{v}\beta 3$ and $\alpha\text{v}\beta 5$ integrin expression on DIPG cells, particularly in spheroid cultures, validates the choice of the RGD4C ligand for phage vector targeting. Comparatively, astrocytes express these integrins minimally.
- **Endosomal Escape Enhancement:** Incorporation of the H5W endosomal escape peptide significantly improves phage vector delivery, resulting in increased transgene expression.
- **TRAIL Efficacy:** DIPG cells demonstrate high TRAIL R-2 expression, with astrocytes expressing lower levels. This suggests TRAIL's potential as a selective therapeutic agent. Both transmembrane and soluble TRAIL induce DIPG cell death, with an even greater effect seen with targeted soluble TRAIL *in vitro*.
- **In Vivo Success:** The phage vector successfully crosses the BBB after intravenous administration in an orthotopic mouse model. It selectively targets tumour cells, demonstrating its ability to home to DIPG and potential for systemic therapeutic delivery.

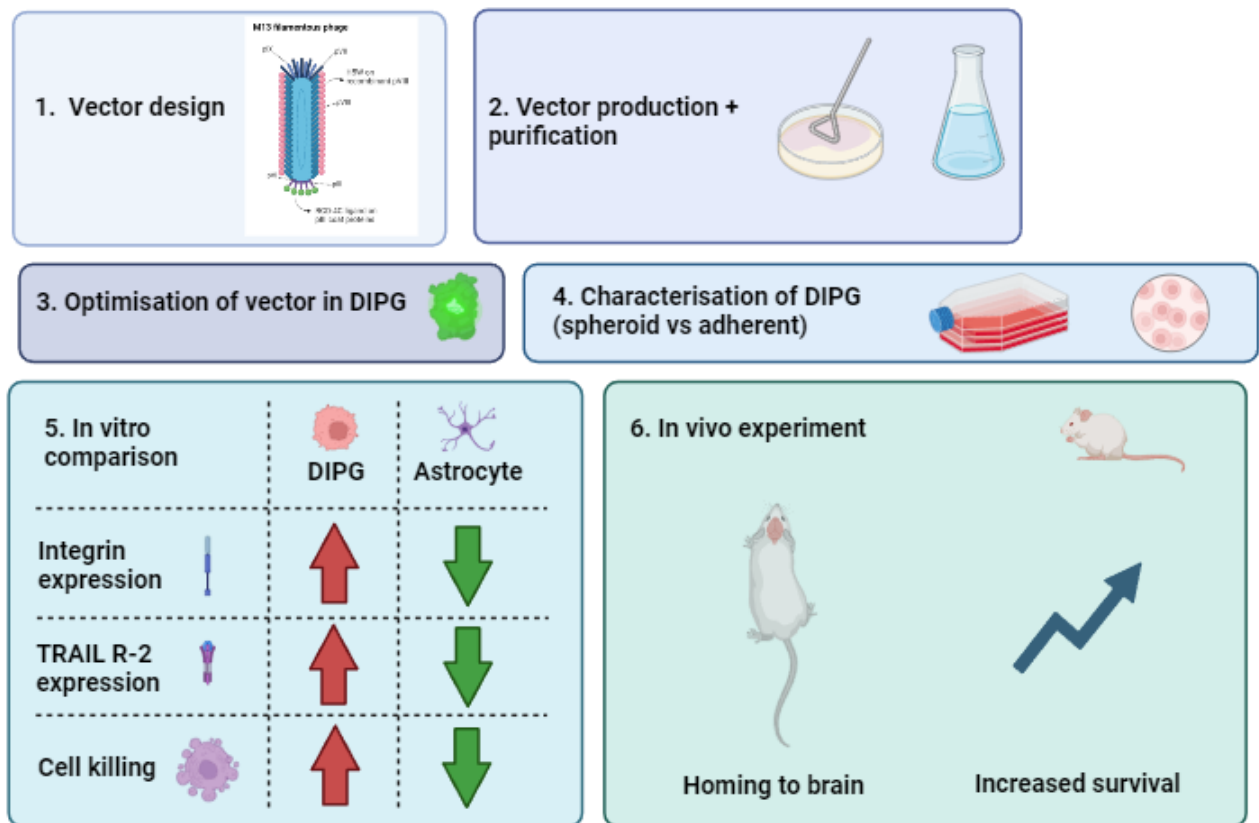


Figure 33. Summary diagram of experimental results. Created with BioRender.com

4. Discussion

DIPG is a devastating paediatric brain tumour with a dismal prognosis. Current treatment strategies, mainly radiotherapy, offer limited survival benefits, highlighting the urgent need for novel therapeutic approaches. Clinical trials testing various agents have not shown benefits significantly surpassing those of radiotherapy. However, enhanced access to patient biopsies has deepened our understanding of the histopathology of DIPG, paving the way for novel therapeutic strategies.

The resistance of treatments derived from adult brain tumours to DIPG can be explained in two ways. Firstly, evidence has shown that DIPGs are molecularly distinct from adult high-grade gliomas, and in itself it is comprised of distinct subgroups characterised by unique mutation patterns and epigenetic traits, which correlate with their biological behaviour and clinical outcomes (230). Secondly, the presence of an intact BBB impedes effective delivery of therapeutic drugs to the brain parenchyma.

This research project investigates the potential of a targeted phage-based gene delivery system as a groundbreaking treatment paradigm for DIPG. The hybrid phage vector, initially conceived by Hajitou and colleagues (100), was constructed by integrating the adeno-associated virus (AAV) into filamentous phage genome to achieve the benefits of both vectors. This was further refined to a new phagemid system, where only therapeutic genes are encoded in its genome while structural capsids are provided from a helper phage, allowing for a even smaller particle size. The vector expresses RGD4C ligand on the pIII capsid protein, allowing it to target $\alpha\beta3$ and $\alpha\beta5$ integrins which are overexpressed on tumour cells and angiogenic blood vessels of solid tumours. Furthermore, we incorporated the H5W endosomal escape peptide on the pVIII capsid protein which enhances transduction efficacy. Unpublished data from the Hajitou group suggests that this induces a proton sponge effect which causes the endosome to destabilise and burst, releasing phage vector particles.

Previously, the members of the TNF superfamily have generated a lot of interest in cancer research due to their ability to activate the extrinsic apoptosis pathway. However, their applications to therapy were limited by their systemic side effects. In this thesis, we have used TRAIL, which has shown preferential toxicity towards cancer cells, thought to be due to an upregulation of its specific death receptors. In contrast, normal healthy cells were thought to be spared due to the presence of decoy receptors (184, 190-192).

We therefore constructed the RGD4C-targeted PAAV vector to deliver the therapeutic TRAIL gene and conducted *in vitro* and *in vivo* investigations to assess its efficacy and translational potential. This construct was established using several rounds of molecular cloning to select the candidate to be used in vector production. Following which, the virus was produced and purified in large titres for research via propagation in yeast cells. Given that one of the main hurdles to using viral vectors in transgene delivery is the difficulty and high cost of production (231), our vector system confers the advantages of ease of production on large scales.

We first validated the use of our phage vector in DIPG cells. Integrins, often overexpressed in cancer cells, serve as ideal targets for directed therapies. The levels of integrin expression can differ significantly between normal and tumor tissues, and this variance is linked to specific cancer types and the tendency for cancer to spread to particular organs. For instance, while integrins $\alpha v \beta 3$, $\alpha v \beta 6$, and $\alpha 5 \beta 1$ are typically found at low or undetectable levels in most normal epithelial tissues, their expression can be substantially increased in a variety of tumours (232). Extensive characterisation of integrin expression across a diverse panel of DIPG cell lines confirmed their robust presence, justifying the use of RGD4C-targeted PAAV.

To maximise intracellular gene delivery, we incorporated the H5W endosomal escape peptide into our phage vector. Using reporter genes *lucia*, we were able to demonstrate a significant enhancement in vector transduction efficiency. This highlights the crucial role of endosomal escape mechanisms in overcoming a common barrier to effective gene therapy.

Next, we focused on the therapeutic potential of TRAIL. The mechanism behind TRAIL preferentially targeting cancer cells while sparing normal cells is theorised to be a result of an up-regulation of TRAIL death receptors in cancer cells while healthy cells express TRAIL decoy receptors. Studies have shown that in colorectal cancer, the over-expression of TRAIL decoy receptors in cancer cells have contributed to treatment resistance (233). Importantly, DIPG cells exhibited strong TRAIL R-2 expression, while astrocytes, representing healthy brain cells, showed lower levels of expression. This differential expression pattern is highly encouraging, as it highlights the potential for TRAIL-based therapies to preferentially target tumour cells while minimising adverse effects on normal brain tissues. In line with this finding, both tmTRAIL and sTRAIL effectively induced DIPG cell death *in vitro*. Notably,

sTRAIL exhibited superior cytotoxic potency when delivered via our phage vector, emphasising the vector's ability to ensure successful intracellular delivery of the TRAIL gene.

A major challenge in treating DIPG is the presence of the BBB, which limits the delivery of therapeutic agents. To address this, we employed an orthotopic DIPG mouse model to assess our system *in vivo*. Excitingly, our results demonstrated that intravenously administered RGD4C-targeted PAAV successfully crossed the BBB and selectively accumulated within tumour cells. This breakthrough highlights the remarkable success of our targeted delivery platform in overcoming a significant obstacle faced by conventional DIPG therapies.

Our research also highlights important aspects of DIPG biology that could guide future drug development efforts. Consistent with previous findings (43), we observed distinct morphological characteristics and differential integrin expression patterns between DIPG cells cultured under adherent and spheroid conditions. Crucially, these phenotypic variations translated into differential responses to our phage-based therapy. This underlines the profound impact of culture conditions on cellular responses and emphasises the critical need for careful cell model selection during drug discovery. Employing both adherent and spheroid cultures, potentially alongside more complex models that incorporate other elements of the tumour microenvironment, could provide a more comprehensive and predictive assessment of the *in vivo* efficacy of new therapeutics.

While our findings highlight TRAIL's potential, further refinement is needed. Future studies should delve into the differences between tmTRAIL and sTRAIL and investigate their effects *in vivo*, perhaps given in combination with other sensitising agents to enhance its efficacy. Additionally, our study establishes a versatile phage-based platform with broad applications for DIPG treatment. This system has the potential to deliver diverse therapeutic genes beyond TRAIL. Future research could explore immunomodulatory genes (eg. Interleukin-12, Interferon-gamma (234)), genes targeting oncogenic pathways (eg. PI3K/Akt/mTOR pathway (235)), or even genes that increase tumour sensitivity to traditional therapies. Since DIPG displays considerable resistance to most single-agent therapies, investigating combination approaches is essential (15, 236, 237). Integrating our phage-based therapy with conventional chemotherapy, multi-pronged immunotherapy, or concurrent chemoradiotherapy could potentiate potent synergistic anti-tumour effects. A recent paper has shown that the combination of an oncolytic adenovirus, Delta-24-RGD, with the dopamine receptor D2 antagonist ONC201 improved the efficacy compared to each agent alone in by

potentiating nuclear DNA damage and thus improving the immune response to each agent alone (238).

The determination of optimal phage vector dosage presents a novel challenge in the treatment of DIPG. While research into other viral vectors such as Delta-24-RGD has shown promise in DIPG models (239, 240), there is no established precedent for dosing with this specific phage vector PAAV. Our initial dosage guidance stemmed from a study investigating the use of this phage vector in human GBM, albeit with the expression of different therapeutic genes (173). This highlights the need for rigorous dose optimisation studies tailored to the specific context of DIPG and the therapeutic payloads used. Optimising vector dosage has the potential to significantly enhance therapeutic efficacy, therefore exploring a range of doses in both *in vitro* and *in vivo* models is crucial. Preclinical *in vitro* work can provide initial insights into dose-response relationships, allowing for the identification of potentially effective doses to be further evaluated *in vivo*. Importantly, *in vivo* studies must carefully consider factors beyond tumour reduction, such as potential side effects, immune responses, and long-term safety profiles of different dosages. This will be further discussed in the limitations section.

Our preliminary *in vivo* results offer encouraging signs that warrant further investigation. The observed survival benefit in mice receiving our phage therapy, even at this early stage, suggests the strategy's potential. Intriguingly, our *in vivo* studies revealed some survival benefit associated with the untargeted TRAIL vector, which surprisingly exceeded that of the targeted vector in this preliminary analysis. This unexpected finding warrants further investigation into potential off-target effects, as well as the role of phage-induced immune responses within the complex tumour microenvironment. It is possible that the phage itself may be triggering beneficial anti-tumour immune mechanisms via systemic administration, a phenomenon that should be carefully explored.

5. Study limitations and future studies

This research represents a pioneering venture into the use of phage vectors for DIPG treatment, with a specific focus on TRAIL as a therapeutic gene. While our *in vitro* results provide strong support for the use of this system, several limitations necessitate further investigation. These limitations highlight key areas where additional research is needed to optimise this treatment strategy, ensure its safety, and ultimately translate it into clinical practice.

A significant consideration is the determination of optimal phage vector dosing. Our initial dosage guidance was derived from studies on human GBM (173), which may not be what is required in DIPG. This underscores the need for rigorous dose-response studies specifically tailored to DIPG models and TRAIL. These studies should explore a range of doses in both *in vitro* and *in vivo* models, assessing efficacy, immune responses, phage clearance, and effects on healthy organs. Understanding the short and long-term consequences of various dosing regimens is paramount for maximising therapeutic potential while minimising potential side effects.

Another fundamental challenge lies in understanding phage biodistribution and its implications for long-term safety. Systemically administered phage vectors are subject to clearance by the mononuclear phagocyte system (MPS), resulting in primary accumulation within the liver and spleen (241-243). The degree of MPS uptake can vary between phage strains (244), and while targeted phage can enhance tumour accumulation, determining the precise percentage of brain-penetrating phage requires further investigation (245). Investigating the amount of phage that reaches the target site is critical, as it influences both therapeutic efficacy and potential off-target effects. Factors like dose, circulation time, delivery route, and potential *in situ* replication all influence phage biodistribution and pharmacokinetics (246). Future studies should prioritize detailed examinations of phage biodistribution, accumulation in various organs, and potential long-term side effects. Strategies to enhance phage circulation time and tumour penetration, potentially through surface modifications or alternative administration routes (e.g., intraventricular), could also be explored.

Since integrins are also expressed on healthy tissues, albeit at lower levels, targeting them with PAAV carries a risk of off-target effects (247). Systemic administration may lead to non-specific accumulation in organs expressing $\alpha v \beta 3$ integrins, potentially leading to adverse effects (247, 248). This includes healthy cells that are involved in angiogenesis, immune function and other physiological processes (248).

Our *in vivo* study did not include collection of other organs, limiting our ability to assess phage distribution and associated toxicity. Future investigations must examine various organs for phage accumulation and unintended side effects, comparing the tumour burden with other tissues to establish a comprehensive safety profile. Refining targeting strategies, such as employing tissue-specific promoters within the phage vector, could potentially mitigate off-target effects.

Our observation of morphological heterogeneity among DIPG cell lines reflects the complexity of this disease and offers an avenue for deeper investigation. Comparing gene expression profiles, particularly those related to cell adhesion, cytoskeletal structure, and cell-cell communication, between adherent and spheroid cultures could elucidate the molecular mechanisms underpinning these differences. Understanding this heterogeneity may allow us to predict cellular responses to various therapies, including our phage-based approach, potentially leading to more personalised treatment strategies for DIPG. Such investigations should ideally incorporate multiple patient-derived cell lines to capture the inter-patient variability that is characteristic of DIPG.

One major limitation of this study is the limited sample size ($n=2$ for most experiments), which affects the breadth of generalisability and the statistical depth of our analyses. Initially, several other investigations were pursued in this thesis, including the use of combination therapy of phage vector and chemotherapeutic agents, comparing the effect of phage vector on DIPG cells obtained from other patient biopsies and investigation of the expression of stem cell factors. This limited the ability to conduct multiple repeat experiments at the start. Repeat experiments were planned towards the end of the experimental period, but unfortunately could not be done due to time constraints, as there was limited laboratory access during the COVID pandemic. This was also the reason why the *in vivo* experiments could not be completed in thorough. Future studies should include expanded sample sizes to corroborate our results.

In conclusion, this thesis lays a strong foundation for developing a novel phage-based, TRAIL-mediated therapeutic approach for DIPG. Our results demonstrate the targeting potential and efficacy of this system using both *in vitro* and preliminary *in vivo* results. These findings warrant further optimisation and refinement of this strategy, with a focus on addressing the limitations highlighted above. Future work should be aimed at maximising the therapeutic benefits while ensuring patient safety. The ultimate goal is to translate this groundbreaking treatment paradigm into the clinic, offering new hope for patients and families affected by this devastating disease.

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Appendix

Nucleic acid sequence for *IL2-sTRAIL*

>EF00374956 (clone 8)

ATCGAATTGAATTGGATCCATGTACAGAATGCAACTCCTGTCTTGTATTGCACTA
AGTCTCGCACTTGTACAAACAGTGAATTCAGAGAAAGAGGTCCTCAGAGAGTA
GCAGCTCACATAACTGGGACCAGAGGAAGAAGCAACACATTGTCTTCTCCAAAC
TCCAAGAATGAAAAGGCTCTGGGCCGCAAAATAAACTCCTGGGAATCATCAAGG
AGTGGGCATTTCATTCTGAGCAACTTGCACTTGAGGAATGGTGAAGTGGTCATCC
ATGAAAAAGGGTTTTACTACATCTATTCCCAAACATACTTTCGATTTCAGGAGGA
AATAAAAGAAAACACAAAGAACGACAAACAAATGGTCCAATATATTTACAAATA
CACAAGTTATCCTGACCCTATATTGTTGATGAAAAGTGCTAGAAATAGTTGTTGG
TCTAAAGATGCAGAATATGGACTCTATTCCATCTATCAAGGGGGGAATATTTGAGC
TTAAGGAAAATGACAGAATTTTTGTTTCTGTAACAAATGAGCACTTGATAGACAT
GGACCATGAAGCCAGTTTTTTTCGGGGCCTTTTTAGTTGGCTAAGCTAGCTGGCCA
GACATGAGTCGACGATCTACGGGTGGCATCCCTGTGACCCCTCCCCAGTGCCTCT
CCTGGCCCTGGAAGTTGCCACTCCAGTGCCCACCAGCCTTGTCCTAATAAAATTA
AGTTGCATCATTTTGTCTGACTAGGTGTCCTTCTATAATATTATGGGGTGAAGGG
GGGGGGGGA