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RNAi therapeutics for brain cancer:

Current advancements in RNAi delivery strategies

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Abstract

Malignant primary brain tumors are aggressive cancerous cells that invade the surrounding tissues of the central nervous system. The current treatment options for malignant brain tumors are limited due to the inability to cross the blood-brain barrier. The advancements in current research has identified and characterized certain molecular markers that are essential for tumor survival, progression, metastasis and angiogenesis. These molecular markers have served as therapeutic targets for the RNAi based therapies, which enable site-specific silencing of the gene responsible for tumor proliferation. However, to bring about therapeutic success, an efficient delivery carrier that can cross the blood-brain barrier and reach the targeted site is essential. The current review focuses on the potential of targeted, non-viral and viral particles containing RNAi therapeutic molecules as delivery strategies specifically for brain tumors.

Keywords: Brain cancer, glioblastoma, nanoparticles, siRNA, CNS delivery, personalized medicine

1. Introduction

Brain tumors are neoplasms (abnormal tissue mass), which either originate primarily in the brain or as secondary tumors involving the brain as a metastatic site. Based on the type, degree of malignancy and location, the World Health organization (WHO) recognized four categories in the 2007 classification of central nervous system tumors (grade I to grade IV). Around 80% of primary malignant brain tumors are collectively called gliomas, which means originating from the glial cells (astrocytes, oligodendrocytes, and ependymal cells) [1] **(Figure 1)**. The different type of gliomas are; 1) Astrocytoma, which are tumors derived from astrocytes (star-shaped cells) and are graded I to IV (grade I - pilocytic astrocytoma; grade II – diffuse astrocytoma; grade III - anaplastic astrocytoma and grade IV – glioblastoma multiforme), 2) Oligodendroglias, which are formed from oligodendrocytes, the cells that form a protective coating around the neurons. They are usually classified as grade II (oligodendroglioma) and III (anaplastic oligodendroglioma); and 3) the last type of gliomas are called Ependymomas, which are derived from ependymal cells that line the fluid-filled cavities of the brain, the ventricles, and the central canal of the spinal cord. They are classified from grade I to grade III (grade I – myxopapillar ependymomas and subependymomas; grade II – ependymomas; grade III- anaplastic ependymomas) [2]. Among the different types and grades of gliomas, the glioblastoma multiforme (GBM) is the most aggressive form of malignant primary brain tumor [3]. It accounts for 17% of these tumors and is classified as grade IV by World Health Organization (WHO). GBM is highly infiltrative in nature, bears cellular heterogeneity and primarily affects the cerebral hemispheres of the brain. It can present as a primary GBM (90-95% of GBM), developing directly from a precursor cell (most likely radial glia) or evolve from lower grade astrocytoma as a secondary GBM (5-10%) [4]. According to the National Cancer Institute (NCI), GBM is the 12th leading

cause of cancer-related deaths in United States, with 6.5 per 100,000 new diagnosed cases/year, 4.3 per 100,000 deaths/year and a 5-year survival rate of approximately 10% [5]. In addition to the brain tumors originating from glial cells, non-glioma primary brain tumors also exist, such as 1) medulloblastomas, located in the cerebellum; 2) pituitary adenomas, tumors originating from the pituitary gland; 3) central nervous system (CNS) lymphomas; and 4) meningioma's, tumors developing from the membrane that covers the brain and the spinal cord [2].
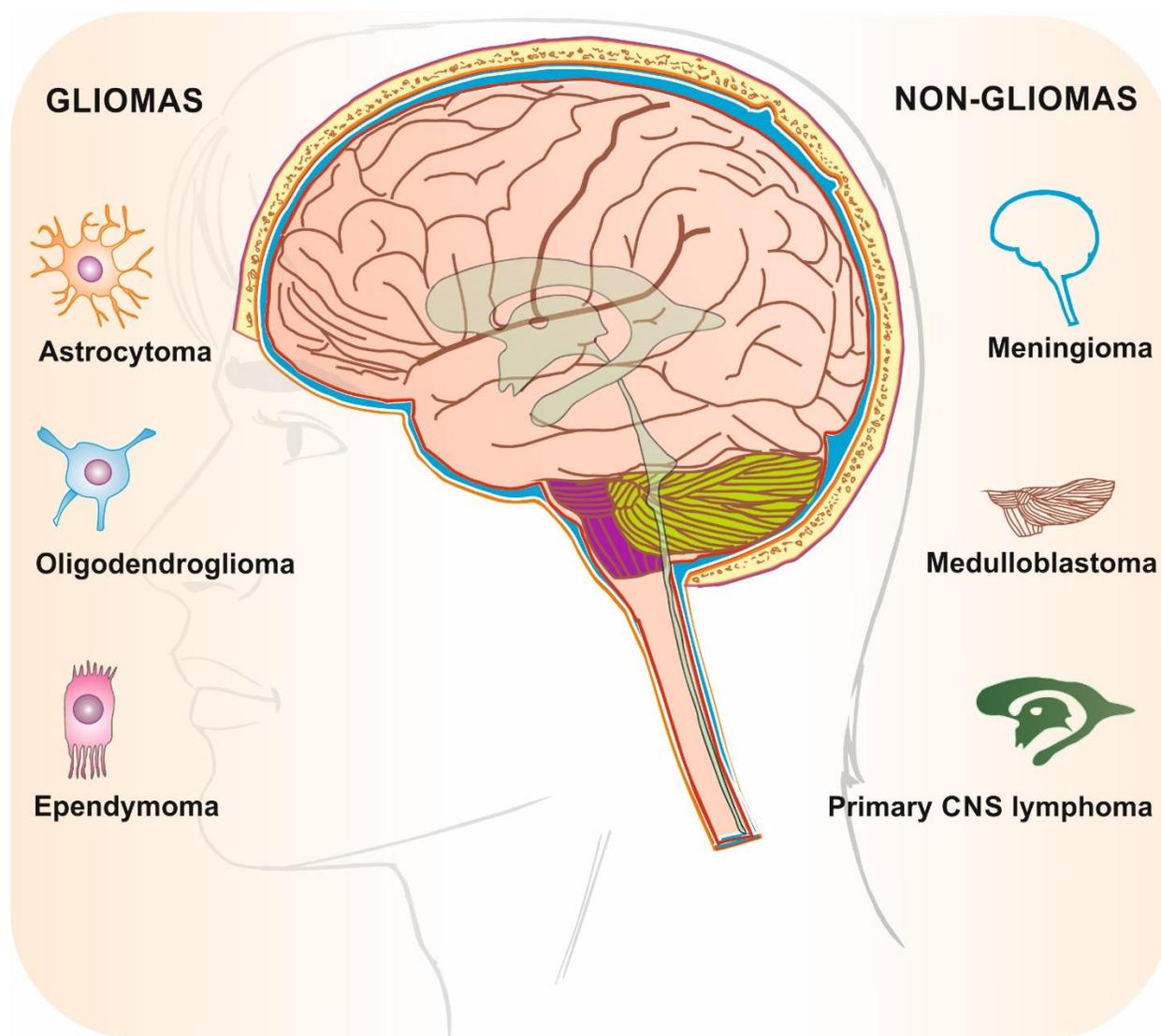

Figure 1: Schematic diagram showing the specific types of cells involved in brain cancer. Specifically, gliomas comprise of astrocytomas, oligodendrogiomas and ependymoma and non-gliomas include, meningioma, medulloblastoma and primary CNS lymphoma.

The conventional treatment methods that currently exist for the treatment of tumors are surgery, radiation therapy and chemotherapy, with surgery being the first step towards the treatment (see section 1.3 below). Apart from this, a recently FDA approved (2011) treatment is the tumor treating fields (TTF) therapy NovoTTF-100A System, which utilizes the placement of electrodes on the patient's scalp to deliver the low-intensity electrical fields to destroy the proliferative cancer cells [6]. In addition to brain tissue loss, the standard methods of treatment bring a risk of post-operative complications (bleeding, blood clots, swelling). Despite the multimodal therapeutic approach with surgery as the first stage of treatment, followed by radiation and chemotherapy, the median survival rate of patients remains at 14.6 months after diagnosis [7, 8]. GBM has a high rate of relapse after surgery, after which the median survival is 6 months [9, 10]. Several complicating factors, such as resistance to conventional therapies (chemo and radiotherapies) and the different response rate of the heterogeneous cell population, limit the treatment of GBMs [11, 12]. As most chemotherapeutic drugs fail to cross the blood-brain barrier (BBB) this further limits the effectiveness of treatment [13]. Selected targeted and anti-angiogenic drugs currently undergoing clinical trials for the treatment of GBM have been reviewed elsewhere [14]. In contrast to the existing chemotherapeutic drugs, which have limited success in treating the heterogeneous gliomas and glioblastomas, a more specific and personalized treatment, such as silencing the expression of genes associated with the disease by using RNAi therapy provides exciting opportunities. The translation of this concept into the clinic is however limited by the barriers to delivery which are unique and significant when targeting the brain.

The present review gives a comprehensive account on the molecular pathology of gliomas, the structural abnormalities associated with the BBB at the tumor site, and advancements and challenges in the design and delivery of RNAi therapeutics.

1.1. Molecular pathology of Gliomas

Gliomas, like other tumor types, are characterized by the acquisition of a number of mutational events that either activate oncogenes or inactivate tumor suppressor genes, ultimately favoring tumor growth and invasiveness. While individual tumors bear different combination of activated or inactivated genes, a number of molecular changes common to a majority of gliomas have been identified [15].

Publication, by the cancer genome atlas network, of a comprehensive molecular analysis of glioblastomas highlighted three pathways involved in the majority of these tumors [16]. Genes with a role in the receptor tyrosine kinase (RTK) / RAS / PI3K pathways are altered in 88% of GBM, leading to increased proliferation and survival of the tumor cells (see **Figure 2** for individual genes). RB1 signalling is affected in 78% of GBM cases either through homozygous deletion of tumor suppressor genes (CDKN2 or RB1 genes) or amplification of CDK4, CDK6 or CCND2, leading to increased cell proliferation (**Figure 2**). Finally, genes from the p53 signalling pathway are mutated, deleted or amplified in 87% of tumors, leading to decreased activation of the cell death pathways (**Figure 2**). It is however reasonable to estimate that alteration of at least one of these pathways is a mandatory step for the development of GBM. In addition, both primary and secondary glioblastoma show a high frequency (80%) of loss of heterozygosity on the long arm

of chromosome 10, suggesting an obligatory tumor suppressor gene inactivation for conversion

to the full GBM phenotype [17].

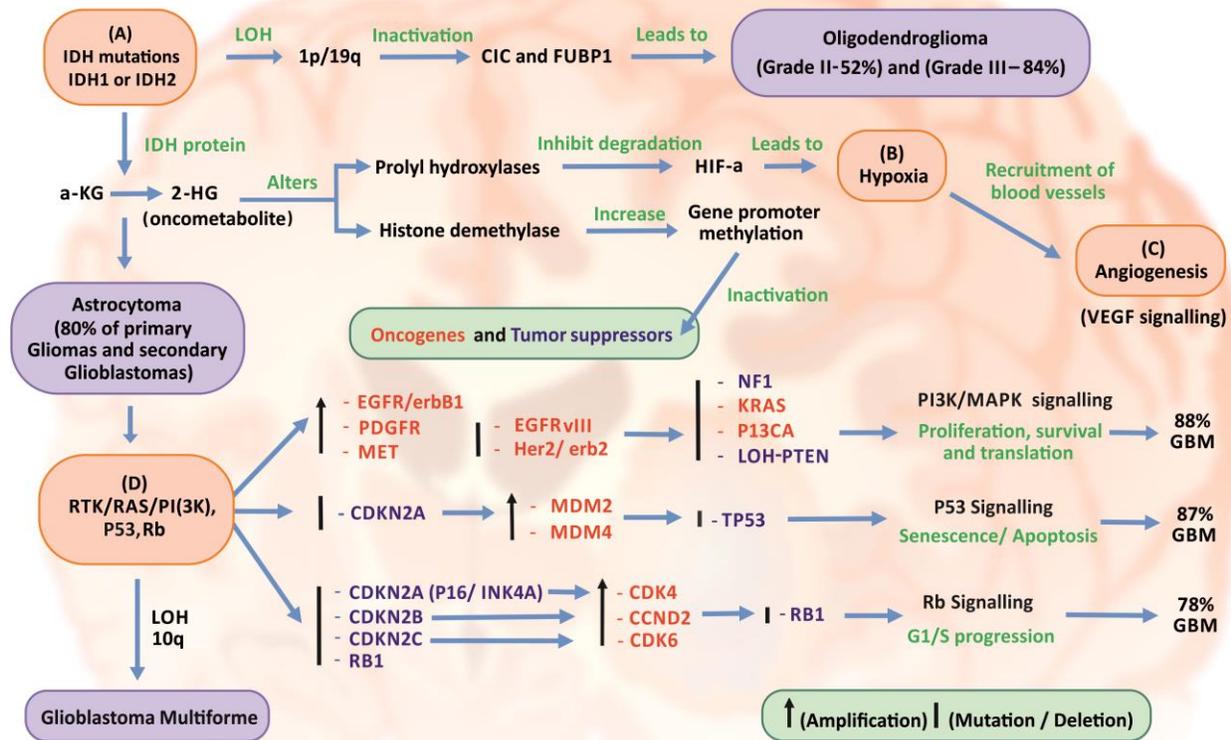## Signalling Pathways Altered in Brain Tumor

Figure 2: Key signalling pathways altered in malignant gliomas. The sequence alterations involved in major signalling pathways **(A)** IDH mutations, **(B)** Hypoxia, **(C)** Angiogenesis and **(D)** RTK/RAS/PI(3K), P53, Rb that result in the formation of Astrocytoma, Oligodendroglioma and Glioblastoma multiforme are shown. The activated oncogenes are represented in red and Tumor suppressor genes are represented in blue.

*Abbreviations: **LOH**, loss of heterozygosity; **GBM**, glioblastoma multiforme; **IDH**, isocitrate dehydrogenase; **a-KG**, α-ketoglutarate; **2-HG**, R(-)-2-hydroxyglutarate; **CIC**, capicua transcriptional repressor; **FUBP1**, FUSE binding protein; **HIF-a**, hypoxia inducible factor; **RTK**, receptor tyrosine kinase; **RAS**, rat sarcoma; **PI(3K)**, phosphatidyl inosital-3-kinase; **P53**, tumor protein 53; **Rb**, retinoblastoma; **EGFR**, epidermal growth factor receptor; **PDGFR**, platelet derived growth factor receptor; **MET**, hepatocyte growth factor receptor; **Her2/erb2**, human epidermal growth factor receptor 2;, **NF1**, neurofibromatosis 1; **KRAS**, Kirsten rat sarcoma viral oncogene; **PTEN**, phosphatase and tensin homolog; **MAPK**, mitogen activated protein kinase; **CDKN2A/B/C**, Cyclin-dependent kinase inhibitor protein; **MDM2/4**, mouse double minute 2/4 homolog; **CDK4/6**, Cyclin-dependent kinase 4/6; **CCND2**, cyclin D2; **VEGF**, vascular endothelial growth factor.*

In addition to the pathways discussed above, mutations in the isocitrate dehydrogenase 1 (IDH1) and IDH2 genes have been identified in a large proportion (80%) of secondary GBM and grade II / III glioma, and to a much lesser extent in primary GBM [18, 19]. IDH proteins are thought to be one of the early, if not the primary, event in the formation of glioma. IDH1/2 mutations are associated with loss of heterozygosity (LOH) by co-deletion on chromosomes 1p and 19q, one of the molecular characteristics of oligodendroglial tumours (50-70% of tumors) [20, 21]. Progression to higher tumor grades in oligodendroglioma is often accompanied by mutations in the CIC and FUBP1 genes on the remaining alleles of chromosome 1p and 19q [22]. IDH1/2 mutations have also been associated with the acquisition of p53 mutations in grade II astrocytomas and tumor progression (**Figure 2**) [23].

Interestingly, all mutations occur on the same arginine residue R132 in IDH1 and its equivalent, R172 in IDH2 [24]. This gain of function mutation allow the IDH proteins to process α-ketoglutarate to 2-hydroxyglutarate (2-HG) which has been shown to be an oncometabolite. 2-HG has been shown to inhibit histone and DNA demethylation, leading to gene promoter hypermethylation and inactivation [25].

As the tumors progress and expand, they often outgrow their normal blood supply as evidenced by hypoxic necrosis associated with advanced tumors. Hypoxia stimulates the expression of the transcriptional regulator hypoxia inhibitory factor 1 (HIF1α) gene. This in turn stimulates the production of other proteins, such as the vascular endothelial growth factor (VEGF), leading to the formation of new blood vessels, a process called neovascularization. In addition, there is

some evidence that the presence of the abnormal oncometabolite 2-HG inhibits the degradation

of HIF1α, leading to increased neovascularization and accelerated tumor growth [25, 26].

While some of the events described above illustrate the commonality of some molecular events,

the genetic heterogeneity and variations in response to therapy remind us that each tumor carries

a unique set of molecular changes. New personalised approaches such as inhibition or

stimulation of specific genes, through RNAi for example, may be needed to achieve efficient

treatment.

1.2.   Structural abnormalities of blood brain barrier at the tumor site

The BBB is very important in maintaining the homeostasis of the brain microenvironment. It

separates the CNS from the circulatory system. Specifically, it is formed by the endothelial cells

that line the microvessels of the brain, along with astrocytes and pericytes [27, 28] (**Figure 3**). The

most important feature of the BBB is the presence of tight junctions (TJs) that restrict the

paracellular diffusion of hydrophilic molecules [29, 30] allowing a low degree of transcytosis. The

transport of other compounds, which are essential for brain energy metabolism are catered for

via specific transport proteins [31].

Astrocytes play a key role in maintaining the normal BBB by stretching their end-feet beneath

the basal lamina surrounding blood vessels and at the adjacent surface of the brain [27, 32]. These

astroglial membranes are characterized by the presence of orthogonal arrays of particles (OAPs),

which carry water channel protein aquaporin-4 (AQP4) [33]. The formation of OAPs is specifically

directed by the presence of a proteoglycan protein called agrin [32]. The presence of aquaporins

along with potassium (K^+) channels maintain the healthy polarisation between the astrocytes and the endothelial barrier [34, 35]. In addition to the astroglial network, the presence of TJ molecules also play a key role in maintaining the microenvironment of the BBB and characterize its permeability and transepithelial electrical resistance (TEER). The BBB TJs consist of transmembrane proteins, such as claudin-3, -5, -12, occludin, other scaffolding proteins and the presence of junctional adhesion molecules [36].

The brain tumor, especially glioblastomas are characterized by an excess of abnormal or irregular-shaped cells that multiply aggressively and induce excessive vascularization [37, 38]. These morphological alterations have been shown to affect the BBB [39]. They include an increased variability in the thickness of the subendothelial basal lamina, an increase in the perivascular space, and abnormal recruitment or morphology of the pericytes [40] **(Figure 3).** Brain tumors have been shown to over-express VEGF [41], which is responsible for increased angiogenesis at the tumor site; leading to the formation of incomplete or disorganised vascularisation.

The thinning of TJs of the BBB at the tumor site have been previously reported [42]. The loss of TJ proteins claudin-1 and -3 and the downregulation of claudin-5 and occludin have been reported in the microvessels of glioblastomas [42, 43]. In addition, the loss of anti-agrin immunoreactivity was also reported in glioma vessels, which was further associated with the absence of occludin from the TJs [44]. This finding suggested that the presence of occludin was dependent on the presence of agrin. The loss of agrin has shown to redistribute AQP4 over the cellular surface [45], which is found to be upregulated in brain tumors [46, 47]. A striking

inconsistency in the immunoreactivity is found with AQP4 and the OAPs in healthy and cancerous brain tissue. In healthy brain tissue, only the OAP-crowded endfoot membrane and not the parenchymal membrane is immunoreactive against AQP4. However, in the case of tumor tissue, the entire glioma cell is AQP4 immunoreactive. This upregulation of AQP4 in gliomas is believed to be a compensatory mechanism for the loss of the endfeet and the increase in the perivascular space, which further leads to the formation of edema [48].

The loss of agrin in the endothelial membrane near the tumor site has been suggested to be associated with the upregulation of matrix metalloproteinase (MMP) enzymes. MMPs are responsible for degrading extracellular matrix (ECM) proteins and have been linked with cell proliferation, migration, differentiation and angiogenesis [49]. MMP-2, -9 and -12 have been found to be upregulated in glioma cells [50, 51]. The upregulation of MMPs not only cleaves the ECM protein such as, agrin but also the proteins of the TJs. Thus, the MMP mediated degradation alters the integrity of the BBB, including the loss of polarity and edema formation. The formation of edema can lead to intracranial pressure resulting in clinical complications such as, decreased cerebral blood flow, ischemia, brain herniation and death [48, 52].
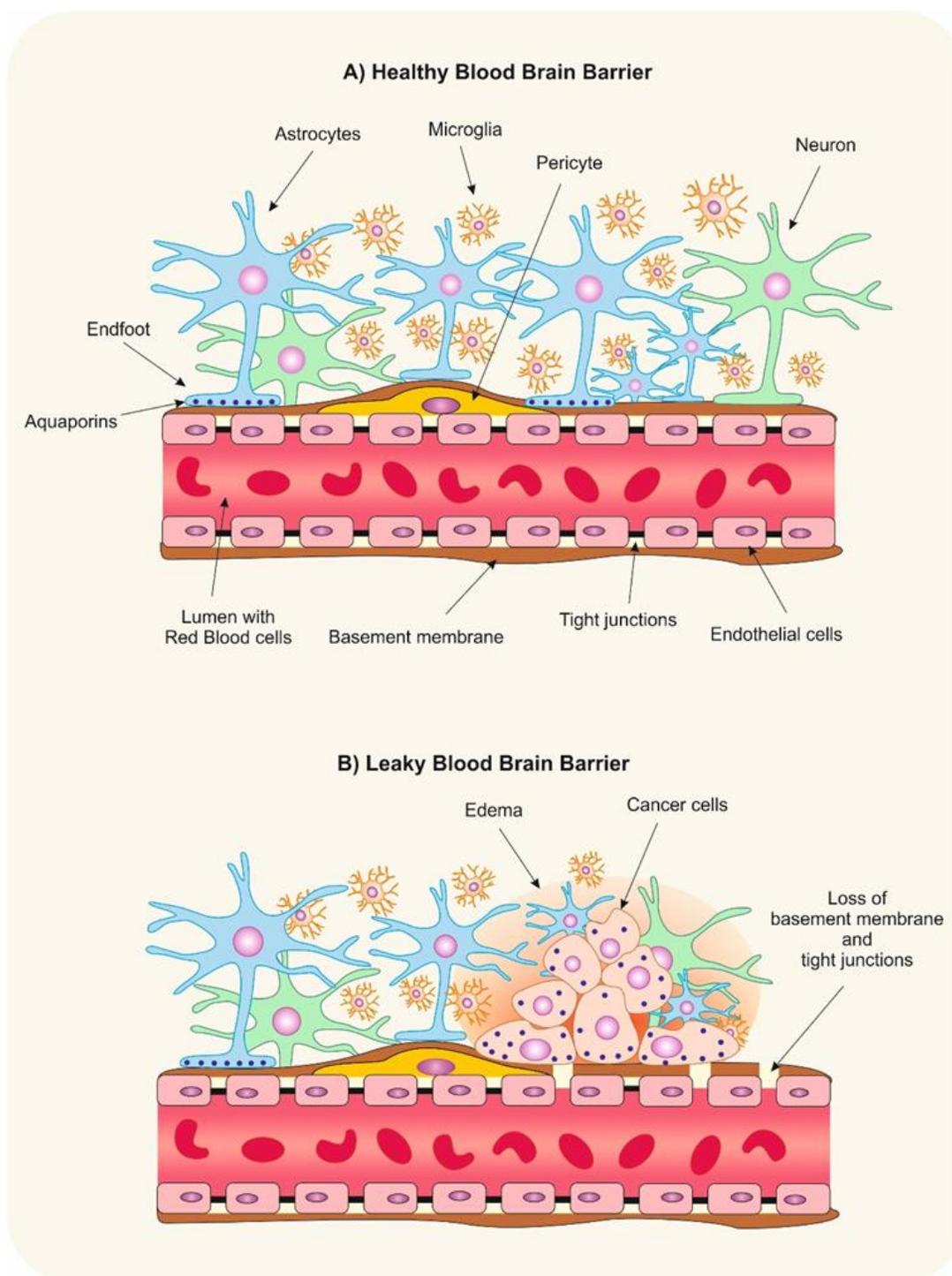
Figure 3: Schematic diagram of the traverse section of the Blood brain barrier (BBB) with the presence of endothelium, basement membrane, pericytes, astrocytes and tight junctions. **(A) The healthy BBB** shows the localisation of aquaporins on the endfoot of astrocytes, surrounding the blood vessels. **(B) The leaky BBB** represents the breakdown of basement of membrane and loss of tight junctions due to the migration of glioma cells along the blood vessels, which displaces the astrocytic endfeet and in turn, leads to leakage of serum components (edema) into neural parenchyma.

1.3. Conventional therapeutic delivery approaches for gliomas

Effective chemotherapy requires that sufficient concentrations of a chemotherapeutic drug be delivered to the tumor site. Regardless of its lipophilicity, size or ionic nature, less than 1% of therapeutics administered systemically reach the brain tumor site [53]. In addition, the permeability, stability, concentration and the intracerebral metabolism of the drug further determine its convection and diffusion in the brain parenchyma [54, 55]. Larger molecules (>800Da) have been shown to more efficiently move by convection, while the smaller molecules move by diffusion. The movement of the drug from the interstitial spaces to the tumor is dependent on the interstitial fluid pressure (IFP), which is directly related to the intratumoral convection currents [56]. The elevated IFP, typical of larger tumors, generates an outward pressure gradient working against drug diffusion by pushing it towards the periphery of the mass, therefore impeding its central delivery. Because of these limitations, convective delivery of therapeutics is preferred or the use of vectors that allow passage across the BBB and brain-tumor barrier (BTB). The current drug delivery strategies include invasive techniques that mechanically breach the BBB to deliver drugs, such as intracerebro-ventricular (ICV) delivery or convection enhanced delivery (CED) [57]. The limitation with ICV is the delayed parenchymal diffusion of the drug from the CSF in comparison to CSF clearance, which eventually drains the drug into the systemic circulation, thereby reducing its efficacy. CED involves placements of drug loaded catheters that actively pump the drug into the brain parenchyma [53, 58]. Though, this method has shown better drug diffusive characteristics, the invasiveness of the treatment and their limitation to reach distant infiltrating tumor cells have become the biggest challenges for an effective treatment [57]. Thus, the limitations of these methods restrict their translatability to clinical trials.

Newer delivery methods include; 1) Interstitial chemotherapy, which uses disc-shaped polymer wafers (Gliadel wafers) soaked with a chemotherapeutic drug (carmustine) [59]. The wafer is implanted after the tumor has been removed surgically [60, 61]. These biodegradable polymers deliver drug by diffusion and hydrolytic polymer degradation. However, the restricted diffusion of drugs, only 1 – 2 cm away from the implant site, limits the therapeutic efficacy of such an approach [60]; 2) Intrathecal chemotherapy, wherein the chemotherapeutic drugs are directly delivered into the spinal cord; 3) Intraarterial chemotherapy, in which the chemotherapeutic drugs are delivered into the arteries of the brain using tiny catheters and 4) Intracavitary drug delivery, wherein the therapeutic is implanted using a reservoir drug device into the resected tumor area or injected using a motor pump that performs a continuous intracerebral delivery [62]. **Table 1** shows a list of CNS tumor types and the standard treatment and chemotherapeutic drugs recommended for use in a clinical setting. The most common chemotherapeutic drugs recommended for brain cancer are temozolomide, carmustine, a PCV drug combination (procarbazine, lomustine, and vincristine) and platinum based drugs – cisplatin, carboplatin. In addition to the chemotherapeutic drugs, targeted (biologic) therapies are also being used, such as, bevacizumab, an angiogenesis inhibitor (Avastin®), or everolimus, an mTOR inhibitor (Afinitor®). Immunotherapies, such as CDX-110 (Rindopepimut), which targets EGFR and DCVax-Brain, is used to stimulate antitumoral immunity, have also reached phase III clinical trials.

2. RNA Interference

2.1. RNAi gene silencing mechanism

RNA interference (RNAi) refers to an endogenous pathway that enables regulation of gene expression by interfering with RNA stability and translation, thus, playing an important role in development, but also in restraining the expression of parasitic invaders [63]. The effects of such intracellular pathway were unexpectedly observed in petunia flowers [64], a few years later generalised to animal systems through experiments in *C. elegans* [65] and also in mammalian cells [66, 67]. In summary, this pathway utilizes micro RNA (miRNA) and short interfering RNA (siRNA) molecules to facilitate targeting of specific mRNA transcripts inducing gene silencing effects.

Endogenous primary(pri)-miRNA derive from primary precursors encoded in the genome and are non-coding RNAs [68]. These long double stranded RNA (dsRNA) molecules are processed by Drosha (RNase III nuclease) into pre-miRNA with ~60-70 nucleotides (nt), which are then transported from the nucleus to the cytoplasm by exportin-5 [68]. In the cytoplasm pre-miRNA are further processed by Dicer generating small dsRNAs with ~21-25 nt and containing nt mismatches. These miRNAs bind to a RNA induced silencing complex (RISC), a multimeric protein complex, which is activated by the unwinding of the miRNA and selection of the antisense strand [69]. Activated RISCs actively search the transcriptome for matching mRNA targets, marking them for degradation or ribosomal arrest. In the specific case of miRNAs, and due to the existence of sequence mismatches, miRNA-activated RISCS are able to silencing multiple mRNA targets acting mainly as translational repressors [68, 69].

The specificity of the RNAi pathway may be artificially hijacked using synthetic siRNAs or short hairpin RNA (shRNAs) [70, 71]. Synthetic siRNAs are chemically synthesized macromolecules of ~14 kDa and carry a net negative charge. When successfully delivered and released into the cell's cytoplasm synthetic siRNAs bypass nuclear processing by Drosha and follow essentially the same pathway as miRNAs [71]. Despite inducing potent gene silencing effects, siRNAs are not replicated intracellularly and a dilution effect might be observed in proliferating cells, which may result in transient effects [72]. On the other hand, shRNAs are usually encoded within an expression vector (plasmid DNA or viral vector), and these constructs require suitable promoters for transcription by RNA polymerase II or III in the nucleus [70]. Moreover, in order to achieve post-transcriptional gene silencing pri-shRNAs need to be processed by Drosha, transported to the cytoplasm and further processed by Dicer. Despite the fact that expression of shRNAs within the nucleus can be associated with replication and that they are more resistant to cellular metabolism, the need for translocation of constructs to the nucleus may restrict the application in quiescent cells, such as neurons [71, 73]. Thus, a key determinant for artificially inducing gene silencing is the selection of an adequate siRNA or shRNA approach, taking into account the target tissues and the available delivery systems [74, 75].

Experimentally RNAi technology has been widely applied as a research tool for target validation, providing insights to gene and protein functions [76], but also as a mean to generate *in vivo* models of disease which would not be currently possible to engineer through other techniques [77, 78]. However, and more importantly, harnessing the RNAi pathway has shown promise as a therapeutic approach for incurable diseases of the central nervous system (CNS) [79, 80], such as

brain cancer [81]. The following sections of the review will focus on the application of RNAi as a

therapeutic approach for the most prominent form of brain cancer, brain glioma.

2.2.   RNAi-based therapeutic approach brain cancer therapy

The possibility of artificially interfering with endogenous gene expression through the RNAi

pathway has emerged as one of the most exciting areas for the development of disease-modifying

treatments in several therapeutic areas, including the CNS and cancer.

2.2.1 <u>Gene targets for RNAi therapeutics in brain glioma cancers</u>

Continuous advances in the understanding of cancer cell biology have permitted the

identification of particular molecular targets, which are dysregulated and which may constitute

valid targets for RNAi therapeutics (see section 1.3 and **Figure 2**). Indeed, to-date several proof-

of-concept *in vitro* and *in vivo* studies have already been carried out specifically targeting genes

involved in carcinogenesis, in tumor-host interactions and in conferring resistance to

conventional therapies [82]. The targeting of tenascin C, an ECM protein using RNAi therapy has

shown promising results for the treatment of high grade human glioma [83-85]. Tenascin C is a

glycoprotein, which is only present in the high-grade gliomas and not in normal brain. At

present, this is the only clinically based study reported for siRNA-based glioma treatment,

conducted in 46 patients, which showed improvement in survival and quality of life [84]. **Table 2**

and 3 summarises different therapeutic gene/protein targets that have been considered in the

development of RNAi therapeutics for brain glioma.

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Therapeutic targets in the carcinogenic pathway include gene products involved in oncogenesis, cell cycle regulators, apoptosis (APOP) pathway, cell senescence, and protein stability and degradation. From the oncogenesis pathway, dysfunctional or mutated Protein Tyrosine Kinase (PTK) receptors, such as epidermal growth factor receptor (EGFR), may cause cellular transformation and lead to pathological alteration of downstream signaling [86]. These receptors have been successfully targeted with RNAi therapeutics to inhibit cell proliferation and cell survival [87]. On the other hand, the inactivation of cell cycle regulators, such as the retinoblastoma tumor suppressor protein (RB1) and p53, results in excessive cell proliferation or the loss or delay of cellular senescence. Thus, RNAi targeting of gene products involved in the inactivation of RB1 and p53 has resulted in apoptosis and reduction of cell proliferation in cancer cells [88]. Similarly, targeting anti-apoptotic proteins, such as Fas-associated death domain-like interleukin-1 beta-converting enzyme-like inhibitory protein (FLIP), Bcl-2, Bcl-xL and survivin, may constitute an effective strategy to control proto-oncogene activation [82]. Furthermore, silencing the expression of the human telomerase RNA (hTER) template and heterogenous nuclear ribonucleoparticulate (hnRNP) A1/A2 proteins (which bind to telomeres) using siRNAs led to a cell growth arrest and apoptosis, demonstrating the usefulness of targets related with cellular senescence [89, 90]. Finally, within the carcinogenesis pathway, other targets associated with protein stability and degradation, and involved in the proteosome-dependent pathways have also been considered. Knockdown of such molecules has led to inhibition of tumor growth [82].

In order for tumors to grow, endure and spread, interactions with the host are crucial. Thus, several gene targets involved in tumor-host interactions have been identified and include gene products associated with neoplasic angiogenesis, degradation of ECM, invasion and metastasis,

and/or cell adhesion. Silencing proangiogenic genes, such as VEGF, resulted in reduced neovascularisation and inhibited tumor growth in a xenograft mouse model [91]. In addition, other studies have demonstrated that silencing molecules involved in ECM degradation, such as MMP-9 and cathepsin B, delays tumor progression and even led to total regression of intracerebral glioma tumors in preclinical models [92]. Finally, immunosuppressive cytokines, such as interleukin 10 (IL-10), have also been considered as valid target for RNAi due to their anti-apoptotic effects. IL-10 is secreted by many tumors and facilitates tumor evasion from the immune system [93], and its suppression has been shown to induce apoptosis.

Genes that confer resistance to conventional chemotherapeutics have also been considered good targets for RNAi therapies. Silencing the expression of multidrug resistance (MDR) genes, such as ABCB1 (MDR1 or P-glycoprotein), reversed drug resistance and increased sensitivity of glioma cancer cells to doxorubicin and vincristine [94, 95]. On the other hand, targeting DNA repair mechanisms, including excision repair cross-complementing 1 (ERCC1), which are overexpressed in cancer cells has also been proven to be effective rendering cancer cells sensitive to chemo/radiotherapy [82]. Expression of such molecules in cancer cells confers resistance to therapy-induced DNA damage and RNAi-based therapies may knockdown the expression of such repair proteins.

In summary, RNAi may be used as a therapeutic strategy to intervene in one or multiple pathways. Furthermore, this approach may be used in combination with current chemotherapy or radiotherapy approaches, allowing the development of more individualized and targeted therapies.

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2.2.2 *Advantages of RNAi versus conventional therapies*

RNAi therapeutics present a great advantage over conventional cancer therapeutics, since through this approach it is virtually possible to target any gene, with known sequence, that has been linked to brain cancer [82]. Furthermore, the identification of potent and highly selective synthetic siRNAs/shRNAs may result in a much faster drug development track than the discovery of new chemical entities [96]. Additionally, nowadays short-sequence oligonucleotides may be engineered and synthesized on a large scale basis at a low production cost. This provides a particular advantage over protein and antibody therapeutics where manufacturing is extremely costly [97]. Moreover, RNAi therapeutics may be used in combination with existing conventional therapies, based on chemotherapy and/or radiotherapy, to enhance their efficiency [82]. For example, this approach may be used to enhance BBB penetration of chemotherapeutics by silencing gene products, such as MDR1 [98] which confer particular resistance to these drugs.

2.2.3 *Limitations of RNAi as a therapeutic approach for brain cancer*

Despite the therapeutic potential of the RNAi-based approach for brain cancer treatment, translation of such technology to the clinic still presents some challenges. The risk of off-target effects and non-specific immune stimulation, the saturation of the endogenous RNAi machinery, the development of resistance to RNAi, but also the lack of efficient nanosystems for delivery to the brain are the main obstacles in the progress of such technology.

Off-target effects may arise from nonspecific hybridization of the antisense strand of siRNAs (or in some cases sense strand) to non-target mRNA transcripts [99]. In fact, partial complementarity may lead to unwanted silencing effects if one or two perfect matches occur between the 2nd – 7th

nucleotide of the antisense strand and the 3'-UTR of an unrelated mRNA [99, 100]. This partial complementarity may lead to miRNA like silencing effects. In addition, in some cases, the unintentional leading of sense (passenger) strand to RISC can also lead to downregulation of critical genes [101, 102]. Therefore, rational algorithmic design should be used to generate highly complementary siRNA/shRNAs to their target mRNAs but also to ensure correct loading of the antisense strand to the RISC, based on the thermodynamic profiles of the antisense strand [103-105]. In addition, rational design may also be used to avoid "danger motifs" such as CpG and UG rich regions in pDNA and siRNA respectively, since these may induce unwanted immunostimulatory effects by activating the interferon response, which in turn triggers the global degradation of mRNA and proteins [106, 107]. Such responses have been observed to be associated with high siRNA to cell ratio or specific sequence composition of the siRNAs [108, 109].

Another limitation associated with RNAi is the saturation of the RNAi machinery, which consequently leads to dysregulation of the endogenous miRNA function. This effect has been shown to occur with both synthetic siRNAs [110, 111] and shRNAs [112-114]. Indeed, previous studies have demonstrated that shRNAs may saturate nuclear exportin-5 preventing the maturation of endogenous miRNA, leading to a global shutdown of the miRNA pathway and to lethal toxicity in mice [112, 113]. Hence, the selection of adequate promoters that enable modest expression of shRNAs, or the co-expression of recombinant exportin-5, or the use of a miRNA scaffolds may represent possible alternatives to overcome this issue [112, 115, 116]. On the other hand, and despite that synthetic siRNAs bypass nuclear processing, circumventing the issue of overloaded nuclear transport, the use of the lowest dose possible is key to avoid saturation of RISC components, which are also used by miRNAs [117].

Additionally, several mechanisms of resistance may affect the efficacy of the RNAi therapeutic strategy. Mutations and small changes in target sequences may turn previously effective siRNAs into an inadequate option; in addition, the presence of complex RNA secondary structures may render mRNA sequences inaccessible, limiting the gene silencing effects [82]. Thus, the use of up-to-date algorithms for predicting secondary structures within the target mRNA and the design of multiple siRNAs should be considered to avoid resistance. However, it is worth noting that despite the fact that well-designed siRNAs may enable powerful gene silencing, these are not able to interfere with pre-existing proteins, and consequently resistance may also arise when targeting gene products with very long half-lives [82]. Furthermore, the enhanced cell division inherent to tumor growth may cause an effect of dilution and lead to short-living gene silencing effects in proliferating cells [118, 119]. Hence, to maximize gene silencing effects, RNAi therapies must target proteins with high turnover, and appropriate dosing schedules for tumor growth inhibition must be designed [120].

Furthermore, stability and nuclease degradation are amongst the major hurdles for naked nucleic acids, such as siRNAs and pDNA coding shRNAs. Exposure to serum and tissue endonucleases leads to degradation of these macromolecules, enhancing elimination and limiting efficacy. In addition, penetration through the BBB and specific targeting of brain tissue still constitute key challenges for most delivery systems. However in the last decade substantial progress has been made in the strategic design of nanovectors to overcome many of the *in vivo* barriers to delivery [121-126].

3. Delivery of RNAi therapeutics for the treatment of brain cancer

3.1 Non-viral versus Viral approaches

Nucleic acids, such as siRNAs and pDNA coding shRNAs, are highly hydrophilic macromolecules (>14 kDa) and have poor cell penetrating proprieties. As a result of their physicochemical characteristics, they present unfavourable pharmacokinetic profiles when administered intravenously and are rapidly eliminated by renal glomerular filtration, mostly in the form of degraded fragments [127, 128]. Hence, several approaches have been evaluated in order to improve delivery to the brain, and these include chemical modifications in siRNAs, and the use of viral and non-viral delivery systems [121].

Chemical modifications have been introduced to siRNAs to enhance stability but also for improving delivery [96, 129]. Such modifications have been reviewed extensively elsewhere, and we refer the reader to those publications for further information [130-132]. Despite the utility of chemical modifications, there are limits to which modifications can be carried out without affecting the potency, and unfortunately this approach has shown limited success in penetrating the BBB after systemic delivery [129, 130].

Recombinant viral vectors have been widely used for gene therapy approaches [133] including, mediating RNAi in the CNS [134]. Adeno-associated viruses (AAV), lentiviruses (LV) and Herpes simplex viruses (HSV) are by far the most widely used for shRNA delivery in the CNS [135]. AAV systems consist of small ~20 nm ssDNA viruses with great tropism across large areas of the brain and with a relatively low immunogenic profile [134, 135]. In spite of avoiding integration into

the hosts genome, sustained expression of transgenes and shRNAs have been reported in the brain with good expression levels [136, 137]. On the other hand, LV-based systems allow integration into the host genome, and thus permit long-term/stable expression of the transgene/shRNA in the brain [138]. These are ssRNA retroviruses, which are capable of transducing post-mitotic cells to undergo retrograde transport [139]. Selected pre-clinical studies using viral particles for delivery of RNAi therapeutics in the treatment of brain glioma are summarised in **Table 2.** Viral vectors are the most widely investigated method of delivery for gene and RNAi therapeutics [140]. However, despite the high level of utilization and tropism in a wide number of cell types, viruses have been associated with extreme adverse reactions [133]. The first human fatality occurred after intravenous administration of an adenovirus vector, which triggered an inflammatory response with multi-organ failure [141]. Thus, caution has been recommended when translating the use of such delivery systems to human therapy.

As a less toxic and less immunogenic alternative, several non-viral delivery systems have been developed to enable delivery of RNAi-based therapeutics to the CNS [121]. These delivery systems are commonly engineered from natural and synthetic materials.

3.2 Non-viral nanodelivery strategies for siRNA mediated gene silencing in gliomas

The application of nanoparticles for delivery of nucleic acid and chemotherapeutic drugs has been widely explored for various therapeutic applications. The design and formulation of the nanoparticle will dictate the stability in the physiological conditions and it will be influenced by the route of administration chosen to reach the target organ. The successful delivery of RNAi is a

challenge due in large part to the physiochemical properties including the anionic and hydrophilic nature of the molecule which result in poor permeability of cell membranes [142].

Nanoparticles can be synthesized using a range of materials including lipids, polymers and inorganic materials and are usually in the size range of 10-200 nm. Nanoparticles possess tremendous flexibility in terms of design and may be modified with a blend of materials and/or targeting ligands, which can influence the overall surface characteristics of the nanoparticles. Ideally materials used to synthesize nanoparticles should be biocompatible, biodegradable, non-immunogenic and non-toxic in nature. Depending on the materials chosen, the surface charge of the nanoparticles can either be cationic, anionic or neutral. The surface characteristics can determine the route of cellular uptake of nanoparticles, which could be adsorptive, receptor-mediated or carrier-mediated. The ideal concept of a nanoparticle revolves around the "make and break" strategy. In essence, the nanoparticle should enable encapsulation of the therapeutic payload (siRNA) in sufficient quantities, protect it from enzymatic degradation (serum nucleases), avoid hepatic and renal clearance and, once inside the cell, should facilitate endosomal escape and release of the therapeutic into the cytoplasm or nucleus to produce a clinical response [74, 143].

It is generally accepted that tumors, including brain tumors possess a leaky vasculature, which facilitates diffusion and retention of nanoparticles (100-800 nm) [144, 145]. Studies have shown that nanoparticles of 10-12 nm can passively extravasate the BTB over time and maintain peak blood concentration [146, 147]. At the tumor site, nanoparticle delivery *in vivo* is mediated via both active and passive transport. The passive transport of therapeutics into the tumor site is enhanced due to

increased permeability of blood vessels in tandem with poor lymphatic drainage and this process is referred to as the "enhanced permeability and retention effect" (EPR) [148]. In addition, the presence of targeting moieties on the surface of nanoparticles equip them to adhere to specific cell surface receptors and enable receptor-mediated active transport.

However, this enhanced permeation effect is only observable at later stages of high grade brain gliomas [149]. The BBB still poses a major challenge to RNAi therapeutics at early stages of brain cancer development thus significantly restricting delivery of anticancer therapies [149]. In such cases, additional strategies that have been employed to improve the permeation of nanoparticles/therapeutics across the BBB include, osmotic disruption of the BBB [150], the use of vasomodulators [151], or the use of potassium channel agonists to increase the formation of transport vesicles [151].

Several nano-based strategies for delivery of bioactive molecules have been proposed for brain tumor therapy [152]. Selected pre-clinical studies using non-viral particles for delivery of RNAi therapeutics for the treatment of brain glioma are summarised in **Table 3.** In the case of non-viral delivery strategies, liposomal nano-formulations have been widely used and have been shown to increase the circulation time of the nucleic acid therapeutic. Improvement in mechanical stability and permeability have been made by incorporating cholesterol molecules in the phospholipid bilayer [153]. Other modifications include the incorporation of a hydrophilic polymer polyethylene glycol (PEG), which provides a stealth character to the nanoparticles, making the nanoparticle invisible to the molecular and cellular components in the plasma, avoid clearance by the reticulo-endothelial system and thus significantly enhancing the blood circulation time [154, 155]. In the past,

cationic lipid nanoparticles were widely studied and many of these formulations have demonstrated activation of the immune system upon systemic administration [156]. To help overcome this issue, solid lipid nanoparticles, prepared with a combination of cationic and fusogenic lipids with small size, low surface charge and stability in serum were prepared and demonstrated reduced toxicity following *in vivo* administration with siRNA [157].

Targeted nanoparticles with specificity towards the receptors expressed on neuronal cells, such as, insulin receptor [158], transferrin receptors [159], leptin and EGF receptors [160] have been used for delivering siRNA, antisense oligonucleotides etc. across the BBB. Other targeting strategies include the use of cell penetrating/targeting peptides [161] or conjugating the particles with endogenous molecules, such as apolipoproteins [162]. The presence of such targeting molecules on the surface of nanoparticles act like a "key" to unlock specific receptors on the surface of diseased cells. Thus, unlike traditional chemotherapy, which affects both the healthy and the diseased cells, nanodelivery approaches can be tailored to achieve target specificity. The targeting ligands can be attached onto the surface of nanoparticles via various methods. The covalent attachment method may include, for example, amino reactive linkages or disulfide bond linkages. In some other cases, the targeting ligands are attached on the terminus of PEG chains, conjugated to the nanoparticles. Thus PEG is used as a spacer between the ligand and the nanoparticle. This approach minimizes the steric hindrance and improves the target binding affinity of the ligand to the cell surface receptor. For example, dual targeted, PEGylated immunoliposomes, conjugated with two monoclonal antibodies, specific for the transferrin receptor (to cross BBB) and the insulin receptor (targeting glioma cells) have been proposed for delivering shRNA against human EGFR gene [163]. The formulation was delivered systemically,

once weekly, targeting the intracranial GBM cells in an animal model of glioblastoma and resulted in suppression of EGFR immunoreactivity and increase in survival of animals. In addition, delivery of a biotinylated siRNA conjugated to the TfR monoclonal antibody via a biotin-streptavidin linker decreased luciferase gene expression in the intracranial brain tumor of rats [164]. Another study utilizing the dual targeting approach is a multifunctional lipid nanoparticles, modified with Angiopep-2 and tLyP-1 peptides to target glioma cells both *in vitro* and *in vivo*. The incorporation of Angiopep-2 peptide served as a targeting ligand and increased the binding affinity to the low-density lipoprotein receptor expressed on brain tumors and the incorporation of the neuropilin-1 receptor (tLyP-1) specific peptide was used to enhance tumor penetration, [165]. The dual targeted lipid nanoparticles showed improved siRNA delivery in comparison to untargeted and single peptide targeted nanoparticles. In addition, the formulation showed efficient gene knockdown of VEGF mRNA when co-delivered with docetaxel [165].

In recent years, the use peptides derived from the protein of viral capsid such as the trans-activating transcriptional activator (TAT) peptide (derived from Human Immunodeficiency Virus-1), have been extensively used to enhance penetration of the BBB [161]. In addition, these peptides have a specific sequence to exploit the nuclear localization signal which can facilitate delivery into the nucleus [57]. Another recent study has investigated the effects of incorporation of the L1 papillomavirus type-16 capsid-derived lipopeptide on the surface of lipid nanocapsules [166]. This peptide was shown to exhibit similar properties to the TAT peptide, in terms of binding negatively charged nucleic acids and enhancing cell membrane penetration.

Targeted nanoparticles take advantage of the receptors present on the surface of the cell membrane. The arginine-glycine aspartic acid (RGD) peptide has been used to target integrins expressed on endothelial cells of tumor vasculature such as glioblastomas [167, 168]. Two recent studies have utilized RGD tagged dendrimers, for siRNA delivery against the EGFP and luciferase genes [169, 170]. The studies indicated enhanced delivery of siRNA in an in vitro 3D spheroid model of brain cancer [169]. Moreover, a 75% increase in the gene knockdown was observed, when co-delivered with Dox [170]. Other receptors that can be used for targeted delivery are the VEGF receptor VEGFR2, as VEGF levels are higher at the site of gliomas associated endothelial cells than normal endothelial cells [171]. A recent study, published by our group utilized surface functionalized amphiphilic cyclodextrins, co-formulated with PEGylated and RVG tagged cyclodextrins, specifically targeting the acetyl cholinesterase receptors present on the human glioblastoma (U87) cells, *in vitro*. The study indicated successfully siRNA delivery and gene knockdown of an endogenous, GAPDH gene with no cellular toxicity [172]. Other studies for siRNA delivery targeting brain cancer cells *in vitro* and/or *in vivo*, include the use of polymeric nanoparticles, fabricated using polyethyleneimine (PEI) [173, 174], chitosan [175], and poly(lactic-co-glycolic acid) (PLGA) [176]. **Table 3** presents a comprehensive list of delivery strategies used for siRNA delivery for brain cancer therapy including polymeric and non-polymeric NPs and alternative technologies such as DNA aptamers [177], carbon nanotubes [178].

Another interesting approach that is gaining momentum for delivery of nucleic acids, such as mRNAs and microRNAs, is the use of exosomes [179]. They are naturally occurring cell-derived vesicles, 40-100 nm in size, and derived from endocytic cellular pathways through inward budding of the late endosomal membrane, which fuses with the plasma membrane. Exosomes

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can be prepared *ex vivo* and be used to systemically deliver therapeutics across the BBB, targeting the brain tissue [180]. Though, this technique has to date only been explored to target neurodegenerative diseases like Alzheimer's disease, the future application of this technology to target brain cancer has significant potential.

4. Conclusions and Future Perspectives

Targeting brain cancer with RNA interference therapeutics, represents a promising strategy that may combat the ineffectiveness and un-intended side effects of conventional therapies. Although viral-based systems have been found to be effective in delivering therapeutics to the hard-to-transfect neuronal cells, the questionable safety issues pertaining to the use of viral particles, such as cytotoxicity and immunogenicity, pose a serious limitation to their use in a clinical setting. To address this issue, non-viral, nanodelivery formulations that biomimic the surface properties of viral particles without reproducing the negative attributes have been investigated.

Nanodelivery systems for RNAi therapeutic delivery have proven to be effective for other types of cancer and in some cases have advanced to clinical trials. However, to date this is not the situation for brain cancer therapy where the scientific advances made are yet to be translated into the clinic. The reasons for the delayed clinical advancement include the lack of convincing levels of penetration across the BBB at the sufficiently high doses needed for the desired duration of activity. In addition, although various chemical modifications strategies have been used to eliminate the undesirable immune stimulation effects of siRNAs and improve systemic stability, the long term toxicity of multi-functional bioactive delivery systems are unknown. There is also an urgent need to establish and validate an in vitro model of the BBB to help screen formulations at an early stage and identify delivery systems with potential for in vivo efficacy.

Brain cancer is a complex heterogeneous disease with poor prognosis, however, genes associated with the different types of brain cancer have been identified. Genetic screening of individual patients is possible and would provide the opportunity to develop personalised medicines with more effective targeting potential. Although RNAi represents an exciting future generation of therapeutics it may not be sufficiently effective as a sole therapy in all cases. The utilisation of combinatorial approaches, where conventional chemotherapy is co-delivered with RNAi therapeutics and/or radiation may also be an attractive therapeutic option to efficiently increase the sensitivity of the diseased cells and thus shorten the long term chemotherapy regime for patients. In conclusion, with the advent of a range of bioactive and biocompatible materials and an increasing level of research activity into neurodegenerative diseases together with increasing knowledge of the genetic basis of brain cancer the future looks promising for the development of a safe and effective RNAi therapeutic.

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Tables

Table 1: Types of Central Nervous System tumors with assigned WHO grade, treatment and recommended chemotherapeutic drugs

Type of Brain Tumor	WHO grade	Treatment	Chemotherapeutic Drug
Neuroepithelial tumors			
Astrocytomas	**I to IV**		
Subependymal giant cell astrocytoma	I	Surgery and Radiation	N/A
Pilocytic astrocytoma	I	Surgery and Radiation	N/A
Pilomyxoid astrocytoma	II	Surgery and Radiation	N/A
Diffuse astrocytoma	II	Surgery with or without Radiation	N/A
Pleomorphic xanthoastrocytoma	II	Surgery and Radiation	N/A
Anaplastic astrocytoma	III	Surgery, Radiation and *Chemotherapy*	Temozolomide, Carboplatin, Cisplatin Combination (Lomustine + procarbazine + vincristine)
Glioblastoma	IV	Surgery, Radiation and *Chemotherapy*	Temozolomide, Carboplatin, Cisplatin, Irinotecan, Cyclophosphamide, Etoposide
Giant cell glioblastoma	IV	Surgery, Radiation and *Chemotherapy*	
Gliosarcoma	IV	Surgery, Radiation and *Chemotherapy*	Combination (Lomustine + procarbazine + vincristine) Bevacizumab Bevacizumab + irinotecan Bevacizumab + nitrosurea Bevacizumab + carboplatin
Oligodendroglial tumors	**II to III**		
Oligodendroglioma	II	Surgery with or without Radiation.	N/A
Anaplastic oligodendroglioma	III	Surgery, Radiation and *Optional Chemotherapy*	Temozolomide, Carboplatin, Cisplatin Combination (Lomustine + procarbazine + vincristine)
Oligoastrocytic tumors	**II to III**		
Oligoastrocytoma	II	Surgery, Radiation and *Optional Chemotherapy*	Procarbazine, Vincristine
Anaplastic oligoastrocytoma	III	Surgery Radiation and *Optional Chemotherapy*	Temozolomide, Carboplatin, Cisplatin Combination (Lomustine + procarbazine + vincristine)

Ependymal tumors	**I to III**		
Subependymoma	I	Surgery and Optional Radiation	N/A
Myxopapillary ependymoma	I	Surgery and Optional Radiation	N/A
Ependymoma	II	Surgery, Optional Radiation and *Chemotherapy*	Etoposide, Bevacizumab, Temozolomide
Anaplastic ependymoma	III	Surgery, Optional Radiation and *Chemotherpy*	Temozolomide, Carboplatin, Cisplatin Combination (Lomustine + procarbazine + vincristine)
Other Neuroepithelial tumors	**I to II**		
Angiocentric glioma	I	Surgery and Optional Radiation	N/A
Chordoid glioma of the thir ventricle	II	Surgery and Optional Radiation	N/A
Tumor of Meninges	**I to III**		
Meningioma	I	Surgery and Otional Radiation	N/A
Atypical Meningioma	II	Surgery and Optional Radiation	N/A
Anaplastic meningioma	III	Surgery, Radiation and *Optional Chemotherapy*	Interferon alpha Somatostatin
Other types			
Choroid plexus tumors	I to III	Surgery, Radiation and *Optional Chemotherapy*	Combination (Vincristine + Cisplatin + lomustine)
Neuronal and mixed neuronal-glial tumors	I to III	Surgery, Radiation and *Optional Chemotherapy*	Combination (Vincristine + Cisplatin + Cyclophosphamide)
Pineal tumors	I to IV	Surgery, Radiation and *Optional Chemotherapy*	Combination (Carboplatin + Thiotepa + Etoposide)
Embryonal tumors including Medulloblastoma	IV	Surgery and Optional Radiation and *Chemotherapy*	Temozolomide Oral Etoposide
Tumors of cranial and paraspinal nerves	I to IV	Surgery and Optional Radiation	N/A
Lymphomas and hematopoetic neoplasms	II to IV	Surgery, Radiation and *Chemotherapy*	Methotrexate. Procarbazine, Leucovorin, Cytarabine, Ifosfamide Methotrexate + Vincristine Rituximab (monoclonal antibody) + Temozolomide
Tumors of sellar region	I	Surgery and Radiation	N/A

Table 2: Selected pre-clinical studies using viral particles for RNAi delivery for the treatment of brain glioma

Viral system	RNAi Target	In-vitro model	In-vivo model	Route	Benefit	Refs
Lentiviruses						
Lentivirus	shRNA – 3'UTR PTPμ mRNA	U87-MG and LN-229	N/A	N/A	↓ 50 to 60% KD of PTPμ Protein ↓ Cell proliferation ↓ Colony formation	181
Lentivirus	shRNA - HIF-1α	U87-MG cells	BALB/c nu/nu mice	s.c.	↓ 80% KD of HIF-1α mRNA ↓ Cell viability ↑ Apoptosis ↓ GLUT-1, VEGF, Bcl-2 and MMP2 expression at both mRNA and protein level	182
Lentivirus	shRNA - RHBDD1	U251 and U87-MG cells	N/A	N/A	↓ RHBDD1 (mRNA and protein level) ↓ Colony formation ↓ 50% Cell proliferation ↓ Cyclin-D1 and Bcl-w	183
Lentivirus	shRNA- SMC1A	U251, U373 and U87-MG cells	N/A	N/A	↓ SMC1A at mRNA and protein level ↓ Cell viability Absence of Colony Formation	184
Lentivirus	shRNA- MGMT	LN18, T98, and VU28 cells	Nude mice	i.t.	↓ 60-80% KD at protein level ↑ Sensitivity to temozolomide ↓ 46% in tumor size	185
Lentivirus	shRNA - ATM	U118, AT5BIVA, C3ABR and AT25ABR cells	N/A	N/A	↑ Sensitivity to ionizing radiation ↓ ATM Protein level	186
Lentivirus	shRNA - ZFX	U87, U251, U373, A172, SHG-44, and C6 glioma cell lines	BALB/c nude mice	Stereotactic injections	↓ 60 – 80% Cell proliferation ↓ 83- 99% Colony formation ↓ Akt and p44/42 (ERK1/2)	187
Lentivirus	shRNA – ChK1 and ChK2	GSC cell line	N/A	N/A	↓ 70 – 85% Chk1 and Chk2 mRNA expression ↑ Radiosensitivity by ChK1 ↑ Apoptosis by ChK1	188
Lentivirus	shRNA - PPM1D	U87-MG cells	N/A	N/A	↓ 76.3% PPM1D mRNA level ↓ 87.0% PPM1D protein level ↓ 47% Cell invasion ↑ Apoptosis ↓ Cell proliferation	189
Lentivirus	shRNA - STAT3	Primary glioblastoma stem cells U251 cells	N/A	N/A	↓ STAT3 mRNA and protein levels ↓ Bcl-2 and Cyclin D mRNA and protein levels ↓ Tumorigenesis ↑ GFAP, MBP and MAP2	190
Lentivirus	shRNA - EGFRvIII	U87Δ cells and U87 cells	SCID mice	s.c.	↓ Cell proliferation, Invasiveness ↓ 80% Colony formation ↓ EGFRvIII expression	191

Lentivirus	siRNA - hTERT	U87-MG cells	BALB/c- nude mice	i.t.	↓ 73% hTERT mRNA expression ↓ TRAP assay Telomerase activity ↓ Invasiveness	192
Adenoviruses						
Adenovirus	siRNA - Ad-MMP-9 construct	IOMM-Lee meningioma cell line	N/A	N/A	↓ Expression of MMP-9 ↓ 98% Migration and invasion of cells ↑ 80 – 90% Apoptosis ↑ phospho ERK and phospho Akt levels	193
Adenovirus Ad-Delo3-RGD	siRNA - YB1	U87-MG, U373-MG, LN-18 cells and SV-GA cells Brain cancer stem cell lines (R11, R28, R40, and R49)	Nude mice	i.t.	↓ 58% KD of YB-1 protein ↓ copy number of adenoviral vectors in R28 cells ↑ Apoptosis ↑ Survival	194
Adenovirus	shRNA - VEGF	U343 and U87-MG cells	Male athymic nu/nu mice	i.t.	↓ VEGF protein levels ↓ Angiogenesis both in-vitro (50%) and in-vivo (60 – 75%) ↓ 80% tumor growth ↑ Apoptosis	195
Adenovirus	siRNA - bFGF	U251 cells	N/A	N/A	↓ Cell proliferation ↑ Apoptosis ↓ STAT3 phosphorylation ↓ Activation of ERK1/2 and JAK2 ↓ IL-6 secretion ↓ CyclinD1 and Bcl-xl	196
Adenovirus	siRNA – bFGF and Vpr gene	U251 cells	Nude mice	i.t.	↑ Apoptosis ↓ Cell proliferation	197
Adenovirus	siRNA bFGF and BCNU and VM-26 chemotherapeutic drugs	U251, A172, and LN229 cells	N/A	N/A	↓ Cell proliferation and Migration ↓ 85% BFGF mRNA expression ↑ Apoptosis ↓ Bcl-2 and Bcl-XL protein levels ↑ Bax protein level ↑ Sensitivity to BCNU and VM-26 drugs	198
Adenovirus	siRNA - bFGF	U251 cells	N/A	N/A	↓ bFGF expression level ↑ Connexin 43	199
Adenovirus	shRNA - COX-2 and Akt1	U251 cells	BALB/c (nu/nu) mice	i.t.	↓ COX-2 and Akt1 ↓ 73.11% Ki-67, 62.34% CyclinD1, 54.45% MMP-2, 48.86% MMP-9 and Bcl-2 ↓ Cell proliferation, 50% invasion and apoptosis.	200
Adenovirus	shRNA - COX-2, Akt1 and PIK3R1	U251 cells	N/A	N/A	↓ 13.2% COX-2, 26.6% Akt1 and 34.9% PIK3R1 protein levels ↓ 20.34% PCNA, 17.34% CyclinD1, 37.65% MMP-2 and 15.60% MMP-9 ↑ 192.45% TIMP2 and 182.65% P53	201

Adenovirus	Survivin promoter	U373-MG, U118-MG, U87-MG, A172 cells	N/A	N/A	↑ Caspase-3 and BAX mRNA levels ↑ Apoptosis	202
Adenovirus	siRNA – MMP-2	U87-MG and U251 cells	Nude mice	i.t.	↓ MMP-2 protein levels ↓ VEGF expression ↑ 50% Apoptosis ↓ 90% Tumor growth	203
Adenovirus	siRNA – MMP-9	Daoy medulloblastoma cell line (HTB 186)	Nude mice	i.t.	↓ MMP-9 mRNA and protein levels ↓ 75-80% Cell proliferation and Tumor growth, 78% Invasion ↑ P16 expression ↓ pRb protein and E2F transcription factor	204
Adenovirus and Retrovirus	shRNA-Hec1	U373-MG cells	Nude mice	i.t.	↓ 52% Hec1 protein levels in cells ↓ 40% Hec1 protein level in tumor	205
Adenovirus	shRNA – uPAR and MMP9	SNB19 cells	Athymic nude mice	Alzet mini pumps	↓ MMP and uPAR protein levels ↓ Cell proliferation ↓ Angiogenesis, Invasion ↓ Phosphorylated ERK, MAPK, and AKT	206
Adenovirus	shRNA - survivin	U251 cells	Nude mice	i.t.	↓ Survivin mRNA and 73% protein levels ↑ Apoptosis ↓ Tumor growth	207
Herpes simplex virus						
HSV-1	shRNA - EGFR	Gli36-Luc cells	Nude mice	i.t.	↓ 50-75% KD of EGFR mRNA level ↓ 40-50% colony formation ↑ 40% Apoptotic cells	208
HSV-1	shRNA - ATM, ATR, ATM/ATR, or MSH6	U87, U373, and T98 cells and GBM4, GBM6, and BT74 cells	Female athymic mice	i.t.	↑ Sensitivity to Temozolomide ↑ Apoptosis ↑ Survival	209
HSV-1	siRNA - KIF23	U87-MG, SF126 and GL261 cells	BALB/c (nu/nu) mice	i.t.	↓ KIF23 protein levels ↓ Cell proliferation ↓ Tumor growth	210

Abbreviations: shRNA, short hairpin RNA; siRNA, small interfering RNA; mRNA, messenger RNA; KD, knockdown; UTR, untranslated region; PTPμ, Protein tyrosine phosphatase mu; HIF, hypoxia-inducible factor; RHBDD1, rhomboid domain containing 1; SMC1A, Structural maintenance of chromosomes protein 1A; MGMT, O-6-methylguanine-DNA methyltransferase; ATM, Ataxia telangiectasia mutated; STAT3, Signal transducer and activator of transcription 3; ZFX, zinc finger transcription factor; GLUT1, Glucose transporter1; VEGF, Vascular endothelial growth factor; Bcl-2, B-cell lymphoma 2; MMP, Matrix metalloproteinase; Bcl-w, Bcl-2-like protein 2; ChK, checkpoint kinase; PPM1D, Protein phosphatase 1D; EGFRvIII, epidermal growth factor receptor variant III; hTERT, human telomerase reverse transcriptase; YB1, Y box binding protein1; TRAP, telomeric repeat amplification protocol; bFGF, Basic fibroblast growth factor; Vpr, viral protein R; Bax, bcl-2-like protein 4; Cox-2, Cyclooxygenase-2; PIK3R1, Phosphatidylinositol 3-kinase; Hec1, highly expressed in cancer; uPAR, Urokinase-type plasminogen activator; pRb, retinoblastoma protein; MSH, mismatch repair protein mutS homolog 6; ATR, ataxia telangiectasia and Rad3-related; KIF23, Kinesin-like protein; GFAP, Glial fibrillary acidic protein; MBP, myelin basic protein; MAP2, Microtubule-associated protein 2; ERK, Extracellular signal-regulated kinases; JAK-2, Janus kinase 2; PCNA, Proliferating cell nuclear antigen; TIMP2, metallopeptidase inhibitor 2; MAPK, mitogen-activated protein kinase; GSC, Glioblastoma stem-like cells; GBM, glioblastoma multiforme; MG, malignant glioma; i.t., intratumoral; s.c., subcutaneous; i.v., intravenous; CED, convection enhanced delivery; N/A, not applicable.

Table 3: Selected pre-clinical studies using non-viral nanoparticles for RNAi delivery for the treatment of brain glioma.

Nano-system	Targeting Ligand	RNAi Target	Other modifications	In-vitro model	In-vivo model	Route	Benefit	Refs
Inorganic Nanoparticles								
Gold nanoparticles (Spherical nucleic acid)	N/A	siRNA - Bcl2L12	N/A	Patient derived TNS line And huBMECs	CB17 SCID mice	i.v and local (i.c)	↓ Bcl2L12 mRNA (26%) and protein (40%) levels ↑ Apoptosis ↑ Effector caspase and p53 activity	211
Iron Oxide magnetic nanoparticle	N/A	shRNA - MDR1	Chitosan Graft	BT325 cells	N/A	N/A	↓ MDR1 mRNA (76.14%) and protein levels ↓ 81.7% P-gp protein expression ↑ Sensitivity of chemotherapy (DOX and VCR)	212
Iron oxide magnetic nanoparticle	Chlorotoxin	siRNA - GFP	PEG and PEI, blocked with citraconic anhydride	C6 glioma cells	N/A	N/A	↓ Polymer induced cytotoxicity on cells ↓ 50% relative GFP expression	213
Bacterial magnetic nanoparticles	TAT peptide	psiRNA - EGFR	PAMAM graft	U251-MG cells	Nude mice	i.t.	↓ 34.87% EGFR mRNA level ↓ Cell proliferation and invasion ↓ p-AKT, MMP2/9, PCNA, VEGF, Bcl-2, and cyclin D1 proteins	214
mPEG-PEI-SPION nanoparticles	N/A	siRNA Pin X1 and Doxorubicin	MPEG and PEI graft	C6 glioma cells	N/A	N/A	↓ 57.7% Pin X1 mRNA level ↑ Inhibiton of glioma cells ↑ Sensitivity (67%) to Dox	215
Magnetofluorescent nanoworms	N/A	siRNA - EGFRvIII	Dendrimers	GBM-6 cells	Swiss Webster mice	i.v and CED	↓ 70-80% EGFR protein level	216
Lipid Nanoparticles and Micelles								
Immunoliposomes	HIRMab/Tf RMAb	shRNA - luciferase	PEG	U87-MG and C6 rat glioma cells	Male Fischer CD344 rats	i.v.	↓ 68% luciferase gene expression ↓ 90% Tumor luciferase expression	217
Immunoliposomes	HIRMab/Tf RMAb	shRNA - EGFR	PEG	U87-MG cells	SCID mice	i.v.	↓ 95% EGFR expression in cells ↑ 88% survival time	218
LipoTrust (commercial reagent)	N/A	siRNA – MGMT and Temozolomide	N/A	U251SP, T98G U251 and 0316-GICs cells	BALB/c nu/nu mice and NOD-SCID mice	i.t. and Alzet osmotic pump	↓ 93% MGMT suppression in tumor ↑ Sensitivity to Temozolomide	219
LipoTrust (commercial reagent)	N/A	siRNA – MGMT and Temozolomide	N/A	N/A	Sprawg dawley rats, Male wistar rats and porcine	CED	↓ Distribution of the nanoformulation in the brain tissue. ↑ DNA repair enzyme level leads to reduced effect of alkylating agents like Temozolomide	220
Cationic liposomes	Transferrin	siRNA - GFP	N/A	U373 cells	N/A	N/A	↓ 45% GFP protein levels ↓ Cytotoxicity	221
Lipid nanocapsules	N/A	siRNA- EGFR	N/A	U87-MG cells	N/A	N/A	↓ 38% Cell proliferation	222

							↓ 63% EGFR protein levels	
Solid lipid nanoparticles	N/A	siRNA- c-Met	PEG	U87-MG cells	Balb/c-nu mice	i.v.	↓ 32.5% c-Met protein level ↓ Cell proliferation and Tumor growth (91%) Absence of systemic toxicity	223
Liposomes	Angiopep-2 and tLyP-1	siRNA – VEGF and Docetaxel	N/A	C6 glioma cells and BMVEC, U87-MG and U251 cells	Balb/c-nu mice	i.t and i.v.	↓ 63.5% VEGF mRNA level ↓ Tumor growth in synergistic manner	161
Lipid nanocapsules	L1 PMV-16 lipo peptide	siRNA CD133 and Bcl2	PEG	U87-MG, Caco-2 and COS-7 cells	N/A	N/A	↓ Bcl2 protei expression ↓ CD133 protein expression N-terminal and L1-LNCs were better in cellular uptake and silencing than the Control Peptide-LNCs	162
Nanobubbles (Polymeric micelles and liposomes)	N/A	siRNA-SIRT2	PEG and PLL	C6 glioma cells	BALB/c nu mice	i.v.	↓ 57.3% SIRT2 mRNA level ↑ 33.5% Apoptosis ↓ 50.3% Cell viability ↓ Tumor volume	224
Lipid nanocapsules	N/A	siRNA - EGFR	Transacylated Chitosan	U87-MG cells	N/A	N/A	↓ 51.95% EGFR expression ↑ 62.55% cell death in combination with Temozolomide	225
Micelles	TAT peptide	siRNA - Raf-1 and Camptothecin	MPEG and PCL graft	C6 glioma cells and RN33B normal neuron cells	Rat model of malignant glioma	i.n.	↑ Nose to brain delivery of the nanoparticles ↓ Tumor cell proliferation with combined therapy	226
Micelles	R3V6 peptide	siRNA - VEGF and BCNU	N/A	C6 glioma cells	N/A	N/A	↑ Transfection efficiency ↓ VEGF expression	227
Dendrimers								
Dendrimers	T7 peptide	siRNA- Luc, pGL3 and pRFP	PEG	BCECs, U87-MG cells	Male ICR and Balb/c-nu mice	i.v.	↑ Accumulation in brain ↑ 1.7 fold higher luciferase expression w.r.t. untargeted NPs ↑ Lucifease KD in-vitro and in-vivo (2.17 fold)	228
Polyamidoamine dendrimers (PAMAM)	N/A	siRNA –NS – Cy3 label and EGFP siRNA	N/A	T98G glioblastoma and J774 macrophages	N/A	N/A	siRNA – dendriplexes are taken up by cells and express silencing only via caveolin mediated pathway.	229
PAMAM	RGD	siRNA- EGFP	N/A	U87-MG cells	N/A	N/A	↑ Delivery of targeted NPs in a 3D tumor spheroid model	165
Dendrimers	RGD	siRNA – Luc and docetaxel	PEG and PLL	U87-MG cells	N/A	N/A	↑ 75% Gene silencing with targeted NP conjugates along with DOX	166
Dendrimers	N/A	siRNA - GFP	N/A	U87-d1EGFP cells	N/A	N/A	↑ Unpackaging of polyplex ↓ cytotoxicity with acetylation of primary amines on dendrimers	230
Polymeric Nanoparticles								

PEI nanoparticles	N/A	siRNA - GAPDH and BDNF	N/A	Neuro2a cells and primary hippocampal neurons	N/A	Voltage controlled-Chemical transfection	Efficient method to screen siRNAs for neuronal cells, which are difficult to transfect	170
PEI nanoparticles	N/A	siRNA - PTN	N/A	U87-MG cells	C57BL/129sv and athymic nude mice	i.p and s.c.	↓ 40% Tumor growth ↓ Cell proliferation	169
Chitosan nanoparticles	N/A	siRNA - MRP1	N/A	C6/VP16 rat glioma cells	N/A	N/A	↑ 83.13% transfection efficiency ↓ MRP1 mRNA and Protein levels	171
Hydroxyapetite nanoparticles	N/A	shRNA - SATB1	N/A	U251 human glioma cells	BALB/c nu mice	i.t.	↓ SATB1, Cyclin B1, MMP-2 and VEGF protein expression ↑ 67.8% Apoptosis ↑ Bax expression and Caspase-9 activity ↓ Cell proliferation, invasion and angiogenesis	231
Poly trehalose (AEMA) nanoparticles	N/A	siRNA - Luciferase	N/A	U87 glioblastoma cells	N/A	N/A	↑ 90% Cellular uptake and 80% gene KD Therapeutic efficiency of siRNA is maintained on lyophilization by having trehalose in the formulation.	232
PBAE (poly beta amino ester) nanoparticles	N/A	siRNA - GFP and DNA	N/A	Primary human glioblastoma cells (GB319 cells)	N/A	N/A	↑ 90% transfection efficacy ↓ 85% GFP knockdown ↓ Cytotoxicity	233
PLGA nanoparticles	EGFP-EGF1	Tissue factor	N/A	Rat BMECs and C6 glioma cells	N/A	N/A	↑ 96.28% transfection efficacy ↓ 4.1 fold Tissue Factor mRNA level ↓ 58.5 % TF proten level ↓ Cytotoxicity	172
PP75 anionic polymer	N/A	siRNA – stathmin and carmustine	N/A	U251 cells	BALB/cAnNCr-nu/nu mice	i.t	↓ Stathmin expression, 81% at mRNA and 90% at protein level ↑ Sensitivity of tumors to carmustine	234
Polydisulfide	N/A	siRNA - luciferase	N/A	U373-MG cells	N/A	N/A	↓ 70% luciferase gene expression	235
Non-polymeric nanoparticles								
Multifunctional surfactant (EHCO)	N/A	siRNA - HIF-1α	N/A	U87-Luc Neo cells	mice	Stereotactic injection / CED	↓ 79% of lower tumor volume in comparison to controls	236
DNA aptamer	N/A	siRNA - c-Met	N/A	U87-EGFRvIII cells	N/A	N/A	↓ 50% c-Met protein level ↓ 68.43% Cell proliferation ↑ Apoptosis	237
PTD-DRBD	Tat peptide	siRNA-EGFP1, EGFP2,	N/A	T98G and U87-MG EGFRvIII cells	BALB/c nu mice	i.t.	↓ EGFR protein level ↓ Akt (1/2/3) protein levels ↓ Cell proliferation	238

		GAPDH, Akt1, Akt2 and Akt3 and EGFRvIII					↑ Apoptosis	
EHCO	c(RGDfK) and bombesin	siRNA – Luciferase, (HIF-1α)	PEG	U87-MG cells	Athymic nude mice	i.p. and i.v.	↓ 60% Luciferase gene in vivo ↓ Tumor growth	239
Multi-walled Carbon nanotubes	PKH26 (red fluorescent dye)	siRNA – Cy3 labelled	Pluronic F108	BV2 microglia and GL261 glioma cells	N/A	N/A	↑ Uptake of MWCNTs without cytotoxicity No changes in cytokine and proliferative profile in-vitro	240
Cyclodextrins	RVG	siRNA - GAPDH	PEG	U87-MG cells	N/A	N/A	↓ 27% GAPDH mRNA expression ↑ Delivery with RVG targeted nanoparticles No cytotoxicity	168

Abbreviations: shRNA, short hairpin RNA; siRNA, small interfering RNA; mRNA, messenger RNA; KD, knockdown; Bcl2L12, Bcl-2-like protein 12; huBMECs, Human bone marrow endothelial cells; MDR1, multidrug resistance; P-gp, p-glycoprotein; PEG, polyethylene glycol; PEI, poly ethyleneimine; TAT, transactivator of transcription; EGFR, Epidermal growth factor receptor; PAMAM, poly (amidoamine) dendrimers; GFP, green fluorescent protein; MMP2/9, Matrix metalloproteinase; PCNA, Proliferating cell nuclear antigen; VEGF, Vascular endothelial growth factor; Bcl-2, B-cell lymphoma 2; Pin X1; EGFRvIII, epidermal growth factor receptor variant IIl; HIRMab, human insulin receptor monoclonal antibody; TfRMAb, transferrin receptor monoclonal antibody; MGMT, O-6-methylguanine-DNA methyltransferase; SCID, Severe combined immunodeficiency; c-Met, hepatocyte growth factor receptor; tLyP-1, neuropilin-1 receptor; L1 PMV-16, L1 papillomavirus type-16; SIRT2, sirtuin-2; Raf-1, serine/threonine-protein kinase; PCL, Polycaprolactone; NPs, nanoparticles; RGD, Arginyl-glycyl-aspartic acid; PLL, poly-L-lysine; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; BDNF, Brain-derived neurotrophic factor; MRP1, multidrug resistance; SATB1, special AT-rich sequence-binding protein-1; Bax, Bcl-2-associated X; HIF, hypoxia-inducible factor; Akt1/2, Protein kinase B1/2; PTD-DRBD, peptide transduction delivery domain to a dsRNA-binding domain, LNCs, Lipid nanocapsules; EHCO, 1-(aminoethyl) iminobis[N-(oleicylcysteinylhistinyl-1-aminoethyl)propionamide]; i.t., intratumoral; s.c. subcutaneous; i.p., intraperitoneal; i.v., intravenous; GBM, glioblastoma multiforme; N/A, Not applicable.