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MicroRNA-based Drugs for Brain Tumours

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Abstract:	<p>MicroRNAs (miRNAs) are key regulatory elements coded by the genome. A single miRNA can downregulate expression of multiple genes involved in diverse functions. As cancer is a disease with multiple gene aberrations, developing novel approaches to identify and modulate miRNA-pathways may result in a breakthrough for cancer treatment. With a special focus on glioblastoma, this review provides an up-to-date understanding of miRNA biogenesis, role of miRNA in cancer resistance, essential tools for modulating miRNA expression, emerging list of clinically promising RNAi delivery systems and how they can be adapted for therapy.</p>

MicroRNA-based Drugs for Brain Tumours

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26 **Abstract (91/120 words)**

27 MicroRNAs (miRNAs) are key regulatory elements coded by the genome. A single miRNA can
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34

35 **Keywords:** glioblastoma, cancer resistance, RNAi, drug delivery, brain targeting, argonaute,
36 exosomes, nanoparticles

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MicroRNA therapeutics for Glioblastoma

Glioblastomas (GBM) are aggressive grade IV primary tumours of the central nervous system [1, 2]. Standard therapy consists of maximal surgical resection followed by external radiotherapy and chemotherapy with temozolomide that confers a median survival of about 15 months [2]. Tumour-treating fields (TTFields) is the latest improvement in GBM treatment which uses a non-invasive external device (Optune® transducer-array) to create a low intensity electric field of intermediate frequency (~200kHz) which interferes with mitosis, hinders cell division and consequently induces cell death in dividing cells [3]. The combination of TTFields plus temozolomide after the standard chemoradiotherapy significantly increased the overall median survival to 20.5 months, with better quality of life [3]. Other treatment options for GBM include chemotherapy other than temozolomide, immunotherapy and therapies targeting specific oncogenic pathways, for review see Seystahl *et al* [4]. All currently available treatments are palliative, not curative [2, 3]. Therapeutic failure of GBM can be attributed to the sub-optimal delivery of drugs and aberrant expression of multiple genes resulting in tumour heterogeneity, aggressive infiltrative behaviour and treatment resistance of GBM cells. It is important to identify multiple drug targets for developing effective therapies.

MicroRNAs (miRNAs or miRs) are natural RNA interference (RNAi) molecules produced by the cells (for Biogenesis and Functions of miRNAs, see **Box-1 and Figure-1**). MiRNAs are involved in GBM pathophysiology, tumor plasticity, and resistance to therapy, see **Figure 2** [5-7]. Differential expression of miRNAs is observed in the tumour mass versus control brain tissue between glioma subtypes [8, 9], and even between different cell subpopulations of the same tumour [7]. These differences in miRNA expression can be used as diagnostic and prognostic

biomarkers [7, 10]. The major therapeutic advantage of miRNAs arises from the fact that a single miRNA can target multiple genes involved in distinct cellular functions. For example, increasing the expression of a tumour-suppressor miRNA (ts-miRNA) like miR-128-3p [11-17] or miR-145-5p [18-23] in GBM can block cell proliferation, self-renewal, invasion, metastasis, angiogenesis and drug resistance by selectively down-regulating the expression of multiple genes, **Figure-2**. Similarly, repeated systemic treatment with miR-138 blocks multiple key immune-checkpoints proteins in T-cells (CTLA-4, PD-1 and FoxP3), resulting in significant T-cell mediated tumour regression and increased survival in orthotopic brain-tumour model expressing PD-L1 ligand [24]. Conversely, inhibiting the function or blocking the expression of an oncogenic miRNA (oncomiR) in cancer cells can reactivate multiple tumour-suppressor genes leading to tumour regression (e.g. miR-21-3p [25] or miR-21-5p [26]) or even to tumour eradication, as in the case of miR-10b [27]. These examples illustrate that the targeting of miRNAs in GBM either by inhibition, replacement or modulation of their activity is a promising therapeutic approach [28, 29], **Figure-2**. Shea et al reviewed an extensive list of GBM relevant miRNAs [30].

The miRNA database (miRBase, version 21) enlists 1881 precursors and 2588 mature human miRNAs [31], of which several of them are identified preferentially as tumour-suppressor miRNA (ts-miRs), as oncomiRs, or as both, depending on the cell type and function of miRNAs in those cells [19, 32]. For example, Fareh et al showed forced expression of miR-302/367 cluster in GBM cells resulted in repression of self-renewal properties and in vivo inhibition of tumour development [33]. Conversely, Guo et al reported that self-renewal and tumour-promoting properties were enhanced by the expression of miR-302/367 cluster in prostate cancer cells [34, 35]. On the other hand, blocking oncogenic miR-21 expression in immune cells,

instead of in tumour cells, might block T-cell activation and the anti-tumour immune response [36]. Hence, it is important to understand the holistic context of miRNA activity [32] and direct the miRNA therapeutics towards the target cells to maximize therapeutic success. Nanomedicines provides essential delivery systems to optimize the biodistribution and activity of miRNA-modulating drugs [37, 38]. Here, we review an up-to-date understanding of miRNA biogenesis and functions (**Box-1 and Figure-1**), role of miRNA in GBM and treatment resistance (**Figure-2**), various tools available for modulating miRNA expression (**Figure-1**), emerging list of clinically promising RNA delivery systems and how they can be adopted for brain-tumour treatment (**Figure-3**).

Strategies to Modulate MicroRNAs

MiRNA could be modulated either by affecting its biogenesis or mode of action, see **Figure-1b**.

Intervening on microRNA biogenesis

Global reduced levels of mature miRNAs are a common feature of cancer, and is usually associated with aggressive metastatic phenotypes, drug resistance and poor prognosis [61-63]. Mutations in p53 are a common occurrence in cancer. Mutant-p53 sequesters cofactors of Drosha (e.g. p68, p72) and interferes with the nuclear cleavage of certain pri-miRNAs, leading to epithelial to mesenchymal transition (EMT), cell migration and survival [63]. Hypoxia downregulates the expression of both Drosha and Dicer and reduces miRNA biogenesis, resulting in tumour progression and infiltration in ovarian and breast cancer [64]. Targeting either Hypoxia-inducible factor 1-alpha (HIF1a) or combined blockade of transcription factors Avian Erythroblastosis Virus E26 Oncogene Homolog-1 (ETS1) and ETS Transcription Factor (ELK1) rescued miRNA biogenesis and reduced tumour growth in ovarian cancer [64].

Exportin-5 (XPO5) acts as a tumour suppressor by mediating the nucleocytoplasmic export of pre-miR and increasing the levels of mature miRNA in the cytoplasm [61]. Activation of Extracellular Signal-Regulated Kinase 2 (ERK) in liver cancer reduces XPO5 activity, resulting in tumour progression and drug resistance[62]. Inducing XPO5 expression by pharmacologically blocking ERK restores sensitivity to chemotherapy and reduces tumour progression [61, 62].

Although restoring miRNA biogenesis in cancer is beneficial in many instances, abrogating microRNA biogenesis may also have beneficial effects. For example, knockdown of argonaute-2 (AGO2) by siRNA in myeloid leukaemia induces cell apoptosis [65]. However, such treatments may cause several side effects in clinical settings since they can affect the homeostasis of

miRNA biogenesis in the entire body. Hence, miRNA-biogenesis blocking treatments should only be used if the treatment benefits outweigh the risks. Conventional chemotherapy acts by damaging the DNA, hence causing a permanent damage to the body. It is possible that these miRNA-biogenesis blocking drugs could be safer compared to conventional genotoxic drugs. Similarly, drugs that selectively inhibit the biosynthesis of specific microRNAs can be used. For example, a synthetic peptide blocks pre-miR-21 [66] and TargapremiR-210 blocks pre-miR-210 biogenesis [67], see **Figure 1**. Alternatively, chemicals that activate the expression of microRNAs can also be of use. Phenformin, an anti-diabetic drug, increases the expression of let-7, miR-124 and miR-137 resulting in the inhibition of GBM stemness and growth [68]. Silibinin, a plant based flavonoid, treatment upregulated miR-494 in head and neck cancer, which downregulated A Disintegrin And Metalloprotease Domain 10 (ADAM10) and B Lymphoma Mo-MLV Insertion Region 1 Homolog (BMI1) resulting in inhibition of tumour growth and self-renewal properties. Blocking the protease activity of ADAM10, an enzyme responsible for Neuroligin-3 (NLGL3) secretion, potently inhibits *in vivo* glioma growth [69]. Hence, silibinin may be useful in GBM treatment. Erismodegib, a phase III drug for medulloblastoma, upregulates the tumour suppressor miR-128, upregulates miR-200 family which suppresses epithelial to mesenchymal transition (EMT), and suppresses the anti-apoptotic miR-21 in glioma-initiating cells [70]. An epigenetic modifier drug decitabine (5-aza-2'-deoxycytidine) and a polyphenolic compound curcumin upregulate miR-145 expression [21, 71]. Curcumin also blocks the expression of a long-non-coding RNA, LncRNA-ROR, a competitive endogenous RNA (ceRNA) that blocks miR-145 activity [72]. Klinger *et al* points out the importance and desperate need of clinical trials with curcumin for glioblastoma and other brain tumours [73]. Taken together, some Food and Drug Administration (FDA)-approved small-

molecule drugs have the ability to control the expression of beneficial miRNAs. Small molecule drugs usually have better absorption, stability and biodistribution compared to nucleic acid therapeutics, hence preferably used for miRNA modulation.

Gene therapy

Clustered Regularly Interspaced Short Palindromic Repeats/ CRISPR-associated 9 (CRISPR/Cas9) Genome editing enables permanent changes in specific locations on the genome. Cas9 is a bacterial endonuclease guided by a dual-RNA system comprising a CRISPR RNA (crRNA) and a trans-activating crRNA (tracrRNA). Jinek *et al* engineered a single guide RNA (sgRNA) to replace the dual RNA [74]. sgRNA/Cas9 nucleoprotein complex creates double strand breaks (DSBs) in a specific genome location complementary to sgRNA and a “NGG” triple-nucleotide protospacer adjacent motif (PAM) [74]. These DSBs are fixed by one of the two DNA repair mechanisms, error-prone non-homologous end-joining (NHEJ), or template-mediated homology directed repair (HDR). NHEJ creates insertion/deletion mutations (INDEL). Such mutations in the stemloop structure of a specific pri-miRNA disrupt its biogenesis and this strategy is used for selective knock-out of onco-miRNAs [27, 75]. For example, ablation of miR-10 expression in GBM caused the death of tumour cells but not of normal cells [27]. Similarly, a missing gene (e.g. ts-miRNA) can be introduced by harnessing the HDR mechanism and supplying a DNA template. However, CRISPR/Cas9 mediated gene insertion is relatively less common due to the challenges associated with the co-delivery of DNA-template [76, 77]. The limitation of viral-vectors is the random gene insertion into the genome. For *in vitro* applications, electroporation can be used for delivering Cas9 encoded

164 plasmid DNA and minimize the risk of gene insertion [77]. Hirosawa *et al* developed miR-Cas9-
165 ON and OFF systems for cell-type specific genome editing controlled by endogenous miRNA
166 levels [78]. This study also used Cas9 to cause several DSBs using a sgRNA targeting an
167 abundant repetitive DNA sequence found in the genome (*Arthrobacter luteus* restriction
168 endonuclease cleavable region, Alu1)[78].

169 To develop GBM treatments using genome editing tools, it would be desired to direct the Cas9-
170 expression only in desired set of tissues (e.g. in cancer cells) when using viral or non-viral
171 vectors. As human miRNA expression profiles and miRNA promoters are mapped, it is possible
172 to engineer tissue-specific expression of Cas9-gene. For example, if Cas9 expression is not
173 required in a specific tissue type (e.g. normal brain cells), a miRNA that is abundantly expressed
174 in that tissue can be identified (e.g. miR-128, a brain enriched miRNA) and its miRNA-target
175 site can be introduced in the Cas9-gene. This strategy can be multiplexed for different tissue
176 types by introducing several miRNA target site [78]. Conversely, RNAi-Inducible Luciferase
177 Expression System (RILES) is a selective gene expression system responding to the presence of
178 specific miRNAs. This system uses a bacterial transcriptional repressor, Cysteine metabolism
179 repressor (CymR), to suppress a gene-of-interest under the control of an operator sequence. By
180 introducing a miRNA target sequence in the CymR-gene, CymR expression can be reduces in a
181 specific miRNA rich environment, which in-turn leads to the expression of the gene-of-
182 interest[79]. Zika virus has shown glioblastoma stem-like cells (GSC)-specific replication and
183 oncolytic activity [80]. If the molecular mechanism behind selective viral replication in GSCs are
184 identified, those strategies also can be used for directing other viral vectors to selectively target
185 GBM cells. T-VISA-miR-34a plasmid system that overexpresses the encoded miRNA is also
186 modified to specifically express the miRNA only in the cells that express high levels of hTERT,

hence targeting cancer cells [81]. This system has a prolonged miRNA activity at the therapeutic level, one week compared to 2 days for miRNA mimics approach [81]. MicroRNA-sponges are artificial sequences encoded in a plasmid that potentially inhibit miRNA function like the natural ceRNAs [82].

Oligonucleotide Therapy

MiRNA replacement therapy aims at increasing the expression of a specific miRNA in a target cell. This can be achieved by introducing synthetic double stranded RNA molecules called miRNA mimics with identical sequence as natural miRNAs. Yu *et al* showed that free metabolically stabilized single-stranded-RNA (ssRNA) can function like ds-siRNA. When stereotactically injected into mouse brain ss-RNAs combined with AGO2 and inhibited target genes without any carrier system [83].

Conversely, anti-miRNA therapy aims to block the expression or inhibit the function of an oncogenic miRNA (oncomiR). Anti-sense oligonucleotides (ASOs) are ssRNAs that binds to mature miRNA and compromises its function [84]. ASOs are referred by different terminologies such as antagomiRs, anti-miRs, anti-microRNA oligonucleotides (AMOs) or Locked Nucleic Acids (LNA). Small RNA zippers are a new class of ssDNA-LNA designed to block a miRNA by connecting the 3'-end sequences of a mature miRNA to the 5'-end sequences of the adjacent miRNA molecule [85]. Most of the clinical trials with synthetic oligonucleotides (ODNs) are conducted with naked RNA, i.e. without a carrier system. ODNs are chemically modified to achieve desired properties for RNA-delivery to target cells [83, 86]. Most often, they contain a mix of chemical modifications optimised for function [83, 86-88]. To be clinically active, these ODNs must survive endo and exonucleases in the body, avoid immune activation, avoid

sequestration by the reticuloendothelial system, stay longer in circulation, access its target site (passive diffusion), be preferentially taken up by the target cells (active targeting), and access the cytoplasm (site of action) [86]. Additional requirements for effective delivery of miRNA-mimics includes successful RISC-loading, passenger strand separation, target interaction, translational suppression, and release [86].

Cancer cells can develop resistance to miRNA replacement therapy. The possible mechanisms are discussed here. After being released, miRNA mimics might require RNA editing enzymes like ADAR (Adenosine deaminase acting on RNA) and APOBEC1 (apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like-1) to function like natural miRNAs [89]. ADAR2 acts on dsRNA and mediates adenosine-to-inosine (A-to-I) editing, which changes the miRNA target specificity and is essential for stability and normal functions of certain miRNAs [89]. ADAR2 is downregulated in GBM resulting in reduced conversion of A-to-I editing of miRNA[89]. This problem can be overcome if the supplied miR-mimics already contain inosine at appropriate positions. Some other problems associated with miRNA-resistance includes the expression of competitive endogenous RNAs (ceRNAs) [72] and competitive endogenous RNA-binding proteins (ceRBPs) can bind to miRNA-target sites (MRE) preventing access to RISC and hence block miR-mediated gene-silencing [90]. In a 3D *in-vivo* setting, gap-junction (connexin) mediated intracellular transport of miRNA [52] could dilute the miRNA concentration in the target cells, but also could help to spread the gene-silencing activity to neighbouring cells [52]. All these points must be considered to achieve successful miRNA function. Chemical modifications are discussed in **Table 1** [84, 86-88, 91] and clinical trials are discussed in **Box 1**.

Blood and extracellular matrix contain abundant extracellular miRNA stabilized by exosomes and argonaute protein complexes and they are biologically active after cellular entry [50, 51, 92] indicating that chemical modifications can be beneficial but not a requirement to achieve RNAi effects when combined with RNA-stabilizing nanocarriers derived from both natural and synthetic sources.

It is very difficult to understand what is being presented and discussed in this entire section. It needs to be heavily re-written.

Natural Nanomedicine

Exosomes and microvesicles are naturally secreted miRNA loaded vesicles that act as natural delivery and signalling systems in cell to cell communication [93]. These vesicles can be charged with miRNA, either by transfecting large amounts of exogenous miRNA into the packaging cells or by directly transfecting the exosomes by electroporation or chemical methods [93-95]. Cell mediated miRNA delivery has been reported where mesenchymal stem cells (MSCs) or glioblastoma cancer cells were modified ex vivo to over-express certain tumour-suppressor miRNAs. These modified cells produced exosomes loaded with the specific ts-miRNA. When these cells were injected in the tumour vicinity, they secreted exosomes loaded with ts-miRNA and inhibited tumour growth [33].

Argonaute-2 (AGO2) is a the major functional element of miRNA. The evidences showing that AGO2 can be used for miRNA delivery is discussed in Box-2.

Bio-mimetic Delivery Systems

Bacterial-mediated RNAi delivery (transkingdom RNAi) has also been explored. The microRNA of interest can be expressed in bacteria through a plasmid encoding its hairpin loop structure as described for *in vitro* siRNA delivery [97]. The virus-like particles (VLPs) via bacteriophage MS2 can deliver a miRNA. MS2 VLPs are biocompatible, biodegradable, stable and the synthesis is simple [98].

Synthetic Nanoparticles

Spherical nucleic acids (SNA) consist of a core gold nanoparticle (AuNP) with its surface densely packed with oligonucleotides attached via thiolate-Au interaction. The passenger strand bearing a thiol (SH) group can react with gold nanoparticles to deliver miRNA or siRNA. The guide strand can be separated from the passenger strand inside the cells by the RNAi machinery [99]. These nanoparticles show high transfection efficiency, low toxicity and ability to cross the blood brain barrier (BBB) without a targeting ligand [99].

Cell penetrating peptides (CPP) and other cationic peptides can be complexed with nucleic acids to form highly efficient delivery systems. Introducing a thiol group in the peptide helps to create a bio-reducible polymeric peptide that can be specifically released in the intracellular environment and deliver both siRNA and plasmid DNA [100].

A *liposome* containing siRNA-CPP complexes and magnetic nanoparticles were used where magnetic field was used to concentrate the nanoparticles at the site tumour and later the release of siRNA-CPP was triggered using an electric field [101]. This strategy is interesting for selective release of RNAi molecules using external triggers.

276 *Lipid nanocapsules (LNCs)* have an oily hydrophobic core stabilized by surfactants.
277 Oligonucleotides like LNAs can be delivered with a cationic peptide grafted on LNC's surface
278 [102].

279 *Polyethylenimine (PEI)* is a gold standard cationic polymer used in the nucleic acid delivery. Use
280 in the clinic is limited by toxicity [103]. Toxicity can be overcome by chemically modifying
281 more toxic primary and secondary amines into less toxic secondary and tertiary amines. High
282 molecular weight (HMW) and branched cationic polymers show high toxicity while the low
283 molecular weight (LMW) and linear polymers show poor nucleic acid binding and transfection
284 efficiency [103]. One way to overcome this is by using hybrid polymers, e.g. linear PEI-chitosan
285 hybrid nanoparticles show better transfection efficiency and improved safety profiles [103].
286 Another way is by cross-linking LMW polymers with bio-reducible disulphide linkages. For
287 example, thiol cross-linked LMW-PEI polyplexes conjugated with brain targeting rabies virus
288 glycoprotein (RVG) were useful for the delivery of miR-124a [104]. These nanoparticles
289 displayed low toxicity and brain targeting capabilities.

290 Endosomal escape and release from nanocarrier are two major factors that impact the efficiency
291 of synthetic nanocarriers. The polymeric delivery systems can destabilize the late endosomal
292 (LE) compartment by *proton-sponge effect* during its acidification (pH 5 – 6) [105]. Therefore,
293 endosomal escape of polymer-nucleic acid complex should accompany the escape of
294 oligonucleotides from the polymers for a functional activity. Similarly, the fate of ~70 % of
295 siRNA delivered by lipid nanoparticles reach late endosomes (LE), packed into exosomes for
296 exocytosis [106] and the remaining would be degraded after fusion with lysosome [105, 107].
297 Only a small fraction (1 – 2%) of the oligonucleotides reach the cytoplasm that account for the
298 functional activity. This cytoplasmic release was predicted to happen either by direct fusion of

liposomal delivery systems with cell membrane or destabilization of the endosomal bilayer causing the oligonucleotides to leak out into the cytoplasm [107, 108]. Hence, strategies that allow the intra-cellular release of oligonucleotides after internalization should be addressed more seriously to achieve highly functional delivery systems [109].

This section needs to be heavily re-written for clarity and grammar.

Crossing the BBB and Locoregional Delivery

The blood brain barrier (BBB) prevents the access of drugs and nanoparticles from the blood stream to the brain, and represents a major challenge to deliver therapeutic amounts of drugs to intracranial tumours. Several strategies have been devised to overcome the BBB including ligand-mediated transcytosis [110], temporary physical or chemical disruption [111], or convection enhanced delivery [112]. Yu *et al* created an optimised bi-specific antibody targeting human transferrin receptor that crossed the BBB in mice and monkeys, while also targeting an intra-brain enzyme β -secretase [110]. Optimal affinity between ligand and receptor is an important factor to increase brain uptake, as it prevents receptor degradation and allows multiple rounds of transcytosis [110]. In clinical trials, Carpentier *et al* implanted an ultrasound (US) transducer in the skull of GBM patients and achieved safe, reversible, loco-regional BBB opening and observed no dose-limiting toxicities to increasing intensities of ultrasound [111]. US-mediated BBB opening enabled the crossing of gadolinium contrast agent (1kDa) and hydrophilic carboplatin (0.3kDa) [111]. As this study indicates that the intensity of ultrasound can be safely increased further, this technique can also be used to facilitate brain entry of ASOs (8kDa), miRNAs (~15kDa) or nanoparticles (>100kDa).

siR-LODER is a PLGA based biodegradable polymeric implant loaded with siRNA polyplexes that releases siRNA-polyplexes in the local environment for extended period [2 – 5 months] [113]. A phase I study reported good progress and Silenseed Ltd. is starting a Phase II clinical trials with siG12D-LODER (siRNA against mutated KRAS oncogene) in combination with gemcitabine for pancreatic cancer patients (NCT01676259). Such long-term release systems can be very helpful for loco-regional delivery of RNAi molecules for GBM treatment.

Concluding Remarks

GBM patients have a median survival of less than two years due to the lacks effective curative treatments. The importance of miRNA and its therapeutic benefits in GBM are increasingly documented in pre-clinical studies. Further developments in this field can lead novel treatments for GBM. MiRNA-modulating strategies can either act as stand-alone therapeutics or be used in combination with conventional therapies as sensitizing agents. Many of these strategies use plasmid DNA or oligonucleotides and delivering nucleic acids to cells is an important technical problem. Simpler delivery strategies are more likely to enter clinical trials, hence naked RNA delivery is attractive. Chemical modifications on RNAi molecules have improved *in vivo* stability of naked RNA, however further improvements in cellular uptake and intracellular release are needed to expand its applications beyond liver. Synthetic nanoparticles often show promising results in *in vitro* and *in vivo*, but fail in human applications due issues with stability, toxicity, targeting and efficacy. Use of patient derived exosomes might be more biocompatible and safe in clinical applications. The potential of AGO2 to excel as a RNAi delivery system is high (refs?) [114-116]. If argonaute-mediated miRNA delivery is properly explored, AGO2 can be a breakthrough for RNAi, like CRISPR-Cas9 is for genome editing. AGO2 will not however

344 solve the delivery concerns with ASOs, hence other strategies such as chemical modifications
345 and nanoparticle mediated RNA delivery are equally important. Such as?? Combining RNAi,
346 nanomedicine and locoregional delivery can result in effective cancer therapeutics for GBM.
347

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Conflict of Interest

The authors declare that they have no conflict of interest.

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Legend - Figure 1: Biogenesis and Modulating Strategies of miRNAs

(a) Canonical Biogenesis of miRNA: (adapted and modified with permission [117]) MiRNA genes are transcribed by RNA polymerases in the nucleus forming large primary-miRNA (> 500 bases) which harbours one or more stem-loop structures [29]. The RNA-binding protein (DiGeorge syndrome Critical Region gene 8, DGCR8/Pasha) and a RNase III endonuclease (Drosha) recognizes the stemloop structures and releases the stem-loop Precursor miRNA (Pre-miRNA, ~70 nucleotides) [41]. Pre-miRNA is then transported out of the nucleus through nuclear pore complexes (NPC) by binding to Exportin-5 (XPO5) and Ran-GTPase [42]. The pre-miRNA has two arms (5' and 3') each might encode an active miRNA sequence named as miR-X-5p (Red strand) or miR-X-3p (Black strand) respectively. Once in the cytoplasm, the pre-miRNA loads into a pre-RISC (RNA induced silencing complex) consisting of Dicer, TAR RNA binding protein (TRBP), one of the argonautes (AGO1 - AGO4) and chaperones (Heat Shock Proteins, HSP70/HSP90) [39, 40, 43]. Dicer cleaves the stem-loop structure creating a ~23bp miRNA duplex which loads into an argonaute (AGO) [39, 40, 43]. Either miR-5p or miR-3p strand loads into the AGO [26, 44]. Binding of seed sequence (2 – 7 nucleotides at the 5'-end of the miRNA, highlighted in green) to the 3'-untranslated region (3'-UTR) of the target mRNA results in the inhibition of protein synthesis either by messenger RNA (mRNA) destabilization (>75%) and translational repression [46, 47].

(b) Other functions of miRNA and RISC: RISC complex also exerts nuclear functions by shuttling between the cytoplasm and the nucleus via Exportin 1 and Importin 8 [48, 49, 118], and possibly also exerts mitochondrial functions [50]. MicroRNA can be secreted from the cell, either in extracellular vesicles or bound to AGO2 [7, 50, 51], and function as endocrine signalling molecules.

664 **(c) MicroRNA Modulation Strategies:** Genome editing [27, 75]; Small Molecule miRNA
665 Inhibitors (e.g. TargapremiR) [67]; MicroRNA mimics and ss-siRNA [83, 86]; Antisense
666 Oligonucleotides (ASO) [85]; MicroRNA Sponges (Gene Therapy) [82]; MicroRNA Masks
667 [119].

668 **(d)** Can recombinant-AGO2 with targeting ligands acts as a RNAi delivery system?

Legend - Figure 2: Role of miRNA in the Hallmarks of Cancer and Treatment Resistance

(a) Role of miRNA in Treatment Resistance: Standard glioblastoma treatment involves radio and chemotherapy which damage DNA and create double strand breaks (DSBs). Various resistance mechanisms lead to treatment failure: (1) Efflux pumps reduce intracellular drug concentration [22]; (2) Detoxifying enzymes inactivate the drug [5]; (3) DNA repair reverses lethal DSBs and DNA damages; MGMT is a DNA repair enzyme targeted by miR-181d and miR-409 [60]; (4, 5) Damaged DNA induces cell death signals and stop cell cycle progression. For detailed list of resistance genes and mechanisms, see review [5]; (6) Tumour-microtubes and connexin mediated invasion through exchange of resistance factors (e.g. miRNAs) helps cancer cells to adapt to genotoxic treatments and surgery [120, 121].

(b) Role of miRNA in other cancer functions: (1) Role of ts-miRNAs (miR-128 [11-17], miR-145 [18-23], miR-7 [54], miR-16 [55]) and oncomiRs (miR-21-5p [25] and miR-21-3p [26]) in controlling other hallmarks of cancer. (2) Exosomal miRNAs (especially miR-21 and miR-29a) can directly bind to Toll-like receptors (TLR7 and TLR8) in the endosomal compartment of macrophages resulting in pro-metastatic inflammation [57];

(c) Intrinsic and Extrinsic Regulation of miRNA function: (1) Intracellular competitive endogenous RNAs (ceRNAs) and competitive endogenous RNA binding proteins (ceRBPs) can inhibit ts-miRNA [72, 90]. Lnc-RNA-ROR, a ceRNA inhibiting miR-145 is presented as an example. Curcumin inhibits Lnc-RNA-ROR and hence restoring miR-145 function; (2) Small molecule drugs controlling the expression of ts-miRNAs and oncomiRs [21, 67, 68, 70, 72];

(d) Role of Argonaute-miRNA complex: Vascular endothelial cells express neuropilin1 (NRP1) which acts a receptor for AGO2-miRNA complexes [114-116]. Functional significance of AGO2-miRNA complexes in GBM is yet unknown.

(e) Legend explaining the symbols.

Abbreviations: EGFR (Epidermal growth factor receptor); PDGFR (Platelet-derived growth factor receptor); BMI1 (B Lymphoma Mo-MLV Insertion Region 1 Homolog); E2F3 (E2F Transcription Factor 3); DCX (Doublecortex), RELN (Reelin); OCT4 (Octamer-Binding Transcription Factor 3); SOX2 [SRY (Sex-Determining Region Y)-Box 2]; KLF4 (Kruppel-Like Factor 4); JAM-A (a junction adhesion molecule), ZEB2 (Zinc Finger E-Box Binding Homeobox 2), ABCB1 (ATP Binding Cassette Subfamily B Member 1), HIF- α (hypoxia-inducible factor 1); TRAIL (TNF-related apoptosis-inducing ligand); XIAP (X-linked inhibitor of apoptosis protein); BCL2 (B-Cell CLL/Lymphoma 2), CDK6 (Cyclin-dependent kinase-6); PHACTR4 (Phosphatase and actin regulator 4); FASLG (FAS ligand); TLR7 and TLR8 (Toll-like receptor 7 and 8); KDM1B (Lysine Demethylase 1B); Cx43 (Connexin 43); GAP43 (Growth Associated Protein 43).

Legend - Figure-3: RNA nanomedicine based strategies for GBM treatment

Solid GBM tumours are facing two main situations, unresectable tumour and the resection cavity. Two major modalities of treatment using innovative nanomedicines may impact the modulation of miRNA contingents and thus GBM outcome: (a) loco-regional treatments and (b) systemic delivery.

(a) For loco-regional treatment, although intratumoural stereotaxic infusion (a1) is explored, intrathecal, intranasal and CSF delivery can also be used for infusion of natural (a2) or synthetic nanomedicine (a3).

(a2) Natural Delivery Systems: Argonaute-2 (AGO-2)-miRNA complex and exosomes are presented as examples. *Pros:* Biocompatible and safe. *Cons:* Delivery and targeting efficiency needs further improvement.

(a3) Synthetic Delivery Systems: Gold nanoparticles (AuNPs) have a metallic core. The RNA is usually loaded on the surface using strong thiol-gold interaction (S-Au); *Pros:* Facile synthesis and biocompatible. *Cons:* Require chemical modification as the RNA are exposed to the surface [99]; Lipid nanocapsules (LNCs) have an oily hydrophobic core. LNC's surface can be modified with cationic polymers or peptides which be used for RNA binding. Alternatively, lipoplexes (cationic lipid+RNA complexes) can be prepared and embedded into its core; *Pros:* High cellular uptake. *Cons:* Low RNA loading capacity and toxicity from surfactants. Chemical conjugates are chemically modified naked RNA linked to a ligand to facilitate cellular uptake and increase delivery efficacy; *Pros:* Simple design. *Cons:* Optimization needed to reach organs other than liver. Liposomes can hold the RNA in its shell or in the aqueous core; *Pros:* High cellular uptake. *Cons:* Low endosomal escape. Cationic polymers condense the nucleic acid by

electrostatic interaction to form nanoparticles. *Pros*: Can load very high quantities of RNA per nanoparticle due to ionic condensation. *Cons*: Toxicity and release of RNA from the polymer after cellular entry is not clearly understood. The surface of synthetic and natural nanoparticles can be easily engineered with polyethylene glycol (PEG) and desired ligand.

(a4) Modalities of administration: As in the case of delivery of radiopharmaceuticals {Vanpouille-Box, 2011 #5048}, it is important to reach optimal therapeutic index and to define therapeutic time windows where the treatment is more efficient. Drug distribution and clearance information help to decide dose fractionation and schedule of administration of miRNA-nanomedicine {Ezzine, 2013 #567}. The volume of distribution would depend on the mode of administration (Bolus or CED). (Part of the image adapted from Servier Medical Art, available under creative commons attributions 3.0).

(a5) Alternatively, long term release implant might be used for sustained release like siRLODER [113].

(b) Systemic delivery and crossing the blood-brain barrier (BBB): The brain endothelium with tight junctions and the surrounding supporting cells form a selective barrier isolating most of the blood components from accessing the delicate brain tissue. Ultrasound transducers placed inside the cranium can reversibly disrupt the BBB causing the leakage of blood components into the brain tissue [111]. This strategy can be used for facilitating the entry of intravenously injected nanoparticles into the brain tissue. (b2) Nanoparticles conjugated with anti-transferrin antibodies can enter the brain tissue by transcytosis through the endothelial cells [110]. Pink dots represent nanoparticles without any ligand on its surface, while the Green dots represent transferrin-conjugated nanoparticles.

Box 1: Biogenesis and functions of miRNAs

The biogenesis and functions of human miRNAs are represented in **Figure 1** [39, 40]. MiRNA genes are transcribed mainly from the nuclear genome (Primary-miRNA transcript, pri-miR) [29], processed and transported out of the nucleus (Stem-loop structured Precursor-miRNA, pre-miR) [41, 42], trimmed in the cytoplasm (Duplex miRNA, miR-5p/miR-3p) [43] and loaded onto argonaute proteins to form the RISC complex (single-stranded Mature miRNA). Either or both miRNA-strands can be loaded onto individual RISC complexes [26, 44]. MiRNA-guided RISC binds to target messenger-RNAs (primarily in the 3'-untranslated region, at specific sites called miRNA recognition elements (MREs)) and blocks protein expression by mRNA destabilization (~80%) and repressing translation (~20%) [45-47]. The RISC complex also exerts miRNA-mediated gene-silencing in the nucleus [48, 49] and mitochondria [50]. MiRNAs can also function like endocrine or paracrine signalling molecules as they can be transported to adjacent cells via connexins [52] or secreted from the cell, either in extracellular vesicles (EVs) or bound to argonaute proteins (e.g. AGO2) [7, 50, 51].

Box 2: Argonaute as a miRNA Delivery System

Extracellular microRNAs are extremely stable and abundantly found in all body fluids, either bound to argonaute proteins (especially AGO2) or encapsulated in extracellular vesicles (EV) [50, 51, 92]. Also, AGO2 stabilizes many of the miRNAs in exosomes [92]. EV-associated circulating miRNA and its role in cell-to-cell communication is increasingly becoming evident while the functions of AGO2-associated miRNAs are explored very less. Ferreira *et al* showed that AGO2 increased miRNA uptake in selective human endothelial cells (EC), derived from cerebral arteriovenous malformation (AVM), normal and glioma endothelium, without the need of any transfection agent [116]. AGO2 also protected miRNAs from degradation after cellular entry and they were functionally active under both *in-vitro* and *in-vivo* conditions [116]. In an intracranial glioma model, intravenously injected AGO2-miR-18a complexes inhibited angiogenesis by specific internalization by brain endothelium [116]. The specificity of AGO2-miR uptake strongly indicates the involvement of receptor mediated endocytosis. Prud'homme *et al* showed that neuropilin-1 (NRP1) acts as a receptor mediating translocation of free miRNAs and productive uptake of AGO2-miRNA complexes [114]. NRP1 is highly expressed in vascular endothelial cells, GBM, and other cancer cells which makes them ideal targets for AGO2-mediated miRNA delivery and might explain the results from Ferreira *et al* [114-116]. The AGO2-mediated RNAi delivery can be expanded to other tissues by attaching ligands on its surface or producing recombinant AGO2-ligand fusion proteins, see **Figure 1**. Also, AVM-EC can be used for AGO-miRNA delivery as it is known to secrete AGO2. Unlike other delivery systems where the guide strand must be loaded into endogenous AGO to be functional, AGO2-RNAi would be functional once reaching the cytoplasm. Pre-loading exogenous AGO would minimize the toxicity associated with overloading and sequestering of the endogenous RNAi

789 machinery by exogenous RNAi molecules [124]. Also, loading AGO2 with a mature guide
790 strand eliminates the non-specific effects associated with the passenger strand. AGO2 protects
791 the RNA from degradation, hence no chemical modifications are necessary; AGO2 is conserved
792 across all humans and hence the native form of recombinant-AGO2 is probably safe for systemic
793 delivery. Exploring the endocytosis and endosomal-release pathways of AGO2-miRNA
794 complexes are needed. With proper exploration, AGO2 can become the solution for RNAi
795 delivery as it has the potential to overcome several barriers.

796

797

Table-1 Clinical Trials with AMOs and MicroRNA mimics

Miravirsen was the first AMO in human clinical trials. Miravirsen development was discontinued due to poor performance in liver, attributed to the lack of uptake enhancers like targeting ligands or nanocarriers [122]. RG-101, the next generation GalNAc conjugated antagomiR against miR-122 is under phase II clinical trial conducted by Regulus Therapeutics (EudraCT Number: 2015-001535-21). The targeted RG-101 has shown significant reduction in the viral load with a single subcutaneous injection at 2 and 4 mg/kg of dosing [123]. These clinical trials emphasise the importance of cellular uptake enhancers is required for the activity of ODNs *in vivo*. MRX34 by Mirna Therapeutics is the only miRNA therapeutic in clinical trials and uses SMARTICLE® lipid based formulation. Phase I clinical trial (NCT01829971) evaluating maximum tolerated dose was completed. The clinical trial with MRX34 and all other pipeline R&D programmes were voluntarily discontinued by the Mirna Therapeutics due to serious immune related adverse events. This clinical study emphasises on the safety of RNA-delivery systems is paramount for achieving success in clinic.

Table – 1 MiRNA and GBM

MiR-128 is highly expressed in normal brain tissue, but down-regulated in GBM [53]. There exists an inverse correlation between miR-128 expression and grade of glioma [11]. MiR-128 reduces tumour growth (EGFR, PDGFR [12]), stemness (BMI1 and E2F3 [13, 14]), invasion (DCX, RELN [15]), induces apoptosis [16] and senescence [17]. miR-128 was expressed at low levels in proneural-GSCs (Glioma Stem-like Cancer cells) and no expression in more aggressive mesenchymal-GSCs [14]. However, overexpressing miR-128 inhibited tumour growth in all molecular-subtypes of GSCs [14]. miR-145 is implicated in the reduction of cancer stemness and invasion abilities by controlling several genes involved in self-renewal (OCT4, SOX2, NANOG), a junction adhesion molecule (JAM-A) which has the potential to rescue stemness by the activation of pAKT [18] and EMT (e.g. ZEB2) [19, 20]. It also targets efflux pump, ABCB1 (or P-gp) [22], cell cycle regulators CDK6 and SP1 thereby involved in chemo-sensitization [21]. Both miR-128 [11] and miR-145 [23] targets p70S6 kinase1, an important down-stream effector of PI3k/ AKT/ mTOR pathway, highly expressed in glioma and promotes angiogenesis by activates hypoxia-inducible factor 1 (HIF- α) [11]. MiRNA-7 sensitized GBM to TRAIL-mediated apoptosis (TNF-related apoptosis-inducing ligand) by directly downregulating XIAP (X-linked inhibitor of apoptosis protein) [54]. MiR-16 inhibited tumour proliferation and invasion by targeting multiple genes BCL2, CDK6 (Cyclin-dependent kinase-6), cyclin D1, cyclin E1 and SOX5 at protein level, however seed base-pairing with isolated target sites yielded only faint but statistically significant downregulation [55]. Similarly, overexpression of oncomiR-10b did not show any downregulation of predicted targeted genes even at protein-level, however, the therapeutic impact was evident upon blocking its expression [56]. Abolishing miR-10 expression by genome engineering did not affect normal brain cells while eradicating tumour

[27]. MiR-21-5p is widely studied oncomiR, while relatively less expressed miR-21-3p oncogenic role was recently identified [26]. MiR-21-3p targets PHACTR4 (Phosphatase and actin regulator 4) resulting in rapid cell proliferation and oncogenesis; This study indicates the importance of studying the miRNA functions without strand-bias [26]. MiR-21-5p decreases apoptosis by targeting FASLG (FAS ligand) [25]. miRNA-21 and miR-29a loaded exosomes are released by tumours cells which activates Toll-like receptors (TLR7 and TLR8) in macrophages causing inflammation and promoting tumour proliferation and metastasis [57]. miR-215 targets epigenetic regulator KDM1B (Lysine Demethylase 1B) and provides GBM growth adaptation under hypoxic condition [58], while reintroducing hypoxia suppressed miR-124 induces cell death by suppressing TEAD1 (Transcriptional Enhancer Factor 1), MAPK14/p38 α and SERP1 (Stress-associated Endoplasmic Reticulum Protein 1) [59].

Trends Box

- miRNAs are involved in GBM development and resistance to conventional therapies.
- MiRNA based drugs and nanomedicine can revolutionize GBM treatment.
- Pioneering clinical trials on miRNA inhibitors and miRNA-mimics show the need for optimization of delivery systems.
- AGO2 is emerging as a RNAi carrier and can become an important tool for RNAi delivery.
- Initial success in PLGA-based long-term siRNA delivery systems in solid tumors provides new hope for developing miRNA-based locoregional treatments for GBM.

Outstanding Questions Box

- What is are the most appropriate microRNA networks and pathways to target in glioblastoma?
- Can miRNA targeting solve radioresistance and occurrence of the the yet inevitable recurrence?
- How to get successful spatial and temporal delivery of miRNA antagonists or agonist into glioblastoma cells?
- How can we achieve long-term delivery of microRNA in the brain?
- Can therapeutic weapons dedicated to miRNAs greatly benefit from innovative nanomedicines? Can argonaute become a solution for RNAi delivery?

Fig 2 - Role of miRNA in the Hallmarks of Cancer and Treatment Resistance

(a2) Natural Delivery Systems

Biocompatible and Safe

(a3) Synthetic Delivery Systems

Easily tunable

AGO2/miRNA (~5 nm)

Exosome (50 - 200 nm)

Gold Nanoparticle (10 - 50 nm)

Lipid Nanocapsule (25 - 100 nm)

Liposome (20 - 200 nm)

Chemical conjugate (> 10 nm)

Polymeric Nanoparticle (10 - 200 nm)

RNA

Ligand

PEG

(a) Loco-regional treatments

(b) Systemic delivery - Crossing the blood-brain barrier (BBB)

Table 1 Important Chemical modifications

Location	Examples of Chemical Modification	References
5'-end	(E)-vinylphosphonate	[87]
3'-end	Aptamer conjugates	[90]
3'-end	hydrophobic moieties	[86]
backbone	phosphorothioate (PS)	[86]
backbone	peptide nucleic acids (PNAs)	[85]
backbone	morpholino	[85]
ribose sugar	2'-O-Methyl	[86]
ribose sugar	2'-O-Fluoro	[86]
ribose sugar	2'-O, 4'-C methylene bridged locked nucleic acids (LNA)	[85]
ribose sugar	2'-O, 4'-C-ethylene-bridged nucleic acids (ENA)	[83]

Trends Box (900 characters, including spaces, required)

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