Tailoring Drug and Gene Co-delivery Nanosystems for Glioblastoma Treatment

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Abstract

Glioblastoma is the most common primary and aggressive brain tumour, with an increasing incidence worldwide. The prognosis of this disease is still poor, with a median survival time not exceeding two years. Standard-of-care therapy includes surgical resection, radio- and chemotherapy, but nearly all patients experience progression of the disease. This may be ascribed to the heterogeneity, invasiveness and resistance of tumour cells, along with the struggle that many chemical drugs present in effectively crossing the dual blood brain-blood brain tumour barrier.

Considering the hurdles associated to traditional therapeutic approaches, there is a pressing need to improve patient care, as treatments currently available have little effect on the overall survival. Therefore, the use of adjuvant chemotherapeutics in combination with temozolomide, a first-line drug, and novel molecularly-targeted approaches against both tumor and stem cells and respective microenvironment are under investigation. This chapter addresses the development of innovative multi-target nanomedicines, comprising complementary chemo- (e.g. temozolomide) and gene therapeutic (antimiR and miRNA mimic) agents, combined with targeting ligands within a single nanostructure directed at the treatment of glioblastoma multiforme. The approach aims at providing significantly improved therapeutics, as treatments currently available have little effect on overall survival.

Keywords: Co-delivery, Drug delivery, Gene therapy, Glioblastoma, miRNA delivery, Nanoparticle, Nanotherapy.

1. Glioblastoma

The World Health Organization has recently reclassified central nervous system (CNS) tumors in 17 categories, according to well-defined molecular and histological (Louis et al., 2016). Glioblastoma, a group of IV grade lesions, is generally originated by the differentiation of astrocytic cells. It is characterized by cellular pleomorphism, nuclear atypia, a diffuse growth pattern, a strong mitotic activity, microvascular proliferation and may or may not display necrosis. This disease may be classified as primary/glioblastoma isocitrate dehydrogenase (IDH)-wildtype, (which includes glioblastoma IDH-wildtype and the variants giant cell glioblastoma, gliosarcoma and epithelioid glioblastoma), secondary/glioblastoma IDH-mutant or glioblastoma NOS, considering its development and absence or presence of IDH mutations (Louis et al., 2016). On the first case, there is no lower grade precursor lesion nor IDH mutation, whereas on the second a low grade malignant glioma with an IDH mutation evolves to glioblastoma multiforme (Urbańska et al., 2014). Glioblastoma NOS is an exclusion diagnosis where IDH mutation status cannot be fully evaluated. Of all glioblastoma diagnosed tumours, 90% are IDH-wildtype and approximately 10% are IDH-mutant tumours. IDH-wild type variants and glioblastoma NOS represent the remaining identified cases and, therefore, considered not common. Taking into consideration the expression profiles of glioblastomas, Verhaak et. al. reported the existence of four subsets, proneural, neural, classical and mesenchymal, from which the proneural subtype presents the better prognosis, by expressing genes associated to normal brain activity and neurogenesis (Verhaak et al., 2010). Over 90% of IDH-mutant glioblastomas present a proneural expression signature, suggesting that IDH-mutant glioblastomas correspond to a group of relatively homogeneous tumours. In contrast, IDH-wildtype glioblastomas have distinct expression profiles, and are thus considered heterogeneous (Verhaak et al., 2010).

Among brain tumors, glioblastoma is the most common, deadly and aggressive type. In fact, its diffuse infiltrative behaviour on neighbouring structures impairs a successful surgical resection and promotes recurrences (Ohgaki and Kleihues, 2005). Glioblastoma affects 3-4 people in every 100,000 North Americans, Europeans and Australians, most of which are older adults (Ohgaki and Kleihues, 2005, Ostrom et al., 2014). Primary glioblastomas are more frequently diagnosed in 59 years old male patients (163 male in every 100 female diagnosis). On the contrary, secondary tumours are commonly diagnosed in 43 years old female adults (96 male in every 100 female diagnosis) (Oh et al., 2016, Lai et al., 2011). Despite these tumors are rare in young patients, which include children and adolescents, they do not usually present significant morphological differences from those of adults. However, they grow and proliferate more rapidly (Urbańska et al., 2014).

Primary glioblastoma presents a widespread distribution in the brain, commonly affecting the frontal, parietal, temporal and occipital lobes, whereas secondary glioblastoma tends to establish in the frontal lobe, a regular site for the growth of diffuse anaplastic astrocytomas and oligodendrogliomas (Zlatescu et al., 2001, Ohgaki and Kleihues, 2013). The glioblastoma proliferation and invasion is commonly limited to CNS organs. Among these, they are rarely found in the spinal cord. In addition, tumor cells are not frequently found in the bloodstream in individuals who have been submitted to surgical resection, hence why metastasis are not common in these patients (Lun et al., 2011).

Glioblastoma standard treatment combines a tripartite strategy that includes surgical resection, radiotherapy and orally administered chemotherapy, using temozolomide as first line medicine. Nevertheless, the strong tumor mitotic activity, drug resistance mechanisms and the presence of cancer stem cells that escape surgical resection

and lethal doses of radio- and chemotherapy are on the basis of the frequent poor outcomes, with approximately 70% of patients experiencing recurrences within the first year of treatment (Stupp et al., 2005). Primary glioblastoma diagnosed patients that are given the triple standard treatment survive on average 15 months, whereas patients treated with surgical resection and radiotherapy have a life expectancy of only 9.9 months. As for patients who have been diagnosed with secondary glioblastoma, the prognosis is slightly better, although limited to a mean of 31 months (vs. 24 months if no chemotherapeutic regimen is followed) (Yan et al., 2009). Novel therapeutic strategies, which include the use of targeted drug delivery nanosystems (Basso et al., 2018a), delivery of genetic material (Kwiatkowska et al., 2013) and repositioning of drugs with proven activity over glioma cells (Basso et al., 2018b) are being thoroughly studied, as they remain encouraging approaches to improve the prognosis of this disease.

2. MicroRNAs

MicroRNAs (miRNAs) are a class of non-coding endogenous RNA molecules, with only 20-25 nucleotides, that play a central role in cellular differentiation, proliferation and resistance to apoptosis. In fact, miRNAs bind to complementary mRNA chains, thus leading to their translational inhibition or degradation and, therefore, to gene expression modulation (Bartel, 2004). Up to date, more than 38,000 miRNAs are identified in the miRbase, from which more than 2,600 correspond to human sequences (Kozomara et al., 2019). Lee et. al. (Lee et al., 1993) have identified, in 1993, a small RNA molecule transcribed from *Caenorhabditis elegans* lin-4-locus with partial antisense complementarity to the 3' untranslated region (UTR) of lin-14, responsible for regulating the developmental timing in *C. elegans*. Despite being considered, at the time, a peculiar event, in only seven years, hundreds of miRNA and other non-coding RNAs

were described in *C. elegans* and other animals, including humans (Lindow and Kauppinen, 2012). There is consistent evidence that miRNAs and the related bio-machinery are involved in several pathological conditions, including cancer, due to genomic events or biogenesis defects (mutations, deletion, amplification or transcriptional changes, misregulation of miRNA regulating proteins, etc) (Bartel, 2004, Rupaimoole et al., 2016, Ha and Kim, 2014, Lin and Gregory, 2015).

2.1 miRNA biogenesis

miRNA biogenesis (**Figure 8.1**) has been extensively studied and involves, in humans, four key enzymes: Drosha, exportin 5, Dicer and argonaute 2 (AGO2), the latter being part of the RNA-induced silencing complex (RISC). Briefly, a miRNA is transcribed by RNA polymerase II from a gene encoding miRNA (normally located in intronic regions with own promoter regions), thus producing a primary-miRNA (pri-miRNA) with a nucleotide sequence up to a thousand of monomers. Pri-miRNAs are usually 7-methylguanosise capped at the 5' end and poly-adenylated at the 3'end, like mRNAs, and form a hairpin-shaped stem-loop secondary structures before being cleaved by a microprocessor complex (500-650 kDa), involving Drosha, a RNase III endonuclease, and the DGCR8/Pasha co-factor, a protein containing two dsRNA binding domains. The cleavage of the pri-miRNA by the complex forms a pre-miRNA, with 60-70 nucleotides, a 5' phosphate end and a 3' 2 nucleotide overhang. The transport from nucleus to the cytoplasm of the pre-miRNA is mediated by the complex exportin 5-RAN.GTP. In the cytoplasm, the RNase III Dicer and TAR RNA binding protein (TRBP) bind to the pre-miRNA, cleaving the terminal loop, thus forming a shorter double strand miRNA:miRNA* duplex. The processing of this duplex is mediated by AGO2, in combination with several cofactors. After unwinding and selection of the leading strand,

the mature miRNA is incorporated in RISC and the mature miRNA* strand is rapidly degraded. The leading mature miRNA strand is then able to recognize complementary sequences of mRNAs (via Watson-Crick base-pairing), hence leading to translational repression or mRNA degradation (Zhang et al., 2007, Rupaimoole and Slack, 2017). Generally, miRNAs regulate gene targeting by binding to 3'-UTRs of mRNAs (Bartel, 2004). This argonaute-catalyzed cleavage requires an extensive, but not complete, complementarity between the miRNA lead strand and the target mRNA, and multiple binding sites for the same miRNA may be found within the same mRNA. In fact, the activity of miRNAs depends on the "seed" sequence, an arrangement of only 6 nucleotides (miRNA 5' nucleotides 2 through 7 or 8). However, miRNA targeting may also occur in mRNA coding regions (Fang and Rajewsky, 2011, Zhang et al., 2007). Taking into consideration that miRNA pairing to mRNA requires only a short sequence of nucleotides complementarity, the same miRNA may be responsible for the repression of numerous genes and, consequently, for off target effects (Bartel, 2004). As such, it is possible, by targeting a single miRNA, to modulate the expression of several gene networks.

INSERT FIGURE 8.1 HERE.

Figure 8.1. Overview of microRNA biogenesis in mammals. Reproduced from (Broderick and Zamore, 2011) with permission from Springer Nature.

3. MicroRNA activity in glioblastoma

Gliomagenesis remains an unknown phenomenon, although some environmental factors (exposition to ionizing radiation or vinyl chloride) and genetic causes (overexpression of oncogenes and mutations or deletions of tumor suppressor genes) may

induce tumorigenesis. In addition, there is solid evidence that miRNAs play an important role in the genetic regulation of these genes (Vogelstein and Kinzler, 2004, Zhang et al., 2007). It is known that miRNAs also participate in epithelial to mesenchymal transitions (EMT), required for enhanced migratory abilities and invasiveness, as well as apoptosis resistance (Franco-Chuaire et al., 2013).

Dysregulation of miRNA expression is clearly associated to several diseases, including cancer. The evaluation of the miRNA activity in cancer may be described by comparing the expression of miRNAs in cancer and normal cells. Knockdown or overexpression of a specific miRNA is a common strategy for better understanding the role of the miRNA in cancer pathogenesis. Other biochemical techniques include real-time PCR, Northern blot analysis and miRNA microarray.

Considering the miRNAs expression levels, they are divided in two categories: tumor suppressor miRNAs (with a reduced or non-existing expression) and oncogenic miRNAs (with a markedly increased expression). Tumor suppressor miRNA overexpression in glioma stem cells is known to reduce cell proliferation and promote cell differentiation, as these miRNA are known to have an impact in regulatory and oncogenes expression (Lang et al., 2012). In contrast, overexpression of oncogenic miRNAs, also denoted as oncomiRs, may increase tumor growth and proliferation, and decrease apoptosis, by inhibiting regulatory or tumor suppressor genes (Costa et al., 2012, Ilhan-Mutlu et al., 2012, Park et al., 2012). Most miRNAs are overexpressed in glioblastoma. In fact, at least 256 miRNA molecules, including miR-93, miR-21, miR-17-92 cluster and miR-10b are significantly overexpressed in glioma tissues, whereas at least 95 miRNAs, which include miR-137, miR-128, miR-34a and miR-7, have a reduced expression in these cells, when compared to healthy brain cells (Moller et al., 2013). In addition, miRNA expression in gliomas depends on the stage of the cancer.

During the progression from grade II gliomas to grade IV glioblastomas, 12 miRNAs, specifically, miR-210, miR-140, miR-130b, miR-28, miR-25, miR-21, miR-20a, miR-19a, miR-17, miR-16, miR-15a and miR-9 were found to be overexpressed, while 2 miRNAs, namely, miR-328 and miR-184, were downregulated during the progression of the tumor (Malzkorn et al., 2010). In addition, the expression of two other miRNAs was also found to be dependent on the stage of the disease; miR-182 is upregulated in late stages of gliomas, whereas miR-137 has a lower expression, hence being able to predict prognosis and survival of patients (Jiang et al., 2010, Sun et al., 2013).

Overall, miRNAs are important marks in regulating several developing processes of cells, including cell differentiation, proliferation and apoptosis. In cancer, the dysregulation of such RNAs plays a central role in establishing signals to evade growth suppressors and enable replicative immortality, cell death resistance, angiogenesis, invasion and metastasis (Shea et al., 2016). Furthermore, in glioblastomas, miRNAs are key factors in drug resistance mechanisms, in particular, regarding TMZ. A comparison of the profile expression of temozolomide sensitive (wildtype) and resistant U251 glioma cell lines was conducted in order to infer on the impact of miRNAs in chemotherapy resistance. It was found that, of the three most upregulated miRNAs, miR-455-3p, miR-185 and miR-10a*, the knockdown of miR-185 had the highest impact in enhancing the therapeutic effect of TMZ (Ujifuku et al., 2010). In a similar study using patient derived tumours, miR-222, miR-221, miR-181b, miR-181c and miR-128 were found to be downregulated, while miR-21 was overexpressed in these patients. Of those, miR-181b and miR-181c proved to have the best correlation with TMZ treatment, suggesting their use as predictive biomarkers for TMZ therapy response (Slaby et al., 2010). miR-21 is a highly frequent upregulated oncogene in cancer, and is involved in TMZ resistance through the downregulation of apoptotic events. In addition, this oncogene has also

proved effects on doxorubicin, paclitaxel, sunitinib and teniposide resistance (Shea et al., 2016). TMZ resistance in A172 and U251 glioblastoma cell lines and patients, via upregulation of glycogen synthase kinase 3β (GSK3β) is also a reality, and may be caused by the knockdown of miR-101. In fact, GSK3β plays a role in increasing MGMT expression and activity, a known resistance mechanism that reverts the methylation activity of TMZ in glioma cells. Therefore, the reversion of the loss of function of miR-101 may surpass TMZ resistance in tumor cells (Tian et al., 2016). miR-328, a tumor suppressor that is down-regulated in glioblastoma, is responsible for the modulation of the expression of the protein ABCG2. This multidrug resistance protein, part of the ATP-binding cassette transporter protein family, is frequently found overexpressed in numerous cancers and is responsible for the efflux of several molecules across the cellular membrane, thus promoting chemoresistance. Hence, the restoration of the function of miR-328, using mimics technology, may be an interesting strategy for improving the efficacy of chemotherapeutic regimens (Li et al., 2010).

3.1 miRNAs as biomarkers

Taking into consideration the important position of miRNAs in the differentiation and immortalization of cells, thus leading to tumorigenesis, the premature detection of these molecules holds a great potential. In fact, the expression and signature of some miRNAs may be unique in certain tumor cells, and may be an important characteristic to differentiate gliomas from other CNS tumors, such as meningiomas and pituitary adenomas (Wang et al., 2012). On that basis, miRNA array analysis has become a powerful tool for identifying and classifying tumor subtypes, and distinguishing different types of cancer and their primary tissues of origin. More significantly, these techniques are able to identify early changes and poorly differentiated tumours (Lu et al., 2005). Considering (in)stability, abundance and accessibility of specific miRNAs in tissues,

biological fluids and circulating exosomes, miRNAs are important biomarkers for the detection and stratification of gliomas, thus being powerful tool for early stage cancer diagnosis (Iorio and Croce, 2017, Hermansen and Kristensen, 2013). Note that, due to the secluded location within the brain, the detection of miRNAs in body fluids as biomarkers of glioblastoma are of high importance. Although the mechanisms of cellular release of miRNAs from cancer cells and blood-brain-barrier (BBB) transport are not yet fully understood, release and serum stabilization of miRNAs may be dependent on protein complexes and extracellular vesicles. It has also been proposed that the presence of extracellular miRNAs may be due to cell death and extravasation (Turchinovich et al., 2011). Interestingly, most of the miRNAs determined in plasma samples and cell culture media are bound to AGO2, thus having better physical and chemical stabilities than naked miRNAs (Turchinovich et al., 2011, Arroyo et al., 2011). Yet, extracellular vesicles are also important in tumor progression and migration, as they are a known mechanism of communication between different cells, including neighbour tumor and endothelial cells of the BBB (Skog et al., 2008). Regarding the latter, miR-181c is known to suppress and downregulate the expression of phosphorylated cofilin, thus leading to a weakened BBB in brain metastatic breast cancer (Tominaga et al., 2015). Note that miR-181c was found to be a tumor suppressor gene in some glioma patients, thus suggesting different miRNA functions according to cancer type and tumor microenvironment (Ruan et al., 2015, Shea et al., 2016). Although extracellular vesicles carrying miRNAs from cancer cells may present a harmful behaviour, these structures with origin in noncancerous cells also play an important role against tumor proliferation. In fact, in some tumors, healthy cells are able to effectively deliver miR-143 to cancer cells, thereby increasing the expression of this tumor suppressive gene, while not affecting the expression of the same RNA in other healthy cells (Kosaka et al., 2012).

Several miRNAs have been identified in plasma samples of glioma patients. In these subjects, miR-21 was found to be upregulated, whereas miR-342-3p and miR-128 were expressed in a lower extent (Wang et al., 2012). However, miR-128 was also found to be upregulated in other glioma bearing patients (Roth et al., 2011). Interestingly, it was later discovered a relation between the expression levels of miR-128 in plasma, before and after a surgical resection of the tumor; patients who had their tumors surgically removed had slightly higher miR-128 expression in plasma, when compared to prior surgery levels (Sun et al., 2015). Disease progression and prognosis may be also determined by analysing the expression of miR-205, as it presents a significantly decreased expression in glioma patients. Moreover, the decrease in such levels was correlated with the progression of the disease, while only presenting elevated levels in plasma following surgical removal of the tumor. However, when patients experienced tumor recurrence, miR-205 plasma levels found to be again decreased. Curiously, there may be a relation between miR-205 plasma levels and overall survival times of advanced stages of cancer patients; in the presence of lower plasma levels of this RNA, patients experienced shorter survival times (Sun et al., 2015, Yue et al., 2016).

3.2 miRNA based therapy

miRNAs have important and dynamic functions in cancer, as they regulate gene expression. Taking into consideration their expression profiles in tumor cells, the activity of such RNAs can be modulated to impair cancer formation and development.

Yet, the therapeutic delivery of miRNAs to tumors still remains a challenging approach, since there are some host defensive strategies that impair the successful transfection of RNAs, including endosomal degradation and blood clearance by phagocytosis, kidney filtration and excretion. Additionally, miRNA delivery to CNS tumors is hampered by the presence of the BBB, as it compromises the transport of several

molecules, including RNAs and chemotherapeutic drugs. Despite the increase in BBB permeability induced by gliomas, some areas surrounding the tumor are still tightly protected against foreign substances, thus compromising the efficacy of the current therapeutic approaches. The *in vivo* administration of oligonucleotides via parenteral injections (intravenous and subcutaneous) is the standard route of choice. Despite reaching several tissues after administration, including, liver, kidney, bone marrow, adipocytes and lymph nodes, first and second generation oligonucleotides do not easily reach central nervous system compartments (Geary et al., 2015). To surpass these hurdles, commonly studied strategies include the use of viruses, nanoparticles, liposomes, exosomes, stem cells, transient chemical and physical alterations to BBB permeability and modification of administration routes, with particular incidence in intratumoral, intrathecal, intraventricular, intra-arterial and intranasal delivery (Alam et al., 2010, S Hersh et al., 2016).

Overall, based on the expression levels of the target miRNA, two distinct approaches may be followed: antisense technology can be used to silence the oncogene expression, while mimic strategies can be explored to increase the tumor suppressor gene expression **(Figure 8.2)**. Overexpressed endogenous miRNAs, which may function as oncogenes, can be inhibited through the administration of artificial antisense ssRNA oligonucleotides, known as antimiRs or antagomirs. These antimiRs bind to mature miRNAs through Watson-Crick base-pairing complementarity, hence leading to the degradation of the double-stranded formed RNA and consequent gene silencing. The design of antisense technology should take into consideration the affinity binding to the target RNA, the degradation from endonuclease proteins and its *in vivo* delivery. In addition, due to the fact that a single miRNA may regulate the expression of several genes, antisense RNA technology can result in significant off targets effects and high toxicity

(Bardin et al., 2018). Therefore, some chemical strategies regarding the use of nucleotide analogues, and backbone and terminal modifications are currently being studied (Chen et al., 2015). Among these strategies, locked nucleic acid (LNA) modifications, i.e., conformationally locked nucleotide analogues through the introduction of methylene bridges, appear to be the most promising, due to the high affinity to nucleic acids, thermodynamic stability and strong nuclease resistance (Lindow and Kauppinen, 2012). In fact, miravirsen, a LNA-modified anti-miR-122, has now reached the phase II of clinical trials and is a promising novel strategy against hepatitis C virus infection, without inducing side effects or miR-122 viral resistance (Bardin et al., 2018).

INSERT FIGURE 8.2 HERE.

Figure 8.2. Current common approaches in gene therapy via miRNA modulation. Adapted from (Bardin et al., 2018) with permission from Frontiers.

In parallel to antisense oligonucleotides, target site blockers (TSBs) and miRNA sponges have also been developed. The first are designed to bind perfectly to the 3'-UTR complementary site of the target miRNA, meaning that they aim at blocking the action of the target miRNA by effectively binding specific mRNA molecules, whereas the latter are characterized by multiple target miRNA binding sites, thus limiting bioavailability and action on non-target sites (Bardin et al., 2018). miRNA sponges have proved to be useful in impairing the expression of the oncogene miR-23b in glioma-bearing mice, since it led to the decrease in the expression of β-catenin, HIF1α, VEGF, ZEB1, MMP2 and MMP9, and upregulation of E-cadherin and VHL, hence reducing cell migration, invasion of tumor cells and angiogenesis (Chen et al., 2014).

Opposing antisense gene therapy, miRNA replacement is an effective strategy to restore or amplify the loss of function promoted by the downregulation of tumor suppressive genes. This strategy considers the administration of synthesized miRNA mimics, with an identical sequence of those downregulated during cancer. Once administered and reach the target cells, miRNA mimics will bind to the complementary mRNA, thus blocking the expression of the tumor promoter and restoring biological and healthy cell functions. Note that the extent of the base-pairing of the miRNA mimic and its target blocks mRNA translation by either degrading the miRNA-mRNA duplex (if in perfect complementarity) or by leading to the translation of an aberrant protein (if in partial complementarity).

4. Novel technological strategies for glioblastoma treatment

4.1 Drug delivery nanosystems

As mentioned before, the existing chemo and radio-based treatment strategies fail to effectively kill all cancer cells, either due to resistance mechanisms, inefficient targeting of such cells, cytogenetic heterogeneity of the tumor and its location, or to the presence of the BBB, that impairs an easy delivery of the chemotherapeutic drugs to the CNS. Moreover, the diffuse infiltrative growth pattern commonly leads to an incomplete surgical resection, hence the common recurrences. Therefore, the current advances in comprehending the molecular pathways and functional genetic events is crucial to the identification of new targets and the development of novel therapeutic strategies. The dysregulation of genetic events has been identified as a major cause of treatment failure, with influence in several biological processes, which includes proliferation, resistance to apoptosis and autophagy, invasion, metastasis, angiogenesis, and drug resistance (Shea et al., 2016).

Novel drug-based strategies, that include TMZ and other chemotherapeutic drugs, repositioned drugs or new chemical entities, rely on the use of nanotechnology. Nanoparticles, as drug delivery systems, not only protect drugs from intestinal and hepatic metabolism, but may also improve circulation time within the bloodstream, promote a controlled drug release and may simultaneously deliver more than one drug, hence increasing the probabilities of a more successful outcome (Mudshinge et al., 2011). Moreover, surface engineering of nanoparticles with targeting moieties is now a reality, thus directing these nanosystems to specific receptors overexpressed in target tumor cells. Several nanoparticulate systems such as liposomes, solid lipid nanoparticles, polymeric micelles, polymeric nanoparticles, gold nanoparticles, and iron oxide nanoparticles have been investigated as carriers for therapeutic drugs for the treatment of GB. Table 1 presents an extensive overview of the current state of the art in glioblastoma therapy, mostly using surface modification approaches, which include the use of BBB receptors, like Tfr, OX26 or APMP, and overexpressed receptors in glioblastoma cells, such as FA, RGD, EGFR or Angiopep-2 (Kadari et al., 2018, Kuo and Liang, 2011, Wang et al., 2018, Minaei et al., 2019c, Minaei et al., 2019b, Zhong et al., 2014, Ganipineni et al., 2019). These approaches have several advantages over viral vector-based delivery systems, including flexibility in design, thus allowing functionalization with specific target molecules, decreased immune response and generally considered a safer therapeutic approach.

Table 8.1 - Summary of drug-loaded nanoparticle approaches for brain tumor therapy and their characteristics.

Nanosystem			Drug	Main Achievements/outcomes	Ref.
Type	**Core composition**	**Surface modification**			
	HSPC and CHOL liposomes	Tf and PFV	DOX and erlotinib	The cellular uptake of Tf-PFV-liposomes loaded with erlotinib was roughly 69% in U-87 MG cells, higher in comparison to single ligand or unmodified liposomes. The *in vitro* internalization of Tf-liposomes was lower than the corresponding for PFV-liposomes, thereby suggesting a superior uptake through receptor-mediated transcytosis. Overall, Tf-PFV-liposomes presented a synergistic uptake behaviour by adsorptive and Tf receptor mediated transport.	(Lakkadwala and Singh, 2019)
	HSPC and CHOL liposomes	T7 and ^{D}A7R peptides	DOX and vincristine	T7 and ^{D}A7R peptides improved the internalization of liposomes due to their participation in receptor-mediated endocytosis. The delivery of both drugs by T7-^{D}A7R liposomes promoted a marked increase in cytotoxicity (IC_{50} = 3.54 μg/mL). In fact, the synergistic effect of T7 and ^{D}A7R on the modified liposomes strongly impaired the proliferative activity of C6 cells. *In vitro* and i*n vivo* studies revealed that both T7- and T7-^{D}A7R liposomes efficiently crossed the BBB, thus exhibiting a favorable brain targeting capacity. Also, T7-^{D}A7R liposomes significantly increased the median survival time of mice (34 days), 1.7, 1.6, and 1.3-fold higher than that of PBS, free DOX and VCR, and T7-liposomes, respectively.	(Zhang et al., 2017b)
	PC and CHOL liposomes	^{D}CDX and c(RGDyK)	DOX	IC_{50} (free DOX) = 0.5 μM; IC_{50}(unloaded-liposome) = 83.1 μM; IC_{50}(DOX-loaded ^{D}CDX-liposome) = 75.8 μM; IC_{50} (DOX-loaded c(RGDyK)-liposome) = 13.2 μM; IC_{50} (DOX-loaded ^{D}CDX-c(RGDyK)-liposome) = 12.0 μM. The higher cytotoxicity from DOX-loaded c(RGDyK)- ^{D}CDX/c(RGDyK)-liposomes may be related with the increasing in cellular uptake. *In vivo* biodistribution further demonstrated that DOX-loaded ^{D}CDX-c(RGDyK)-liposome resulted in a precise and high glioma retention. The median mice survival time was longer for DOX-loaded ^{D}CDX- and c(RGDyK)-liposomes-treated groups (32.5 and 30.5 days, respectively) compared to saline group.	(Wei et al., 2015)
	PC and CHOL liposomes	c(RGDyK) and pHA	DOX	c(RGDyK)-pHA-PEG-DSPE-liposomes were preferentially internalized by U-87 MG and HUVEC cells via RGD-integrin interaction. Additionally, the inhibitory effect in the proliferation of U-87 MG cells was significantly superior following the structural modification of the surface of the liposomes.	(Belhadj et al., 2017)

				in vivo results show that the mice treated with DOX-loaded c(RGDyK)-pHA-liposomes had a significantly longer median survival time (35 days) than that of those administered with free DOX, unmodified liposomes, c(RGDyK)- and pHA-liposomes (23, 26.5, 28.5 and 30 days, respectively). The half-lives and mean residence time of free and encapsulated DOX indicated that the liposomes effectively increased the circulation time of DOX by impairing its metabolic elimination.	
	GMS and SA SLNs	Angiopep-2	DTX	For U-87 MG cells, IC_{50} (DTX) = 88.06 ± 1.48 ng/mL; IC_{50} (DTX-loaded SLN) = 56.39 ± 1.47 ng/mL; IC_{50} (angiopep-2-conjugated SLN) = 23.42 ± 1.5 ng/mL. For GL261 cells, IC_{50} (DTX) = 122.10 ± 10.60 ng/mL; IC_{50} (DTX-loaded SLN) = 93.31 ± 2.61 ng/mL; IC_{50} (angiopep-2-conjugated SLN) = 50.69 ± 5.57 ng/mL. Cellular uptake of angiopep-2-conjugated SLNs was 1.34-fold in U-87 MG, and 1.25-fold in GL261 cells, after 2 h. From *in vivo* assays, the AUC0-∞ for DTX, DTX-loaded SLNs and angiopep-2-conjugated SLN were 29 ± 1.3, 52 ± 1.1 and 159 ± 1.4 μg/mL/h, respectively, which suggests an increased systemic circulation of angiopep-2-conjugated SLNs in the body. DTX from angiopep-2-conjugated SLN showed a preferential accumulation in glioma induced tumors (4.13 μg/g) than that from DTX-loaded SLN (1.96 μg/g) and DTX (1.23 μg/g).	(Kadari et al., 2018)
	DPPC and CB SLNs	Apr and anti-MTf	DOX	HBMECs and Has viability was found to be higher in the presence of blank SLNs and DOX-loaded SLNs. However, none of the nanosystems showed a significant toxicity in these healthy cells, demonstrating that SLNs could shell the strong cytotoxicity of DOX. The BBB permeability coefficient for PI was the highest for DOX-loaded Apr-anti-MTf-SLNs. On the contrary, the lowest value was observed for unmodified DOX-loaded SLNs. Overall, when compared with DOX-loaded anti-MTf-SLNs, DOX-loaded Apr-anti-MTf-SLNs carried Apr to target LRP expressed on HBMECs.	(Kuo and Lee, 2015b)
	SA and CB CASLNs	EGFR	DOX	The growth inhibitory effect was found to be in the following order: DOX-loaded anti-EGFR–CASLNs with 100% CB > DOX–loaded CASLNs with 100% CB > DOX–loaded CASLNs with 0% CB > DOX–loaded CASLNs with 50% CB > free DOX solution. The interaction between DOX-loaded anti-EGFR–CASLNs and the membrane of HBMECs promoted a cytotoxic effect.	(Kuo and Liang, 2011)
	PVA, CHOL, ST, GCS and Na-BA SLNs	-	PTX	Permeability studies using hCMEC/D3 monolayers indicate a significant increase in the transport of coumarin, when loaded in SLNs. However, after	(Chirio et al., 2014)

				24h, there were no differences in the transport of uncharged or positively charged SLNs. Cell viability studies using NO3 cells indicate that unloaded SLNs do not induced cytotoxicity, whereas PTX-loaded SLNs increased cell death in, at least, an equal magnitude of free PTX. Co-culture experiments using hCMEC/D3 and glioblastoma cells demonstrated that encapsulated PTX had a stronger cytotoxic effect on cancer cells, due to the increased permeation through the cell monolayer.	
	SA and egg phosphatidyl choline SLNs	PEG	Noscapine	IC_{50} (noscapine) = 40.5 μM; IC_{50} (noscapine-loaded SLN) = 27.2 μM IC_{50} (noscapine-loaded PEG-SLN) = 20.8 μM. Noscapine-loaded PEG-SLNs at ~20 μM of noscapine induced cell cycle arrest and apoptosis in U-87 MG cells in a more significant way, when compared to noscapine-loaded SLNs and free noscapine. *In vivo* results indicate an increase in plasma half-life up to ~11-fold and ~5-fold by noscapine-loaded PEG-SLNs and noscapine-loaded SLN. Noscapine-loaded-SLN and noscapine-loaded PEG-SLN deposited significantly higher concentration of 313.1 μg/g and 410.7 μg/g, and eliminated after 12 h with last detection peaks of 1.2 μg/g and 9.6 μg/g, respectively.	(Madan et al., 2013)
	Dynasan 114, palmitic acid and SA SLNs	APMP and FA	Etoposide	APMP and FA significantly promoted the uptake of etoposide-loaded SLNs by HBMECs, thereby weakening the structure of the BBB. Furthermore, the incorporation of both APMP and FA decreased the viability of U-87 MG cells.	(Kuo and Lee, 2015a)
	Compritol 888 ATO, Cremophor ELP and SPC NLCs	RGD and PEG-DSPE	TMZ	TMZ-loaded RGD-NLCs showed higher cytotoxicity than TMZ-loaded NLCs. In fact, TMZ-loaded RGD-NLCs IC_{50} was 2-fold times over TMZ-loaded NLCs and 10-fold higher than a TMZ solution, thus promoting a stronger activity in reducing the viability of these malignant glioma cells. The *in vivo* antitumor therapeutic effect was also assessed, with the most significant tumor regressions being demonstrated in the TMZ-loaded RGD-NLCs group. The use of NLC containing the RGD peptide was shown to be more effective, when compared to a non-RGD nanoparticle, hereby addressing the importance of active targeting strategies in cancer treatment.	(Song et al., 2016)
	Precirol and Capmul MCM NLCs	-	CUR	IC_{50} (CUR-loaded NLCs) = 9.8 ng/mL and IC_{50} (adrenomycin) = 13.6 ng/mL. The highest *in vivo* concentration was observed following an intranasal administration of CUR-loaded NLCs. In the brain, the C_{max} was 86.2 ± 8.2 μg/g at t_{max} of 120 min, whereas for a free drug suspension, the C_{max} was 5.4 ± 2.1 μg/g at t_{max} of 180min.	(Madane and et al., 2016)

Polymeric	PLGA and PVA NPs	OX26 mAb	TMZ	*In vitro* uptake studies using U251 cells indicate a superior internalization of mAB-NPS after 0.5 and 2 h of incubation, when compared to unmodified NPs. The same trend was observed in U-87 MG cells. The use of the monoclonal antibody for the transferrin receptor, overexpressed in both cell lines, proved to promote a significant uptake of the NPs in these cells, suggesting a selective endocytosis mediated by the transferrin receptor. Cytotoxicity studies also showed a significant inhibition by encapsulated TMZ, when compared to free TMZ, in both cell lines.	(Ramalho et al., 2018)
	PLGA NPs	Poloxamer 188	DOX	DOX-loaded NPs exhibited a high antitumor effect against the experimental orthotopic glioblastoma in rats. Not only the median survival time was increased, but also long-term remission was observed in 25–40% animals, whereas the effect of free DOX was only marginal.	(Pereverzeva et al., 2019)
	PLGA NPs	Poloxamer 188	DOX	*In vitro* analysis of DOX-loaded NPs indicates an efficient clathrin-mediated endocytosis by human glioma cells, and a consequent intracellular release of DOX, following the formation of lysosomes.	(Malinovskaya et al., 2017)
	PLGA NPs	CS	DOX	IC_{50} (DOX-loaded CS-PLGA-NP) = 340.3 nM; IC_{50} (DOX) = 391.2 nM. *In vitro* viability studies indicate a stronger cytotoxic effect of encapsulated DOX over U251 cells. Uptake studies conducted with DOX-loaded CS-PLGA-NPs suggest an active targeting approach to CD44 receptors. support this conclusion. The intravenous injection of this nanosystem improved the pharmacokinetic profile of DOX and, consequently, promoted an strong tumour inhibition in U251-tumor bearing animals, while decreasing the intrinsic cardiotoxicity of DOX. Nonetheless, at 0.5 h following the administration, the NPs were found to accumulate in other tissues, which include the liver, heart, spleen, lung and kidney.	(Liu et al., 2019)
	PAA NPs	PEG	CIS	*In vivo* studies demonstrated that PAA-NPs provided a sustained release of CIS in significant concentrations, thus effectively promoting tumor cell death, while not causing any toxicity-related losses. In addition, the median overall survival of orthotopic glioma-bearing rats was improved by the administration of the brain penetrating loaded NPs (80% long-term survivors vs. 40 days for CIS delivered in conventional unPEGylated NPs-treated group vs. 12 days for CIS alone-treated group vs. 28 days for PBS-treated group).	(Zhang et al., 2017a)
	PAA NPs	PEG	CIS	*In vitro* cytotoxicity assays indicate that a CIS solution displayed a stronger cytotoxicity over F98 glioma cells, than the CIS loaded NPs, which was ascribed to the encapsulation and sustained intracellular release of the drug.	(Timbie et al., 2017)

				Magnetic resonance image-guided focused ultrasound-mediated delivery of CIS NPs showed to be a potential treatment strategy to prevent recurrences, as efficacy studies conducted in tumor bearing animal models showed a significant increase in median survival (31.5 days vs. 27.3 days in control group)	
	PLGA NPs	PEG	Gefitinib and GSK461364A	*In vitro* studies indicate that the use of GSK461364A increased cell death and apoptosis due to a competition with the ATP binding site of PLK-1. In addition, the combination of free drugs within a single nanoparticle promoted a higher cytotoxicity, when compared to free drugs or gefitinib loaded- and GSK461364A loaded-NPs.	(Velpurisiva and Rai, 2019)
	CH NPs	PEG and CTX	TMZ	A 2-fold increase in the intensity of fluorescence was identified in cells treated with TMZ-loaded CTX-NPs, in comparison to TMZ-loaded NPs, thus suggesting and active targeting strategy and uptake promoted by CTX . Following the intravenous administration of TMZ-loaded CTX-NPs, it was demonstrated that the NPs effectively reach avascular areas of the brain. The permeability characteristics of the nanosystem were ascribed to the small size associated to different BBB permeation strategies, via surface tailoring engineering.	(Fang et al., 2016)
	PLGA NPs	RGD	SPIO/PTX	IC_{50} (PTX) = 1ng/mL; IC_{50} (PTX-loaded NPs) = 0.9 ng/mL; IC_{50} (SPIO/PTX-loaded NPs) = 0.8 ng/mL; IC_{50} (SPIO/PTX-loaded RGD-NPs) = 0.9 ng/mL. U-87 MG cells and HUVECs showed a superior internalization of RGD-NPs, once again confirming the use of RGD as an active targeting strategy due to its interaction with αvβ3 membrane receptors. Overall, *in vivo* studies demonstrated a significant reduction in tumor volume in all the treated groups, particularly, in SPIO/PTX-loaded RGD-NPs treated group. In parallel, median survival time was prolonged in all groups, when compared to control.	(Ganipineni et al., 2019)
Metallic	SPIONs	PEG, PBA and FA	TMZ	Following 24 and 48 h of treatment, the IC_{50} values of TMZ-loaded PEG-PBAPEG-FA-SPIONs in C6 cells were, respectively, 2 and 2.37 times lower than those of TMZ-loaded PEG-PBAPEG-SPIONs. The introduction of FA proved to be an effective strategy to increase C6 cellular association and the anti-glioma activity.	(Minaei et al., 2019a)
	Au NPs	CH	Metformin	Metformin-loaded CH-Au NPs have an increased cell internalization and affect the viability of GBM cells compared to control and free metformin. Also, their antitumor effect is not statistically different when compared to	(Aldea et al., 2018)

				unloaded-CH-Au NPs, although a slight tendency to a better response may be observed.	
	Au NPs	cRGD-PEG-b-PCL-LA	DOX	IC_{50} (non-targeting hybrid-DOX NPs) = 50.8 μg DOX/mL; IC_{50} (cRGD-hybrid-DOX NPs + laser) = 6.2 μg DOX/mL; IC_{50} (free DOX) = 5.7 μg/mL. The cells treated with AuNPs without NIR irradiation showed little toxicity, suggesting a high biocompatibility. However, combining radiation therapy, lower cell viability was achieved (58.2% and 30.0% for 5.0 and 10 μg DOX equiv./mL, respectively). Overall, cRGD-hybrid-DOX NPs and hybrid-DOX NPs proved to have a significantly prolonged circulation, when compared to free DOX. Moreover, a higher tumor retention and accumulation of cRGD-hybrid-DOX NPs was verified.	(Zhong et al., 2014)
	Au NPs	PKKKRKV peptide	CIS	CIS-Au NPs showed to be effectively internalized, when compared to free CIS. CIS-PKKKRKV-AuNPs show the highest uptake and superior inhibition of GBM cell growth compared to free CIS. The superior efficacy is most likely due to the combination of AuNPs and the PKKKRKV peptide. *In vivo* studies in healthy mice showed that magnetic resonance imaging guided focused ultrasound increased the BBB permeability and the delivery of CIS and CIS-PKKKRKV-AuNPs to healthy brain tissue.	(Coluccia et al., 2018)

Key: SQ – squalene; PEG – polyethylene glycol; HSPC - hydrogenated soy phosphatidylcholine; CHOL – cholesterol; Tf - transferrin; PFV – cell penetrating peptide PFVYLI; DOX – doxorubicin; BBB – blood-brain-barrier; PC – L-α-phosphatidylcholine; cRGD - cyclic-arginine-glycine-aspartic acid peptide; pHA - p-hydroxybenzoic acid; HUVECs - human umbilical vein endothelial cells; GMS- glyceryl monostearate; SA - stearic acid; SLN – solid lipid nanoparticle; DTX – docetaxel; DPPC - 1,2-dipalmitoyl-sn-glycero-3-phosphocholine; CB – cacao butter; Apr – Aprotinin; anti-MTf - melanotransferrin antibody; HBMECs -human brain microvascular endothelial cells; Has – human astrocytes; CASLN – catanionic solid lipid nanoparticle; EGFR - anti-epithelial growth factor receptor; PVA - poly(vinyl alcohol) ; ST - stearylamine; GCS - glycol chitosan; hNa-BA - sodium behenate; PTX – paclitaxel; APMP - p-aminophenyl-α-D-manno-pyranoside; FA – folic acid; SPC - soybean phosphatidylcholine; NLC – nanostructured lipid carrier; PEG-DSPE - polyethylene glycol-b-distearoylphosphatidylethanolamine; TMZ – temozolomide; CUR – curcumin; PLGA - poly(lactic-co-glycolic acid); NP – nanoparticle; mAb – monoclonal antibody; CS - chondroitin sulphate; PAA - poly(aspartic acid); CIS – cisplatin; CH – chitosan; CTX – chlorotoxin; SPION - superparamagnetic iron oxide nanoparticle; PBA - poly (butylene adipate); MNP - magnetite nanoparticle; Au – gold; GBM – glioblastoma; PCL - poly(ε-caprolactone); LA – lipoic acid.

4.2 miRNA delivery nanosystems

Despite the promising therapeutic application of miRNAs for the treatment of several forms of cancer, including GBM, their *in vivo* delivery remains the most challenging obstacle/condition to achieve therapeutic success. Several reasons are related with the inefficient delivery of naked miRNAs, including low stability due to their degradation in the systemic circulation and their intrinsic negative charge that hinders their transport over the plasma membrane and consequent binding to their target in the cytoplasm, as well as immune responses and their rapid renal clearance. Furthermore, the BBB and BBTB (brain-blood-tumor barrier) represents the major physiological barriers that become miRNA delivery particularly challenging for brain tumors since the poor dissemination throughout extravascular areas of tumor parenchyma. (Nana-Sinkam and Croce, 2014, Allhenn et al., 2012) Consequently, effective carriers to increase stability and to delivery miRNA molecules into the tumor cells need to be developed. Nanoparticles gather interesting physicochemical properties which have boosted their extensive investigation as new approaches able to provide suitable platforms for gene and drug delivery to the brain (Díaz and Vivas-Mejia, 2013). Their biocompatibility and biodegradable properties, the ability to protect miRNAs against nuclease degradation in plasma and stabilize them, combined with the possible surface functionalization with targeting ligands (peptides or antibodies) to enhance the selectivity to tumor cells, makes nanoparticles attractive vehicles for miRNA delivery to GBM cells.

Several nanosystems have been developed and described in the literature as miRNA-based therapeutics for treatment of brain tumors. The most relevant studies for the purpose are summarized in Table 8.2. Among them, there are lipid, polymeric, metallic and other types of nanoparticles. Indeed, cationic lipid-based nanoparticles have been mainly explored for allowing the attachment of negatively charged miRNAs,

enabling a better interaction with cell membranes. Cationic liposomes have been developed as promising targeted delivery systems for GBM treatment in preclinical studies (Costa et al., 2013, Costa et al., 2015). AntimiR-21-encapsulated cationic liposomes, designed as stable nucleic acid lipid particles (SNALPs), were functionalized with chrolotoxin (CTX), a glioma-specific peptide. The attachment of this targeting ligand enhanced the particle internalization into GBM cells and decrease tumor cell proliferation (Costa et al., 2013). However, some drawbacks come from the cationic property of these lipid-based delivery vehicles, specially related with their toxicity due to the contact with negatively-charged components (such as serum proteins and enzymes, opsonins), that may promote hemolysis and the stimulation of the complement system, thereby causing a quick clearance (Senior et al., 1991, Kedmi et al., 2010). Also, an unsatisfactory non-sustained delivery of nucleic acid molecules at the tumor site is frequently identified when no targeting moieties were coupled (Pecot et al., 2011, Safinya et al., 2014).

Other nanocarriers have been developed to overcome such limitations. Polymeric nanosystems, such as poly(lactic-co-glycolic acid) (PLGA)-based nanoparticles, provide some advantages upon the most of other delivery vehicles, including biocompatibility, biodegradability, sustained delivery and long-term stability of encapsulated molecules, low cost and Food and Drug Administration (FDA) approval for their use (Ananta et al., 2016, Ananta et al., 2015, Malhotra et al., 2018b). In the same way, polycationic dendrimers, that present on their exterior several amines, have provided a stable binding to nucleic acids and a satisfactory transfection efficiency (Janaszewska et al., 2012). Poly(amidoamine) (PAMAM) dendrimers, the most important dendrimer type, have been used as miRNA delivery systems applied to GBM treatment with promising preclinical outcomes (Qian et al., 2013, Liu et al., 2013). Thus, miR-7/FA-PAMAM polyplexes were

developed and *in vitro* treatment of U251 glioma cells with these miRNA complexed formulations exhibited higher transfection efficiency compared to miR-7/liposomes group. Moreover, *in vivo* studies demonstrated that FA-targeted PAMAM vector efficiently potentiated the reduction of tumor size and prolonged mice median survival time compared to miR-7/liposomes and control groups. However, this approach displays the same toxicity disadvantaged of cationic-based nanoparticles (Aillon et al., 2009).

On the other hand, different metallic nanoparticles revealed the ability for miRNA delivery into the brain tumor cells, as for example gold (Au) nanoparticles or superparamagnetic iron oxide nanoparticles (SPIONs) and zinc-doped iron oxide nanoparticles ($ZnFe_2O_4$) as magneto-based transfection gene carriers (Kouri et al., 2015, Lo et al., 2015, Yin et al., 2014, Benisvy-Aharonovich et al., 2014). Chondroitin sulfate-polyethylenimine (CS-PEI) copolymer-coated poly(acrylic acid)-bound iron oxide (PAAIO) nanoparticle (CPIO) complexed to miR-128 expressing pDNA demonstrated an enhanced miR-128 *in vitro* transfection and downregulation of target genes and improved tumor accumulation in U-87 MG subcutaneous tumor-bearing mice, under the concomitant application of an external magnetic field. In this study, the CS moiety function as a CD44-targeting approach for GBM therapy, enhancing the nanosystem propensity to cross the BBB, and it was also added to attenuate PEI toxicity (Lo et al., 2015).

Additionally, other carriers for antitumor miRNA delivery have been purposed, including mesenchymal stem cells (MSCs)-derived exosomes, as encouraging strategies (Munoz et al., 2013, Katakowski et al., 2013, Lee et al., 2013). For instance, marrow MSCs were transfected with a miR-146b expression plasmid and the isolated exosomes were intratumorally injected in a rat model of brain cancer, significantly reducing tumor growth 5 days after treatment (Katakowski et al., 2013).

These *in vitro* and *in vivo* studies present positive results against glioblastoma, and are part of a preclinical foundation for future translation to the clinic of miRNA-based targeting therapies.

Table 8.2 - Summary of miRNA-based nanosystems approaches for brain tumor therapy and their characteristics.

Nanosystem			Gene therapy		Other therapy	Main Achievements/outcomes	Ref.
Type	**Core composition**	**Surface modification**	**miRNA**	**Target gene**			
Lipid	Labrafac® WL 1349, Solutol® HS15 and Lipoid S75-3 LNCs	L1 lipopeptide	AntimiR-21 or antimiR-210 LNAs	-	Radiation	The treatment with LNA21/L1-LNC complexes specifically decreased the miR21 levels in U-87 MG cells (45% inhibition) and the cell viability was reduced (60% decrease), with an increased effect when combined with irradiation (80% decrease). However, no effect of any investigated LNA-LNC complex on miR-210 expression was observed and LNA210-L1-NLC complexes displayed a minor effect on cell survival.	(Griveau et al., 2013)
	%molar ratio to total lipid of NPs: 58% ATX, 7% DSPC, 33.5% CHOL and 1.5% DMG-mPEG2000 (LUNAR-301)	-	miR-124	STAT3	-	LUNAR-301 enhanced antitumor effect with a median survival time exceeding 70 days after a single treatment regimen. In addition, the therapy showed immunomodulatory properties, induction of immunological memory and no treatment-related toxicity.	(Yaghi et al., 2017)
	% molar ratio to total lipid of liposomes: 50% DOTAP, 50% CHOL	-	miR-7	EGFR	-	The treatment with miR-7/cationic liposome complex provided significant suppression on primary tumor growth (40% in tumor volume and weight) and metastatic nodules (60% suppression ratio in lung metastases and 80% suppression ratio in lymph node metastases, respectively) before systemic delivery to U-87 MG and U251 subcutaneous tumor xenografts. Furthermore, the miR-7-mediated downregulation of EGFR was identified.	(Wang et al., 2013)
	Cationic lipid, CHOL, phospholipid, and pegylated lipid	-	AntimiR-10b	MBNL1-3, SART3 and RSRC1	-	The treatment regarding to continuous osmotic delivery of lipid NPs formulated with anti-miR-10b, during two weeks, significantly reduced the intracranial human GSC-derived tumor xenografts growth, when compared to control groups. In addition, it decreased tumor cell proliferation and increased apoptosis, despite not presenting noteworthy differences in cell migration and invasion in both treatment and control.	(Teplyuk et al., 2016)
Polymeric	PBAE NPs	-	miR-148a and/or miR-296-5p	Dnmt1 and Hmga1	IR	Intratumoral delivery of miR-148a-loaded polymeric NPs plus IR cooperatively enhanced the inhibition of intracranial iCSCs tumor xenograft growth. Also, the co-delivery of both miR-148a and miR-296-5p into polymeric NPs more significantly decreased the intracranial GBM1A glioma xenografts burden than either miRNA delivered alone, as well as enabled a prolonged median survival time.	(Lopez-Bertoni et al., 2018)

	Polyamide NPs	cRGD peptide	miR-7	Ki-67 and OGT	-	cRGD-coupled polyamide NPs encapsulating miR-7 significantly inhibited tumor growth and angiogenesis in U-87 MG tumor bearing mice. Moreover, an *in vitro* and *in vivo* downregulation of Ki-67 and OGT target genes was observed.	(Babae et al., 2014)
	dPG-NH_2 NPs	-	miR-34a	CDK6, Notch1, BCL-2 and C-MET	-	The treatment with the miR-34a/dPG-NH_2 polyplex in U-87 MG, A172 and T98 human cell lines promoted higher expression of miR-34a, while downregulating the expression of CDK6, Notch1, Bcl-2 and C-MET. Also, its local administration in subcutaneously inoculated U-87 MG tumor inhibited tumor growth and prolonged median survival time (55 days vs. 40 days for negative control miR-treated group vs. 20 days for PBS-treated group).	(Ofek et al., 2016)
	PAMAM dendrimer	FA	miR-7	EGFR, PI3K and AKT2	-	miR-7/FA-PAMAM polyplexes induced lower levels of EGFR, PI3K and AKT2, compared with control and nonsense groups, and prompted higher transfection efficiency in U251 glioma cells compared to miR-7/liposomes group (87.6±7.8% vs. 51.4±6.9%, respectively). Moreover, the tumor size was reduced and the median survival time was prolonged in miR-7/FA-PAMAM group (23.5±2.4 days vs. 19.4±2.1 days for miR-7/liposomes group vs. 6.4±2.2 days for control group).	(Liu et al., 2013)
	Disulfide-modified polyglycerol NGs	Secondary amine group	miR-34a	c-MET, CDK6, Bcl-2 and Notch-1	-	The treatment with miR-34a/NG polyplexes containing different secondary amine-bearing moieties attached to polyglycerol over U-87 MG cells significantly increased miR-34a expression levels after transfection, but miR-34a/NG3 polyplex prompted a more efficient downregulation of its target genes. Furthermore, the intratumoral administration of this polyplex to U-87 MG tumor-bearing mice showed a notable antitumor activity compared to miR-34a/NG4 and significantly reduced tumor volume (379 ± 175 mm^3 vs. 883 ± 580 mm^3 for negative control miRNA group).	(Shatsberg et al., 2016)
Metallic	Au NPs	mPEG-SH	miR-182	Bcl2L12, c-MET and HIF2A	-	In vitro studies demonstrated that miR-182/SNAs complexes effectively penetrated in U-87 MG cells and patient-derived GICs (>90%) and significantly downregulated miR-182 target genes. Also, in vivo studies revealed that their systemic administration in glioma tumor xenografts reduced tumor burden and	(Kouri et al., 2015)

						increased survival time (U-87 MG, 55 days vs. 42 days for control group; GIC-20, 51 days vs. 43 days for control group), with no significant adverse side effects.	
	PAAIOs	CS-PEI CPs	miR-128 expressing plasmid (pDNA)	pAKT, AKT and Bax	-	miR-128 expressing pDNA/CPIO magnetoplexes enhanced the miR-128 transfection into U-87 MG cells, when in the incidence of an external magnetic field (~1.5-fold higher vs no magnetic field; ~2.6 fold higher vs control group) and the suppression of miR-128 targets expression. Furthermore, the intravenous administration in U-87 MG-derived subcutaneous tumor xenografts exposed to the magnet field demonstrated an higher retention at the tumor location.	(Lo et al., 2015)
	$ZnFe_2O_4$ MNPs	DMSA and PEI	let-7a	KRAS, NRAS, c-MYC and IGF1R, PI3K, caspase-3 and HSPs	Magnetic hyperthermia	The MNP-PEI/miR/PEI complex combined the therapeutic effects of let-7a delivery to U87-EGFRvIII cells and magnetic hyperthermia reduced viability (34% vs. 69.8% or 63.14% for let-7a delivery or magnetic hyperthermia alone, respectively). Moreover, the expression of RAS, c-MYC, IGFR1, PI3K, HSPs target genes was significantly downregulated whereas the caspase-3-mediated apoptosis levels were significantly increased, considering the combined therapy.	(Yin et al., 2014)
	Au NPs	-	premiR-145 (pDNA)	CTGF	-	premiR-145 expressing pDNA/GFP/Au NP complexes demonstrated a high uptake efficiency by the A172 glioma cells. Also, the increase in the miR-145 expression and GFP gene and the decrease of CTGF target gene expression in treated cells were observed, compared to the control untreated cells.	(Benisvy-Aharonovich et al., 2014)
Others	3WJ of pRNA RNPs	FA	AntimiR-21 LNA	PTEN and PDCD4		*In vitro* studies demonstrated that FA-3WJ-LNA-miR21 RNPs treatment significantly reduced U-87 EGFRvIII cell viability (3-fold higher compared to negative control RNP). Also, an increased apoptotic cell death in U-87 EGFRvIII cells was observed (49% vs. 6.3% for negative control RNP	(Lee et al., 2017)

						group). *In vivo* studies demonstrated that the systemic administration of FA-3WJ-LNA-miR21 RNPs decreased the tumor growth rate and enhanced survival time (23 days vs. 19 days for negative control RNP group) in GBM30 tumor xenografts. Moreover, a 2-fold reduction of miR-21 expression levels was confirmed, as well as a 4-fold and 2-fold increase in PTEN and PDCD4 levels, respectively, compared to negative control RNP-treat mice.	
	HSA NPs	cmHsp70.1 mAb	Survivin miRNA expressing plasmid	membrane Hsp70 and caspase-3/7	IR	Survivin miRNA plasmid-loaded and cmHsp70.1 mAb-coupled HSA NPs increased cellular uptake and decreased survivin expression in U-87 MG and LN229 cell lines, in comparison to non-target, non-plasmid carrying and scrambled miRNA carrying NP controls. Thus, this nanosystem combined with radiation therapy promoted a higher decrease of clonogenic cell survival and increase of capase-3/7 activity.	(Gaca et al., 2013)
	Marrow-derived MSCs exosomes	-	miR-146b expressing plasmid	EGFR and NF-κB	-	*In vitro* studies revealed that the treatment of 9L gliosarcoma cells with miR-146b-MSC exosomes significantly reduced cells growth compared to normal MSC exosome-treated group, whereas the healthy astrocytes growth was not considerably modified. Further, EGFR and NF-κB expression levels were decreased. *In vivo* investigations demonstrated that intratumoral injection of miR-146b-MSC exosomes also reduced tumor volume in 9L gliosarcoma xenografts, compared to control groups.	(Katakowski et al., 2013)
	Bone marrow, adipose tissue, placenta and umbilical cord- derived MSCs exosomes	-	miR-124 and miR-145	SCP-1 and Sox2	-	miR-124 and miR-145 displayed low expression levels in U-87 MG and A172 glioma cells, in the GSCs and in the different MSCs, while they were significantly expressed in NSCs and NHAs. MSCs efficiently delivered miR-124 and miR-145 mimics into the adjacent cultured cells. The same results were observed by the ipsilateral injection of bone marrow-derived MSCs transfected with miR-124 in U-87 MG glioma xenografts. Also, the decrease of SCP-1 and Sox2 target genes expression was detected, as well as the decrease of migration of glioma cells and the self-renewal of GSCs.	(Lee et al., 2013)

Key: LNC – lipid nanocapsule; L1 - papillomavirus-derived peptide; miR – microRNA; LNA - locked nucleic acid; DODAP - 1,2-dioleoyl-3-dimethylammonium-propane; NP – nanoparticle; ATX – proprietary ionizable amino lipids; DSPC - 1,2-dioctadecanoyl-sn-glycero-3-phosphocholine; CHOL – cholesterol; DMG-mPEG2000 - 11,2-dimyristoyl-rac-glycero-3-methoxy[(polyethylene glycol)2000]; DOTAP - 1,2-dioleoyl-3-(trimethylammonium) propane; GSC – glioma stem cell cell; PBAE - poly(β-amino ester) ; IR – ionizing irradiation; iCSC – induced cancer stem cell; cRGD - cyclic arginine-glycine-aspartic acid; dPG-NH_2 - dendritic polyglycerolamine; GBM – glioblastoma; PAMAM - poly(amido amine); FA – folic acid; NG – nanogel; Au – gold; mPEG-SH – methoxy(polyethylene glycol)-thiol; SNAs - spherical nucleic acids; GIC - glioma-initiating cell; PAAIO - poly(acrylic acid)-bound iron oxide nanoparticle; CS-PEI - chondroitin sulfate-polyethylenimine; CP – copolymer; CPIO - CP-coated PAAIO; pDNA – plasmid DNA; MNPs – magnetic nanoparticles; DMSA - 2, 3-dimercaptosuccinic acid; GFP - green

fluorescence protein; 3WJ - three-way-junction; pRNA – packaging RNA; RNP – RNA nanoparticle; HAS – human serum albumin; Hsp-70 - heat shock protein 70; mAb – monoclonal antibody; MSC – mesenchymal stem cell; NSCs - neural stem cells; NHA - normal human astrocytes.

4.3 Drug and miRNA combined therapy and co-delivery nanosystems

To achieve superior efficacy, it is common in clinical practice to combine more than one anticancer drug, usually with different pharmacological mechanisms and secondary effects, thereby reducing individual doses and achieving better results with minimized adverse effects in normal organs. As mentioned before, nanoparticles have been extensively explored as carriers for miRNAs, thereby protecting them against nuclease degradation and vectorizing them for target sites, while being biocompatible and biodegradable. Combination therapies with encapsulated miRNAs and free drugs have been explored over the last years for glioblastoma, presenting promising results. For instance, the systemic administration of CTX- functionalized and antimiR-21-encapsulated SNALPs in glioblastoma intracranial tumor-bearing mice demonstrated a preferential accumulation within brain tumor, reduction of tumor miR-21 expression levels and an increase of miR-21 target genes levels. The combination of SNALPs with sunitinib, a tyrosine kinase receptor inhibitor, following oral administration, enhanced the cytotoxic effect of this drug, as reflected by the decrease of tumor cell proliferation and tumor size, consequently improving the mice median survival times (Costa et al., 2015).

Combination therapy as a strategy to reduce TMZ resistance mechanisms has also been explored. For this regard, antimiR-10b and antimiR-21 co-loaded PLGA nanoparticles effectively silenced miR-21 and miR-10b expression and increased miRNA target gene levels (HOXD10, PDCD4 and PTEN) in glioma cells, reducing cellular proliferation and invasion and improving cell cycle arrest to G2/M phase, when compared to the conventional chemotherapeutic glioblastoma therapy (TMZ). Moreover, the proposed nanosystem displayed an interesting role in sensitization of glioma cells to TMZ, improving therapy efficacy (Figure 8.3) (Ananta et al., 2016). AntimiR-21/poly(amidoamine) (PAMAM) polyplexes also showed promising results in

decreasing the expression of miR-21 when combined with TMZ treatment and, consequently, enhancing the chemosensitivity of U-87 MG cells to TMZ treatment (Qian et al., 2013).

Despite the interesting results of combination therapies with drugs and miRNAs, off target effects and incomplete efficacy due to the non-encapsulation of the anticancer drugs limit their translation to clinical trials. Taking into consideration the considerable advantages of nanoparticles, that include an extreme versatility, it is possible to design and produce delivery systems that combine, within a single nanostructure, drugs and miRNAs. Their co-delivery usually combines synergistic mechanisms aiming at one or several cellular pathways. However, one key challenge for co-encapsulation lies in the distinct physicochemical properties of small drugs and RNAs, specifically, size, charge, hydrophobicity and stability (Kim et al., 2015). In addition, thermal degradation of miRNAs during the production of the nanoparticles should be avoided. Although more challenging, this approach may efficiently deliver, to cancer cells, both the drug and the miRNA, thus improving the efficacy of the treatment.

Over the last years, various delivery systems, either following separate administrations or co-loaded with both drugs and miRNAs, have been developed against glioblastoma. Considerable advances have been achieved *in vitro* and *in vivo*, and are explored in detail in Table 8.3.

INSERT FIGURE 8.3 HERE.

Figure 8.3 - Co-delivery of antimiR-10b and antimiR-21 and PLGA nanoparticles induced chemosensitivity glioma cells to TMZ therapy. Retrieved from ref. (Ananta et al., 2016) with permission from the American Chemical Society.

Table 8.3 - Summary of nanosystems based on a dual approach, drug and miRNA combined therapy and co-delivery, for brain tum

Nanosystem			Gene therapy		Drug	Main Ach
Type	**Core composition**	**Surface modification**	**miRNA**	**Target gene**		
Drug and miRNA Combined Delivery						
Lipid	% molar ratio to total lipid of liposomes: 25% DODAP, 45% CHOL, 22% DSPC and 8% C16-mPEG2000-ceramide	CTX peptide	antimiR-21	PTEN, PDCD4 and caspase-3/7	Sunitinib	The attachment of CTX onto enhanced particle cellular intern cells, in comparison to unt internalization in non-tumor cel loaded CTX-SNALPs significan cells, increased the tumor suppre activity of caspase-3/7, hence en considering the combined therap
	% molar ratio to total lipid of liposomes: 25% DODAP, 49% CHOL, 22% DSPC and 4% C16-mPEG2000-ceramide	CTX peptide	antimiR-21	RhoB	Sunitinib	CTX-coupled SNALPs enca preferential accumulation within miR-21 tumor expression levels levels of its direct target RhoB. NPs combined with orally-admin antitumor effect of sunitinib, dec 98.2 ± 43.8 mm^3 of untreated), a days vs. 21 days of untreated).
Polymeric	PLGA NPs	-	antimiR-21	PTEN and caspase-3	TMZ	The antimiR-21-loaded PLGA N endogenous miR-21 levels in U antimiR-21 transfection signifi treatment compared to TMZ alo was no significant cell death pro The expression of PTEN and ca increased (in PTEN expression, TMZ vs. 30% for TMZ alone; PLGA NPs vs. no significant inc
	PLGA NPs	-	antimiR-21 and/or antimiR-10b	PTEN, PDCD4 and HOXD10	TMZ	The antimiR-21 and antimiR- reduced cell viability (24% vs. 1 arrest at G2/M phase (2.7-fold hi treatment in U-87 MG cells, imp

						drug. A higher expression of the downstream targets of miR-21 and miR-10b, particularly, PDCD4, PTEN and HOXD10 was also observed.	
	PLGA NPs	cRGD peptide and PEG	antimiR-21 and antimiR-10b	PTEN, PDCD4, HOXD10, p53 and caspase-3	TMZ	cRGD-targeted PEG-PLGA NPs co-encapsulating antimiR-21 and antimiR-10b demonstrated a 3-fold increased cellular uptake in U-87 MG and Ln229 GBM cells at 24h after treatment and a higher chemosensitivity of these cells to lower TMZ concentrations, compared to unmodified NPs. Also, the expression of downstream targets of miR-21 and miR-10b was substantially higher upon the delivery of targeted and unmodified NPs. Curiously, unmodified PLGA NPs containing both antimiRs plus TMZ co-treatment showed a more pronounced reduction of volume in subcutaneous U-87 MG tumor xenografts, in comparison to the cRGD-targeted PEG-PLGA NPs.	(Malhotra et al., 2018a)
	PU-PEI NPs	-	miR-145	Oct4, Sox2, Nanog, Klf4 and Bmi-1	TMZ	*In vitro* studies demonstrated that the miR-145/PU-PEI polyplex delivery enhanced the sensitivity of patient-derived GBM-CD133+ cells to TMZ and irradiation treatments and reduced the expression of drug-resistance genes, increasing the efficacy of the standard therapy. Furthermore, *in vivo* studies revealed that the intracranially delivery of this polyplex in combination with radio and chemotherapy achieved the maximal suppression of tumor progression, improved the survival rate and inhibited the expression of Bmi-1 gene and Oct4, Sox2, Nanog and Klf4 stemness factors (CSC-like properties and stemness signatures) in the GBM-CD133+ tumor xenografts.	(Yang et al., 2012)
	PDL, DES and MDEA based NPs	ApoE	antimiR-21	PTEN	TMZ	NPs superficially modified with ApoE displayed an enhanced and efficient internalization, thus leading to miR-21 suppression and consequent PTEN upregulation and cell apoptosis in GBM cells. *In vivo* results suggest the combined use of miRNAs and TMZ, considering the improvement in survival following the downregulation of miR-21 combined with the anticancer drug. Although the administration alone of antimir-21 NPs led to a slightly increase in overall survival, promising results were achieved only when TMZ was added to the therapeutic regime (26 days for control vs. 28 days for antimir-21 NPs vs 41 days for TMZ vs 50 days for antimir-21 NPs co-administered with TMZ).	(Seo et al., 2019)
	PLA NPs	Sodium periodate modified hyperbranched polyglycerol	antimiR-21	PTEN	TMZ	The co-treatment of NPs and TMZ promoted the highest increase in cell death via apoptosis, which was ascribed to the increase internalization of the NPs, when compared to the other formulations studied. *In vivo* intratumoral administration to tumor bearing rats of antimiR-21 NPs, followed by the	(Seo et al., 2019)

						administration of TMZ in the next day strongly increased the overall survival time (25% of survival over 80 days vs. 0% survival in day 26 for control).	
	PAMAM dendrimer	-	antimiR-21	STAT3	TMZ	AntimiR-21/PAMAM polyplexes achieved the maximal uptake efficiency in U-87 MG cells upon combination with TMZ (58.2% vs. 0.5% for antimiR-21 alone). The lowest miR-21 expression was observed when the tumor cells were subjected to the co-treatment with these complexes and TMZ (9.4% vs. 46.4% for TMZ alone vs. 20.1% for miR-21 alone), enhancing the chemosensitivity of these cells to TMZ treatment (the IC_{50} of TMZ was decreased from 29 μM to7.5 μM).	(Qian et al., 2013)
Others	Bone marrow-derived MSCs exosomes	-	antimiR-9	MDR1	TMZ	The delivery of antimiR-9 from MSCs exosomes to U-87 MG and T98G cells combined with TMZ treatment decreased cell viability and miR-9 expression levels (50% decrease), compared to TMZ alone. Moreover, the sensitization of these cells to TMZ and the decrease of MDR1 expression were shown, as well as the increased of caspase-3/7 activity.	(Munoz et al., 2013)
Drug and miRNA co-delivery							
Lipid	glyceryl tristearate, Tween® 80 and DDAB SLNs	-	antimiR-21	-	Pemetrexed	Free pemetrexed could not remarkably inhibit cell growth, as there were no significant cell death with high concentrations at 24 or 48 h. Yet, at 72 h, free pemetrexed reduced cell viability. SLNs co-delivering anti-miR-21 and pemetrexed showed a higher cytotoxicity when compared with the free solution. Cellular uptake studies demonstrated that SLNs entered U-87 MG cells much more efficiently (65 % vs. 6% for free pemetrexed).	(Berrin and Asuman, 2017)
Polymeric	deoxycholic acid-conjugated polyethylenimine micelles	-	antimiR-21	PTEN, PDC4	CUR	miR-21 level decreased following transfection with both loaded and unloaded micelles. Co-delivery of CUR and antimir-21 showed to be more efficient, with higher anti-tumor effects, than those from the single delivery of scrambled-antimiR-21/CUR micelles or antimir-21 micelles, thus suggesting a synergistic activity between CUR and the miRNA. In vivo administration to tumor bearing mice supports these findings, as scrambled-antimiR-21/CUR micelles and unloaded antimir-21 micelles presented a weaker antitumor effect that that of the miRNA and CUR co-loaded complex.	(Piao et al., 2018)
	CH NPs	FA	miR-218	-	TMZ	The cytotoxicity of TMZ (IC_{50} = 6.2 μM) was strongly potentiated by the cotreatment with miR-218 mimics, as a concentration of TMZ of 1μM doubled the reduction in cell viability. Cellular uptake of the NPs was also increased due to the surface engineering using folic acid. *In vivo* antitumor	(Fan et al., 2015)

						efficacy studies show that the size of the tumor in the treatment group was 20 times smaller, when compared to the saline control group. Furthermore, no significant decreases in body weight were detected in all tumor-bearing animals. However, the same was not observable in the free drug administration group. The optimized NP formulation showed a preferential targeting and accumulation in tumor site, with nearly 95% of the particles reaching the tumor site.	
	Ooleoyl chloride and PEG micelles	-	miR-145	Oct4A, Ocy4B1, and Sox2	CUR	Cell viability studies show that free curcumin or unloaded NPs did not show cytotoxicity after 72h, with loaded NPs being cytotoxic to U-87 MG cells at 24 h (20 μM) and 72 h (10 μM). Cell cycle distribution studies indicate that the NPs effectively target cell division and proliferation pathways, as the cell population in the SubG1 phase significantly increased, while promoting G2/M phase arrest. Caspase activity also increased in U-87 MG cells, with miR-145 expression increasing up to 34%, in comparison with nontreated control cells. The expression of Oct4A, Oct4B1, and Sox2 reduced in an miR-145 concentration dependent manner.	(Mirgani et al., 2014)
Metallic	MSNPs	R8-PNA	antimR221	-	TMZ	At 24h, TMZ-MSNPs and antimR221-MSNPs promoted a modest cytotoxicity over C6 glioma cells. However, when co-delivered in the same carrier system, antimR221-TMZ-MSNPs, there was a significant decrease in cell viability (down to 30%). The same behaviour was visible after 48 h. Thus, the co-delivery of TMZ and anti-miR221 in NPs displayed a synergistic effect in these cells. While the administration of the free drug did not promote high levels of apoptosis, anti-miR221-TMZ-MSNPs induced a significant death in TMZ resistance cells.	(Bertucci et al., 2015)
	Gd-NGO	-	Let-7g	Pan-Ras	EPI	IC_{50} (free drug) = 6.4mg/mL; IC_{50} (Gd-NGO/EPI) = 3.4mg/mL; IC_{50} (Gd-NGO/let-7g/EPI) = 1.3mg/mL. Blank Gd-NGO NPs and let-7g were not cytotoxic towards U-87 MG cells. In vivo biodistribution studies show that Gd-NGO NPs easily accumulates in the brain. Moreover, FAM-labeled let-7g in Gd-NGO NPs showed that the nanosystem was successfully delivered to the brain and, in particular, transfected to cancer cells.	(Yang et al., 2014)
Others	PLGA, PCL and L-α-phosphatidylcholine, trimyristin NPs	-	antimiR-21	-	Pemetrexed	Cytotoxicity studies in U-87 MG cells show that free pemetrexed does not remarkably inhibits cell growth at 24h or 48h. However, at 72 h there is a significant cell viability reduction caused by the drug. Despite the significant cellular uptake of pemetrexed/antimiR-21 NPs (79% vs 6% of free pemetrexed), which was mostly located in the nucleus of the cells, the	(Küçüktürkmen et al., 2016)

						synergism of antimiR-21 and pemetrexed was not evident in all the formulations, at 72 h, due to a low encapsulation efficiency of the miRNA.	

Key: DODAP - 1,2-dioleoyl-3-dimethylammonium-propane; CHOL – cholesterol; DSPC - 1,2-dioctadecanoyl-sn-glycero-3-phosphocholine; C16-mPEG2000-ceramide - N-palmitoyl-sphingosine-1-succinyl[methoxy(polyethylene glycol)2000]; CTX – chlorotoxin; SNALP - stable nucleic acid lipid particle; NP – nanoparticle; PLGA - Poly (lactic-co-glycolic acid); TMZ – temozolomide; cRGD - cyclic Arginine-Glycine-Aspartic acid; PEG - polyethylene glycol; PU-PEI - polyurethane-short branch polyethylenimine; CSC – cancer stem cell; GBM – glioblastoma; PDL - 15-pentadecanolide; DES - diethyl sebacate; MDEA - N-methyldiethanolamine; ApoE – apolipoprotein E; PLA - poly(lactic acid); PAMAM - poly(amido amine); MSC – mesenchymal stem cell; DDAB – didodecyldimethylammonium bromide ; SLNs – solid lipid nanoparticles; CH – chitosan; FA – folic acid; MSNPs - mesoporous silica nanoparticles; R8-PNA - polyarginine-peptide nucleic acid; Gd-NGO - poly(amidoamine) dendrimer-grafted gadolinium-functionalized nanographene oxide; EPI – epirubicin; PCL - poly-ε-caprolactone.

5. Conclusions

Common chemotherapeutic strategies used for glioblastoma treatment are still limited, mostly due to lack of effectiveness, drug resistance mechanisms, off-targets effects and tumor targeting difficulties. Therefore, pursuing molecular pathways and functional genetic events is crucial for finding novel targets and developing new active therapeutic strategies in glioblastoma treatment, either focused on the delivery of small drug molecules or gene therapy.

Novel drug-based strategies include the administration of TMZ and other chemotherapeutic drugs, repositioned drugs or new chemical entities, and often make use of nanotechnology. In this chapter, an extensive description of nanoparticulate based systems for drug delivery, gene therapy, and their combination was comprehensively addressed.

However, despite the positive results of the single administration of nanoparticles containing either chemical drugs or miRNAs, co-delivery approaches, by conveying both actives to tumor cells, have showed a superior efficacy. *In vivo* delivery in glioblastoma remains a challenge in terms of therapeutic success, as they have yet failed to reach clinical trials. Nevertheless, the wide variety of delivery systems developed, and the substantial advances identified so far highlight the potential role these nanotherapies may offer for glioblastoma treatment.

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