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Toward an effective strategy in glioblastoma treatment. Part II: RNA interference as a promising way to sensitize glioblastomas to temozolomide

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RNA interference (RNAi) is a strategy of gene regulation that has opened up many opportunities for the treatment of cancers, especially glioblastoma multiforme (GBM). This strategy reduced the expression of many proteins involved in the resistance of these tumors to anticancer drugs, particularly to temozolomide (TMZ). A significant research effort has gone into RNAi delivery and target selection for clinical application of this new discovery in the treatment of GBMs. However, some limitations must be resolved to enhance the safety of RNAi-based therapeutics and to reduce their immune response. In this review, the mechanism of RNAi will be described. Moreover, the opportunities offered by RNAi strategy to reverse the phenotype of these tumor cells as well as prospects and challenges ahead in the RNAi-based therapy will be discussed.

Introduction

Resistance to anticancer drugs is a problem in many cancers, particularly glioblastoma multiforme (GBM) – the most common type of primary brain tumor [1]. The prognosis of these tumors remains poor with a median survival of 14.6 months despite receiving many therapies including surgery, radiotherapy and chemotherapy [2]. Combining radiation therapy with temozolomide (TMZ) is currently the first-line therapy for GBMs. However, the efficiency of TMZ remains limited owing to inherent and acquired resistance of glial tumor cells. The main resistance mechanisms have been detailed in part I of this review series. Different strategies of inhibiting the effect of these proteins involved in resistance to TMZ were tested. However, the effects obtained are often inadequate or disappointing.

Development of the RNA interference (RNAi) strategy has opened new perspectives for the treatment of these malignancies. Indeed, the principle of RNAi is based on the reduction of expression of a target mRNA into protein. This strategy has been applied with success *in vitro* and *in vivo* in different pathologies including cancers, viral infections and metabolic disorders [3]. However, transport and delivery of interfering RNA requires the use of vectors that must be safe and efficient. In this review, we will initially describe the RNAi strategy mechanism, followed by a description of different nonviral vectors used for the transport and delivery of interfering RNA. Finally, application of this strategy in GBMs and the various challenges and obstacles that must be overcome for successful clinical application will be discussed.

RNAi mechanism in mammalian cells

In recent years, the strategy of RNAi, the principle of which is based on inhibition of protein synthesis by targeting a specific mRNA, was discovered and has opened up new perspectives in human disease treatment. RNAi is an intrinsically cellular pathway that was discovered in 1998 [4,5]. Two classes of small RNAs have been identified to mediate RNAi, small interfering RNA (siRNA) and micro RNA (miRNA).

For siRNA-mediated RNAi, the cellular process begins with long double-stranded RNAs (dsRNAs) cleaved in the cytoplasm by the enzyme Dicer to generate mature siRNAs of about 21–23 base pairs (bp). The resulting siRNAs are incorporated into RNA-induced silencing complex (RISC), which becomes active. Then, the antisense strand remains in the RISC complex and guides the RNAi enzymatic machinery while the sense strand is eliminated from the RISC complex. The RISC-containing guide strand binds to

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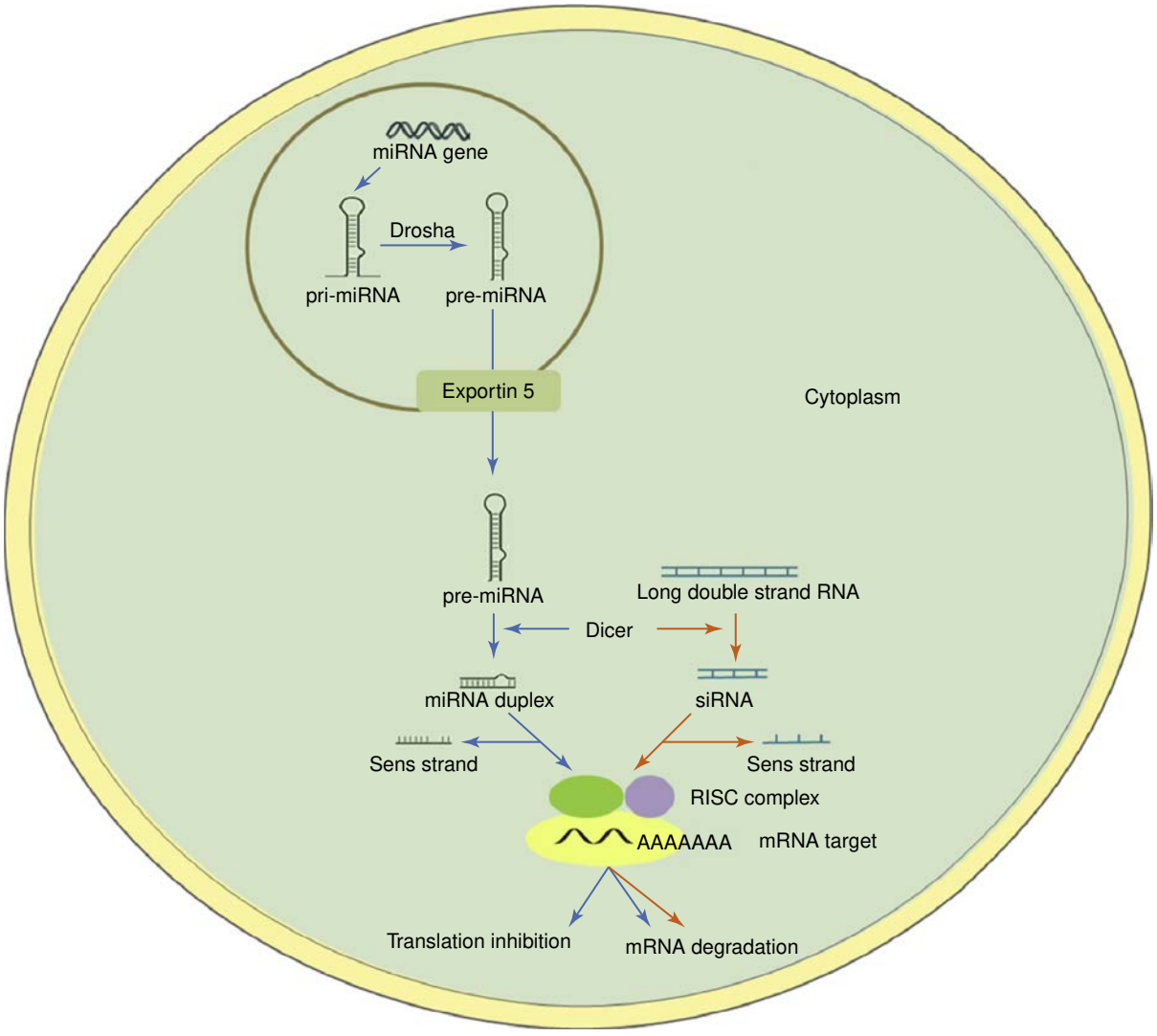

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FIGURE 1
Mechanism of action of RNA interference (RNAi) by silencing RNA (siRNA) or micro RNA (miRNA) in mammalian cells.

complementary mRNA, which causes its degradation by the enzyme argonaute 2 (Ago-2) [6,7] (Fig. 1).

Concerning miRNA-mediated RNAi, genes encoding miRNAs are transcribed into long primary transcripts (pri-miRNAs) that are cleaved by RNAse III Drosha to generate precursor miRNAs (pre-miRNAs) in the nucleus [8,9]. Pre-miRNAs are transported from the nucleus to the cytoplasm by exportin-5 and then processed by RNAse III Dicer to generate mature miRNAs of about 22 nucleotides in length [8]. Then, miRNAs are incorporated into the RISC complex and bind through imperfect complementarity to the 3′ untranslated region (UTR) of its mRNA target, leading to translational repression or mRNA degradation [8,10]. A single miRNA molecule is probably capable of regulating multiple mRNAs, and conversely one mRNA can be regulated by multiple miRNAs (Fig. 1).

RNAi delivery

The use of these small RNA molecules alone as therapeutics is not possible for several reasons. Among these is their negative charge which prevents them from crossing the plasma membrane to reach their target in the cytoplasm [6,11]. The presence of nucleases in plasma and in the cytoplasm are also likely to deteriorate these biopolymers rapidly after injection [12], not to mention the immune response that can be triggered by their presence in the blood [12]. These reasons emphasize the need for a suitable vector able to deliver these small RNA molecules into the desired cells.

Viral and nonviral vectors were developed to carry siRNAs to their site of action located in the cytoplasm [13]. The RNAi induced by viral delivery was demonstrated using different viruses particularly adeno-associated virus (AAV), herpes simplex virus (HSV) and lentiviral vectors [8,14]. Despite their efficiency, viral vectors have some limitations such as their residual pathogenic effect, which represents a potential risk to patients [15]. Moreover, a mutagenesis effect was observed in some clinical trials using these vectors. This is why a lot of research has been focused on the development of nonviral vectors. An ideal nonviral vector has certain attributes that are: (i) to be biocompatible and biodegradable; (ii) to be able to protect nucleic acids against nuclease degradation in plasma and avoid their renal clearance; and (iii)

to allow a reversible binding of nucleic acids and to trigger their release at the site of cellular action.

Different carriers of siRNAs and miRNAs were developed and described in the literature; most of them are positively charged to allow attachment of nucleic acids that are negatively charged and to enable a better interaction with the cell membrane and improve the cellular uptake [16]. Among these vectors, there are polymeric nanoparticles, lipid-based systems and inorganic nanoparticles.

Polymeric nanoparticles

Cationic polymers can interact with the negatively charged phosphate groups of nucleic acids, which create complexes called polyplexes. Natural and synthetic polymers are used for RNAi delivery in mammalian cells [17].

Polyethyleneimine. Polyethyleneimine (PEI) is a synthetic polymer considered to be the most effective polymer for siRNA delivery. The high density of positive charge provided by this polymer creates strong links with nucleic acids, thereby protecting them from enzymatic degradation by nucleases. Factors such as molecular weight (MW) and degree of branching of PEI affect the efficiency and the toxicity of the polymer [18]. Indeed, the high MW chains are more effective for siRNA delivery compared with the low MW chains. Similarly, branched PEI is more efficient for siRNA binding than the linear form. However, the high MW chains and branched chains are the most toxic [18] (Fig. 2).

PEI–siRNA complexes are internalized by endocytosis in various cells. This pathway is composed of vesicles known as endosomes (with an internal pH of approximately pH 5) that mature from early endosomes to late endosomes before fusing with lysosomes, which contain digestive enzymes [19]. The acidic lysosomal environment causes the protonation of the amine groups of PEI that, owing to the proton sponge effect as a result of its high buffering capacity, induces osmotic swelling of the endocytosis vesicle until vesicle rupture, leading to the release of polyplexes into the cytoplasm [20]. The ability of PEI to escape lysozomal degradation enables the release of intact siRNA and miRNA in the cytoplasm and thus provides a high efficiency of gene silencing [19–21].

Chitosan. Chitosan is another polymer used for RNAi delivery. This natural polysaccharide consists of repeating D-glucosamine and *N*-acetyl D-glucosamine units linked by β(1–4) glycosidic bonds [22]. It is obtained by deacetylation of chitin and is considered to be a nontoxic and biocompatible polymer [23].

Chitosan MW, degree of deacetylation (DDA) and N:P ratio (ratio of the protonated amine groups of chitosan on the phosphate groups of the siRNA) are factors that greatly influence siRNA transfection efficiency [24]. Indeed, higher MW chains are flexible allowing them to wrap the siRNAs in stable complexes and protect them from nuclease degradation. However, this stability can be an obstacle to the release of siRNAs in the cytoplasm. In addition, high MW chitosan showed drawbacks such as aggregation and low solubility at neutral pH. Low MW chains enables more-efficient intracellular release but low complexation [24]. Higher DDA enhances electrostatic interaction with siRNAs, which leads to a greater stability of the complexes formed with siRNAs [25] (Fig. 2). The excess of positive charges in comparison with the negative charges increases the stability of the chitosan–siRNA complexes and increases uptake across anionic cell surface [25].

Dendrimers. Dendrimers are synthetic macromolecules; their structure is composed of a central core, branches of repeating units and terminal groups, which can interact with charged molecules such as nucleic acids, imaging agents and anticancer drugs [26,27]. In addition, the internal hydrophobic cavities of dendrimers can encapsulate hydrophobic anticancer drugs [28] (Fig. 2).

Dendrimers with positively charged surface groups are used for RNAi delivery [29,30]. Poly (amidoamine) dendrimers (PAMAM) are the most important type in the dendrimer family [31]. PAMAM possess tertiary amine functions in their structure, which can be protonated in acidic medium and thus confer a proton sponge effect [32], which allows a release of siRNA and miRNA on the cell level while preventing degradation in the endolysosomal compartment [31,33]. Some studies showed that PAMAM dendrimers are excellent nonviral vectors for siRNA and miRNA delivery, thereby producing powerful gene silencing *in vitro* and *in vivo* [34–36]. Despite their transfection efficiency, cationic and higher generation dendrimers have the disadvantage of interacting with blood components, destabilizing cell membranes and causing cell lysis [37].

Lipid-based systems

Liposomes. Liposomes are efficient vectors for the delivery of drugs and nucleic acids [38]. Liposomal forms of doxorubicin

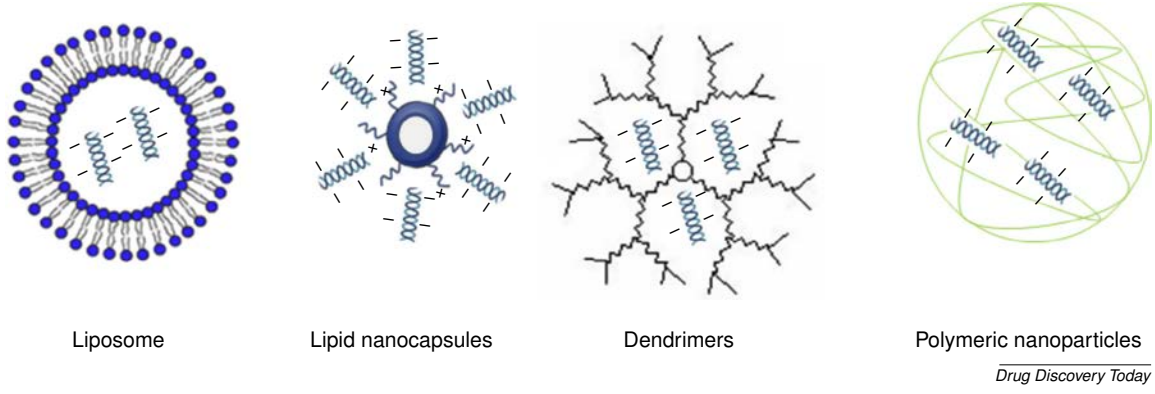

FIGURE 2

Different types of nanocarriers for delivery of silencing RNA (siRNA) used for RNA interference. Negatively charged siRNA interact with positive charges of the nanocarrier components.

and amphotericin B obtained FDA approval for the treatment of Kaposi's sarcoma and fungal infections, respectively [39]. Liposomes are formed by unilamelar or multilamelar phospholipid bilayers. Three classes of phospholipids are used in the formulation of liposomes: anionic, cationic and neutral phospholipids [17,39] (Fig. 2).

Owing to their hydrophilic nature, the nucleic acids can be encapsulated in the core of liposomes. Conversely, cationic lipids are the most efficient for the transport of nucleic acids, because they can interact as cationic polymers that create lipoplexes [20,40]. In addition to their ability to encapsulate drugs, the liposome surface can be functionalized to allow, firstly, an avoidance of recognition by the immune system (i.e. pegylated stealth liposomes) increasing the plasma half-life [41] and, secondly, to target tumor cells specifically through the use of various ligands such as specific antibodies [42]. Despite their transfection efficiency of nucleic acids, cationic liposomes have the drawback of being toxic by their interaction with negatively charged cellular components (i.e. opsonins, serum protein and enzymes) resulting in hemolysis and activation of the complement system, which causes their rapid elimination [43].

Lipid nanoparticles. Various types of lipid nanoparticles have been developed. Among them, the lipid nanocapsules (LNCs) have a structure that is a hybrid between polymeric nanocapsules and liposomes because of their oily core, which is surrounded by a membrane made of tensioactive agents such as PEG hydroxystearate (Solutol®). LNCs have many advantages such as increased safety, high stability and the possibility of lipophilic drug encapsulation (e.g. paclitaxel) [44]. They can be produced without organic solvents by a phase inversion process and with generally recognized as safe (GRAS) excipients and are also genetically modified organism (GMO)-free [45]. The concept of this process uses the specific ability of some polyethoxylated surfactants to modify their affinities for water and oil as a function of the temperature [46]. These nanocapsules have shown their efficiency for the delivery of nucleic acids. Indeed, siRNA can be entrapped into LNCs after formation of lipoplexes with cationic lipids [47]. Another strategy consists of surface modification of LNCs by grafting a cationic polymer such as chitosan to obtain a cationic vector capable of fixing nucleic acids by electrostatic attraction [48] (Fig. 2).

Inorganic nanoparticles

A number of inorganic nanoparticles such as carbon nanotubes, magnetic nanoparticles and gold nanoparticles have been developed for gene therapy [49]. These systems are different from one another because of their composition and the methods of formulation used.

Carbon nanotubes. Some studies have shown that carbon nanotubes easily cross the plasma membrane using an endocytosis-independent mechanism [49]. Several functionalized carbon nanotubes have been designed and tested for siRNA and miRNA delivery [50–53]. The most effective formulations of carbon nanotubes were functionalized with amino groups [54]. Despite their efficiency in the transport of nucleic acids, their toxicity profile caused problems for their use in the clinic [55,56].

Magnetic nanoparticles. Magnetic nanoparticles were developed for tumor imaging and drug delivery. siRNA delivery was tested with different magnetic nanoparticles, such as iron oxide nanoparticles, and has shown promising results [49]. The advantage of these nanoparticles is the possibility to deliver siRNA to the desired tissue by applying an external magnetic field [57].

Gold nanoparticles. Gold nanoparticles are also used as nonviral vectors and present several opportunities for RNAi delivery because of their interesting properties, in particular their biocompatibility and low toxicity [58,59]. Their stability enables avoiding interactions with serum ionic compounds in particular proteins [60]. Gold nanoparticles possess photothermal properties that can be exploited for cancer therapy [60]. In addition, their surface can be functionalized with specific ligands to allow easy fixation of small RNA molecules for gene therapy [60,61].

RNAi delivery system used to sensitize GBM cells to TMZ

Studies that used RNAi to sensitize GBM cells to TMZ are summarized in Table 1. These results show that different targets are to be reached by siRNA for the treatment of GBMs. The administration of RNAi can be done directly into the tumor or intravenously. The results obtained in these studies are very promising. Despite this, only a few nonviral vectors are approved by the FDA and are in clinical trials, demonstrating that there are still some barriers to overcome, particularly in terms of selectivity, efficacy and toxicity.

Among the vectors that have reached clinical trials, the Rondel™ system produced by Calando Pharmaceuticals was used in clinical trial CALAA-01 indicated for the treatment of cancers and solid tumors refractory to conventional therapy. It used an siRNA targeting the M2 subunit of ribonuclease reductase, a key enzyme in the synthesis and replication of DNA that converts ribonucleotides into deoxyribonucleotides. Rondel™ vector consists of alternating β-cyclodextrins and a positively charged polymer to allow attachment of siRNA by electrostatic attraction; the formed nanocomplexes have a diameter of less than 100 nm. Each cyclodextrin molecule contains in its hydrophobic cavity a molecule of admantane, which is covalently bound to one molecule of polyethylene glycol (PEG). The other end of the PEG molecule is linked to a transferrin molecule that will allow the nanocarrier to bind to the transferrin receptor (TfR) overexpressed in tumor cells [62].

For clinical application, RNAi-based cancer therapeutics should specifically recognize cancer cells without affecting normal cells. The selectivity of nanocarriers was improved by their conjugation to specific ligands that can bind the tumor cell receptors. However, to reach their target in cancer cells, it is necessary to increase the circulation time of these particles in plasma by protecting them against a capture by the reticuloendothelial cell system (RES). This protection can be efficiently achieved through a PEG coating that also has the drawback of reducing the tumor cell uptake of these particles [49].

It is crucial to improve vector safety because even though many vectors have proven their efficiency in the transport and delivery of drugs and nucleic acids they often cause a stimulation of the immune response and cytotoxicity that does not allow their clinical use. The safety profile of vectors could be improved through the use of biocompatible, biodegradable and nontoxic compounds [49]. In addition to efficacy, selectivity and safety, vectors must be produced on a large scale to allow their use in clinical trials. However, the production of nonviral vectors at a

TABLE 1

***In vitro* and preclinical *in vivo* RNAi delivery system used to sensitize glioblastomas to temozolomide.**

Delivery system	RNAi used	Targeted gene	*In vitro* model	Efficacy *in vitro*	Animal model	Route	Efficacy *in vivo*	Refs
Viral vectors								
Lentiviral vector	shRNA	MGMT	LN18 and T98 GBM cell lines	Inhibition of 80% of the MGMT protein and increase of TMZ sensitivity in both cell types	Subcutaneous tumors in nude mice	Intratumoral	Tumor size was reduced by 46% in combination with TMZ	[74]
Plasmid	Anti-EGFR shRNA + wild-type PTEN cDNA	EGFR and PTEN	U251 glioma cells	Downregulation of EGFR expression and upregulation of PTEN expression resulted in suppression of cell proliferation, cell cycle and promotion of cell apoptosis	Subcutaneous tumors in nude mice	Intratumoral	The growth of the subcutaneous tumor was significantly inhibited	[75]
Plasmid	siRNA	Mdm2	Not applicable	Not applicable	Nude mice	Intratumoral	Suppression of the HepG2 xenografted tumor growth and reduction of 60% of the Mdm2 protein expression	[76]
Nonviral vectors								
LipoTrust™ liposome	siRNA	MGMT	T98G and U251 GBM cell lines	99% of MGMT knockdown	Intracranial tumors in NOD/SCID mice	Intratumoral	Response to TMZ significantly increased	[77]
ProFection® Mammalian Transfection System	siRNA	Galectin-1	HS683 GBM cells	Inhibition of 60% of the galectin-1 and increase of cytotoxic effect of TMZ in these cells	Nude mice	Intratumoral	Galectin-1 knockdown in orthotopic xenograft-Hs683 enhanced the TMZ effect and increased the survival of mice	[78]
Lipofectamine™	siRNA	Mutant p53	T98G and U138 GBM cells	p53 knockdown led to a fivefold increase in chemosensitivity to TMZ	*In vitro* model	Not applicable	Not applicable	[79]
Chitosan transacylated lipid nanocapsules	siRNA	EGFR	U87MG GBM cell line	Inhibition of 51.9% of EGFR production on U87MG cells and increase of TMZ sensitivity	*In vitro* model	Not applicable	Not applicable	[48]
Polyurethane-short-branch polyethylenimine	miRNA-145	Oct4 and Sox2	GBM-CD133+ cells	Increase the sensitivity of treated cells to radiation and TMZ	Intracranial tumors in nude mice	Intratumoral	Delivery of PU-PEI-miR145 in orthotopic GBM-CD133+ improved survival in combination with radiotherapy and TMZ	[80]

Abbreviations: EGFR, epidermal growth factor receptor; GBM, glioblastoma multiforme; Mdm2, murine double minute 2; MGMT, O^6-methylguanine DNA methyltransferase; NOD, non-obese diabetic; PEI, polyethyleneimine; PTEN, phosphatase and tensin homolog; PU, polyurethane; SCID, severe combined immunodeficiency; TMZ, temozolomide.

large scale has only been demonstrated with a limited number of drug delivery systems, this impedes the use of these promising formulations in clinical trials [63].

One limitation to the use of siRNA in the clinics is the off-target effect due to the imperfect complementarity between the siRNA and the 3′ UTR of the mRNA transcripts. This leads to the inhibition of genes other than the target genes that might have important consequences for cell function [64]. To reduce or eliminate this effect, chemical modifications of the nucleic acids averted complementarity with other mRNAs other than those targeted [64,65]. The short *in vivo* effect of siRNA (about one week) is another limitation for their use that requires repeated administration and causes a massive accumulation of vectors and siRNA into tumors responsible for toxicity [66]. It is thus necessary to obtain the most biocompatible vectors to prevent their toxic effect.

The saturation of the cellular enzymatic machinery by exogenous interfering RNA is another problem that can cause disruption of cellular functions controlled by small RNAs such as endogenous miRNA. Indeed, exportin-5 is a key protein of the miRNA pathway that can be saturated with exogenous miRNA [64,67]. By contrast, competition is also present for the incorporation of interfering RNA in the RISC complex. This competition is problematic because the endogenous miRNA present incorporation kinetics slower than that of exogenous RNA [68].

For GBM treatment using the intravenous route, an siRNA carrier must cross the blood–brain barrier (BBB) to release the interfering RNA. This barrier protects the brain from toxins and drugs presented in the circulation; this protection is ensured in particular by the tight junctions and efflux pumps such as P-glycoprotein (P-gp) that actively remove these molecules from the circulation, which explains the failure of some chemotherapy [69]. Although this barrier seems to be somewhat disrupted in the abnormal vascular networks characterizing GBMs, it frequently remains intact along the infiltrating area where the plurality of recurrences tend to occur [70].

By contrast, the local administration of nanocarriers avoids the crossing of the BBB and allows drug delivery directly into the brain parenchyma. The major drawback of this technique is a poor diffusion of particles in the tumor, which does not allow the drug to reach the area of the recurrence of GBMs located a few centimeters from the original tumor area [71]. Moreover, particle reflux from the tumor area is often encountered which reduces the effectiveness of treatment. This is partly solved by the development of convection-enhanced delivery (CED), which allows injecting the particles under a pressure gradient for more-homogeneous distribution in the tumor [72]. With this technique, the drug concentrations in brain tumors can be superior to those obtained with systemic administration.

In addition, GBMs are very heterogeneous tumors characterized by the parallel overexpression of multiple proteins that are involved in resistance to TMZ. This implies that, for effective treatment by RNAi, it is necessary to adapt the therapy to each patient based on their molecular characteristics. Also, GBM resistance to TMZ can be innate or acquired, which can cause a rapid recurrence of tumor after initial remission. This illustrates that the success of a therapy requires knowledge of the gene expression profile of the tumor at different times to adapt the treatment with siRNA and possibly target other proteins involved in this new induced resistance [70]. Despite its effectiveness, drug administration using CED has several disadvantages including poor distribution distance of the drug to the site of placement of the catheter (<3 cm) which is a problem knowing the infiltrating and invasive character of GBMs [73]. The complexity of brain tumors associated with edema and leakage of drugs into the subarachnoid space are drawbacks that will have to be addressed for a better use of this technique [73].

Concluding remarks

Concomitant administration of TMZ and radiotherapy improved prognosis of patients with GBM by increasing median survival, but this effect remains modest. The development of molecular biology has enabled the understanding of the involvement of certain genes in cancer resistance and allowed the molecular profile of each tumor to be defined. RNAi is one of the most promising strategies to overcome cancer resistance. Indeed, this strategy can be applied to achieve different targets involved in GBM resistance to TMZ. These targets could be identified by genetic screening to find the genes that are out of control for every individual. This concept known as personalized medicine adapts the therapeutic strategy for each patient, which is a major advantage given the heterogeneity of GBMs. In addition, the development of nanocarriers able to transport drugs and nucleic acids provides a synergistic effect with lower effective doses of these drugs and fewer side-effects. Further investigations are still being carried out to improve the safety of these nanocarriers and their effectiveness for clinical application.

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