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Advances in RNA cancer therapeutics: New insight into exosomes as miRNA delivery

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ABSTRACT

We are now entering a new era of RNA therapies, such that mRNA-based vaccines and RNA interference approaches such as siRNA have already been launched on the pharmaceutical market. However, there are no FDA-approved RNA-based therapeutics for cancer treatment. Among RNA molecules, miRNAs represent a promising solution against cancer. Despite the ability to target multiple pathways, miRNA-based therapeutics struggle to reach phase 3 clinical trial. A reason of this delay is linked to complications of selective administration of miRNAs to their target, the tumor cell. Because of this, an efficient delivery system is necessary. In this sense, exosomes are considered the most promising miRNA-based therapeutic carriers in terms of safety and efficient cargo delivery. Furthermore, researchers have developed a new strategy to overcome the tumor capacity by using exosomes to release unnecessary miRNAs, shedding light on a new generation therapy of cancer treatment. The review describes recent advances in the application of miRNAs in the treatment of cancer, the use of exosomes for miRNA delivery, focusing on new approaches to overcome the limits of miRNA-loaded exosomes in clinical applications.

1. Introduction

RNA-based therapies, including messenger RNA (mRNA), RNA interference (RNAi) such as small interfering RNA (siRNA) and microRNA (miRNA) therapeutics, are promising approaches for treating several diseases (Damase et al., 2021). These treatments have been demonstrated to have greater therapeutic efficiency than DNA-based drugs and, compared with protein-based therapeutics, are more cost-effective (Li et al., 2021). Currently, four siRNA and two mRNA-based drugs have been approved and are on the market. However, none of them has been applied for cancer treatment. This is attributable to the complicated pathophysiological environment of cancer including the dense tumor stroma, unstructured blood vessels, immunosuppression, multidrug resistance, and hypoxia (Heinrich et al., 2021). Indeed, several issues need to be addressed to obtain therapeutic effect. In the circulation, 'naked' RNA molecules must avoid enzymatic degradation, renal clearance, and phagocytic entrapment because of their small size, poor stability, and immunogenicity (Kulkarni et al., 2019). Inside the tumor, the physicochemical properties of RNA molecules such as a high molecular weight, negative charge, and high hydrophilicity do not allow them to efficiently cross negatively charged biological membranes and enter the cancer cells. Finally, even after internalization, few RNA molecules can escape endosomal entrapment, since exogenous agents are internalized through endocytic and endo-lysosomal pathways likely resulting in RNA degradation (Singh et al., 2020).

Multiple chemical modifications have been proposed to improve the stability and reduce the immunogenicity of RNA therapies. The formulations approved in clinical practice, Givosiran (GIVLAARI® Inc.), Lumasiran (OXLUMO® Inc.), and Inclisiran (LEQVIO® Inc.), are constructed by conjugating siRNA to N-acetylgalactosamine molecules. However, endosomal escape remains an issue (Dammes and Peer, 2020).

The mRNA-based coronavirus disease 2019 vaccines highlighted the role played by drug delivery systems in RNA-based therapies. Lipid nanoparticles (LNPs) for the delivery of nucleic acid- and mRNA-based therapeutics, are well established. It was reported that mRNA translation after administration of mRNA/LNPs occurred primarily in the liver (Mukai et al., 2022). Therefore, efficient delivery of RNA molecules to

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Abbreviations

2′-F	2′-fluoro
2′-O-MOE	2′-methoxyethyl
2′-O-Me	2′-O-methyl
ADSC	Adipose-derived stem cell
AEs	Adverse events
AMD	Age-related macular degeneration
ASOs	Antisense oligonucleotides
CNS	Central nervous system
CCL2	Chemokine C–C motif-ligand 2
CARPA	Complement activation related pseudo-allergy
CTCL	Cutaneous T-cell lymphoma
CXCL12	CXC chemokine ligand
CMV	Cytomegalovirus
ESCRT	Endosomal sorting complex required for transport
EGFR	Epidermal growth factor receptor
EVs	Extracellular vehicles
FDA	Food and Drug Administration
GBM	Glioblastoma multiforme
HTLV-1+	Human lymphotropic virus type 1
hTERT	Human telomerase reverse transcriptase
ILVs	Intraluminal vesicles
IP	Intraperitoneally
IV	Intravenously
KTZ	Ketoconazole
LNPs	Lipid nanoparticles
LKB1	Liver kinase B1
LNAs	Locked nucleic acids
LDL	Low-density lipoprotein
LDLR	Low-density lipoprotein receptor
MPM	Malignant pleural mesothelioma
MSC	Mesenchymal stem cells
mRNA	Messenger RNA
miRNA	MicroRNA
MVs	Microvesicles
mMCAI	Middle cerebral artery infarct
mSWAT	Modified severity-weighted assessment tool
MLSMR	Molecular libraries small molecule repository
MSC-EXOS	MSC-derived exosome
MM	Multiple myeloma
MVBs	Multivesicular bodies
MF	Mycosis fungoides
NPC	NCGC pharmaceutical collection
nSMase	Neutral sphingomyelinase
PR	Partial response
PHS	Phosphatidylserine
PS	Phosphorothioated
PD	Progressive disease
PKN3	Protein kinase N3
Rab	Ras-associated binding
RME	Receptor-mediated endocytosis
RNAi	RNA interference
RISC	RNA-induced silencing complex
ssRNA	Single-strand RNA
siRNA	Small interfering RNA
SL	Sphingolipid
SMA	Spinal muscular atrophy
SD	Stable disease
SELEX	Systematic evolution of ligands by exponential enrichment
Tf	Transferrin
TfR	Transferrin receptor
TDEs	Tumor-derived exosomes
TME	Tumor microenvironment
UTR	Untranslated regions
VEGF	Vascular endothelial growth factor

tumor site while avoiding accumulation in the liver is the main challenge. The off-target effects, which can induce serious side effects and reduce the therapeutic efficacy, may result from non-specific delivery and inability of LNP to avoid immune surveillance.

Different to mRNA-based therapies, which act by upregulating expression of targeted proteins, RNAi therapies including miRNA and siRNA act by complexing with the RNA-induced silencing complex (RISC) in the cytoplasm to induce cleavage of the mRNA sequence and thus to downregulate the expression of checkpoint proteins for disease treatment (Thody et al., 2020). Therefore, an ideal RNA delivery system for cancer treatment should protect RNA degradation and should possess endosomal escape capability to release RNAs into the cytosol. Moreover, tumor targeting ability and immune system surveillance escape are fundamental features to consider in developing an efficient nanocarrier.

In this review, we provide an overview of current RNA applications in cancer therapy, focusing on miRNA-based therapeutics currently in clinical trials, and discuss recent advances in the use of exosomes as miRNA transfer vehicles. New approaches to overcome the limits of miRNA-loaded exosomes in clinical applications will be described.

2. RNA in therapy

RNA-based therapies present a rapidly expanding class of new drugs that, due to their role in biological processes, have promising clinical applications for prevention and treatment of various human diseases. It is well known that only 1.5% of the human genome encodes proteins, and of these only 10–15% are druggable using conventional proteins or small molecules (Hopkins and Groom, 2002). Compared with established drugs, RNA therapeutics can be used to bind “undruggable” or mutated targets and can be designed to affect any gene or region within the genome, including non-coding transcriptomes (Yu et al., 2019).

Although RNA therapy has many advantages over existing therapies, only a limited number of RNA-based drugs have been approved for clinical use. The development of therapeutic RNAs has required overcoming several major hurdles including unfavourable physico-chemical properties (negative charge, large molecular mass and size), instability and short circulating half-life, and strong immunogenicity (Damase et al., 2021). For this purpose, several chemical modifications of the saccharide and/or phosphodiester backbone are required. In majority of therapeutic oligonucleotides, phosphodiester linkages are replaced with phosphorothioate (PS) to protect them from nuclease degradation and enhance their stability, and to facilitate their cellular uptake *in vivo* (Eckstein, 2014). Among the multitude of chemical modifications sugar-modified oligonucleotides have been shown as most successful. In this regard, modification at the 2′-position of the ribose increases their binding affinity and nuclease resistance (Prakash, 2011). Furthermore, therapeutic RNAs must be encapsulated in appropriate delivery systems to protect RNAs from degradation and to deliver them efficiently into target tissues and cells, and to ensure cellular uptake. To achieve these goals, a variety of selective delivery agents are being development (Bajan and Hutvagner, 2020).

Most RNA therapeutics fall into three broad categories: RNA molecules that target nucleic acids (DNAs or RNAs), RNA drugs that target proteins (aptamers) and RNA drugs that are translated into proteins (mRNAs). The first category includes single-stranded RNAs (antisense RNA) and double-stranded RNAs (small interfering RNAs) (Lieberman, 2018).

2.1. Antisense RNAs

Antisense oligonucleotides (ASOs) are short (15–20 bp) synthetic, single-stranded RNA/DNA molecules, fully complementary to the target RNA sequence. ASOs binding to a specific RNA via Watson–Crick base pairing can alter gene expression via two types of mechanisms: RNA cleavage (RNase H-dependent ASOs) and RNA blockage (RNase H-independent ASOs) (Crooke, 2004). The mode of ASO action depends on the target sequence and the ASO chemistry. The first is more commonly used and is more efficient for inducing protein knockdown. ASOs target RNA, forming ASO-RNA hybrids, with RNase H recognizing and cleaving the RNA strand of the RNA-ASO duplex (Wu et al., 2004; Wada et al., 2021). By contrast, non-RNase-mediated mechanisms result in down-regulation of target protein expression by steric hindrance of ribosomal, destabilization of pre-mRNA (inhibition of 5′ cap formation) or alteration of splicing (splice-switching) (Chan et al., 2006).

ASOs are chemically modified to enhance stability, delivery, targeting and pharmacological properties (Kurreck, 2003; Bennett et al., 2017). These modifications can occur within the ASO backbone or on its side chains; moreover, many ASOs are designed as chimeras, as they contain a mixture of nucleotides with different chemistries (Scoles et al., 2019). In this regard, in the first-generation ASOs, the phosphodiester linkages were replaced with PS, whereas in second-generation ASOs, in addition to a PS backbone, the –OH group at the 2′-position has been substituted with 2′-O-methyl (2′-O-Me) or 2′-fluoro (2′-F) (Eckstein, 2014; Khvorova and Watts, 2017, 2017stergaard et al., 2017). In the third generation, the use of C3′-endo conformation by connecting 2′-oxygen and 4′-carbon of the ribose with a bond bridge, represents a remarkable progress by creating locked nucleic acids (LNAs)-modified antimiRs, characterised by increased binding specificity and resistance to nucleases (Rupaimoole and Slack, 2017). Furthermore, to ensure efficient delivery of antisense oligonucleotides to the target cells, ASOs must be encapsulate in nanoparticles, such as liposomes, polymeric nanocarriers, and metallic particles, or conjugated with various peptides (Yang et al., 2020).

ASOs are not only a useful tool for studies of loss-of-gene function, but they represent a promising therapeutic strategy to treat diseases with dysregulated protein expression, such as cancer, diabetes, AIDS, and cholesterol-linked syndromes (Crooke et al., 2018). For instance, Fomivirsen (Vitravene) is the first drug using antisense technology approved by the Food and Drug Administration (FDA) for the treatment of cytomegalovirus (CMV) retinitis. The drug prevents viral replication, blocking translation of early CMV proteins (Jabs and Griffiths, 2002). Other ASOs approved by FDA include Mipomersen (Kynamro® Inc.) for the treatment of familial hypercholesterolemia (Duell et al., 2016), eteplirsen (Sarepta Therapeutics® Inc.) for treatment of Duchenne muscular dystrophy (Mendell et al., 2013), and nusinersen (Spinraza® Inc.) for treatment of spinal muscular atrophy (SMA) (Khorkova and Wahlestedt, 2017). Although no FDA-approved ASOs exist for cancer therapy, results obtained in pre-clinical studies and clinical trials are encouraging (Xiong et al., 2021).

The antisense technology, based on mRNA of the target protein, may solve the problem of “undruggable” proteins. Thus, ASOs are more versatile, customizable and specific than conventional small drugs. Antisense drugs can be produced quickly and can be targeted into pathological tissues without harming healthy cells. However, disadvantages include toxic side-effects, targeted tissue-delivery (i.e., ASOs generally do not cross the blood-brain barrier), and difficulty of determine the optimal dosage, plus the high cost (Xiong et al., 2021; Crooke et al., 2021).

2.2. RNA aptamers

RNA aptamers are short single-stranded RNAs that bind to a target with high specificity and affinity, and block or alter its function. For these properties, RNA aptamers act as molecular mimics of antibodies (Germer et al., 2013). Aptamers with affinity for a desired target are identified from random pools of RNA by a method named Systematic Evolution of Ligands by Exponential enrichment (SELEX) (Zhuo et al., 2017). RNA aptamers have been developed against a wide variety of targets including small molecules, peptides, proteins, carbohydrates, toxins, and even live cells. Rather than the primary sequence, aptamer binding is determined by its tertiary structure. In fact, RNA molecules fold into various and complex tertiary structures that interact with the target ligand by means of hydrophobic and electrostatic interactions, van der Waals forces, hydrogen bonding, and base stacking (Zhang et al., 2021a).

RNA aptamers represent a viable alternative to antibodies, and they can be used for analytic, diagnostic and therapeutic purposes, and for target validation and drug discovery. The use of RNA aptamers for therapeutic purposes has many advantages over the use of antibodies, such as lack of immunogenicity, better stability, greater structural flexibility, simpler modification, and lower manufacturing costs and time. Furthermore, compared to antibodies, RNA aptamers can bind non-immunogenic molecules (such as small targets or toxins) or poorly accessible binding domains, and to enter biological compartments more easily (e.g., tissues and cells) (Keefe et al., 2010; Lakhin et al., 2013). At the same time, the small size makes RNA aptamers susceptible to renal filtration and to nuclease-degradation. Therefore, therapeutic aptamers are chemically modified to improve the pharmacokinetic profile and the circulating half-life *in vivo* (Kovacevic et al., 2018). In this context, conjugation with polyethylene glycol (PEG) reduces their renal excretion, while modification of the 3′ and 5’ ends confer their resistance to exonucleases (Adachi and Nakamura, 2019).

Therapeutic RNA aptamers have been developed against specific targets to treat a wide range of human diseases such as tumors, macular degeneration, viral infection, inflammation, diabetes, and cardiovascular and coagulation complications (Xiao et al., 2021). Although many RNA aptamers have been identified, to date only nine RNA aptamers have undergone clinical trials. Pegaptanib (Macugen: Eyetech Pharmaceuticals® Inc.), the only aptamer-based drug approved by the FDA, is an RNA aptamer against vascular endothelial growth factor (VEGF), being used for the treatment of age-related macular degeneration (AMD) (Ng and Adamis, 2006). Other RNA aptamers, currently evaluated in clinical trials, are NOX-E36 against the chemokine C–C motif-ligand 2 (CCL2) to treat type 2 diabetes mellitus, NOX-A12 that binds the CXC chemokine ligand (CXCL12) to reduce lymphocytic leukemia cell motility, REG1 that targets factor IX, a protein implicated in coronary artery disease, and ARC1905 acting against complement component 5 for the treatment of AMD (Kaur et al., 2018; Byun, 2021). Despite promising results, further investigation is needed, and several limitations, including their safety and inability to cross the plasma membrane by passive diffusion, must be overcome.

2.3. Messenger RNAs (mRNAs) as therapeutics

Therapeutic mRNA is a synthetic or *in vitro*-translated mRNA that mimics natural mRNA and acts as an intermediate to deliver genetic information to the translational machinery to induce expression of a specific protein (Shin et al., 2018). Structurally, mRNA consists of a single-stranded open reading frame flanked by untranslated regions (UTRs), with a cap at the 5′ end and a 3′ poly(A) tail (Sahin et al., 2014). Main approaches of mRNA-based therapy consist of: replacement therapy, where mRNA is delivered into the patient's cells to compensate for a defective or missing protein; vaccination, where mRNA encodes for antigen(s) that activate(s) the immune system to produce antibodies against a specific pathogen or to recognize pathological cells (i.e., cancer cells); cell therapy, where target cells are manipulated *ex vivo* to introduce therapeutic mRNA (Ouranidis et al., 2021).

mRNA as a therapeutic offers numerous advantages compared to conventional small molecules or recombinant proteins such as low manufacturing cost and time, adjustable gene expression, fast protein production, and the possibility of personalized therapy (Wadhwa et al., 2020). Furthermore, compared with DNA as a therapeutic, mRNA-based

therapy provides greater safety due to lack of genomic integration, no persistence *in vivo*, lack of exogenous elements in the RNA sequence and simple downstream purification (Orlandini von Niessen et al., 2019). Despite this, the use of mRNA for therapeutic application exhibits several problems including short half-life, susceptibility to enzymatic degradation, inefficient delivery to target cells and adverse immune reactions. Therapeutic mRNAs are designed and chemically modified to enhance intracellular stability, protection from nuclease degradation, translation efficiency and specificity, in order to achieve desirable immunogenicity for specific applications (To and Cho, 2021). Interestingly, UTRs can be modified to encode regulatory elements in order to control RNA expression in a cell-specific manner (Wroblewska et al., 2015). At the same time, some RNA base modifications, such as N1-methyl-pseudouridine, increase mRNA translation and reduce its immune-stimulatory activity (Andries et al., 2015). In addition, encapsulation of therapeutic mRNAs in nanocarriers (such as lipidic, polymeric and polypeptidic systems, gold and silica nanoparticles, and dendrimers) may provide more favourable half-life, better-controlled release, and cell type- and organ site-specificity (Lee et al., 2022).

Therapeutic mRNA appears a highly promising and innovative approach for the treatment of many diseases, including cancer, infection, rare diseases, metabolic, cardiovascular and immunology disorders, and any other disorder associated with functional loss of proteins (Weissman and Karikó, 2015; Xiong et al., 2018). The mRNA technology has been used in numerous pre-clinical studies and a low number of clinical trials. Indeed, although the use of mRNA as a therapeutic was discovered a while ago, its development has been slow. Currently, most mRNA-based ongoing clinical trials are still in Phase 1 or 2, and the only two mRNA-based therapies approved by the FDA are vaccines against SARS-CoV-2 (Gómez-Aguado et al., 2020; Pawlowski et al., 2021). However, the development and worldwide utilization of mRNA these mRNA vaccines showcase the enormous potential of mRNA technology. Finally, satisfactory results have been obtained with anticancer-mRNA-based vaccines that stimulate and activate anti-tumor immune responses via different immunotherapeutic approaches, such as the use of therapeutic mRNA encoding tumor antigen, antibodies, or immunomodulators (cytokines and co-stimulatory) (Beck et al., 2021).

Even though promising results have been obtained using mRNAs as therapeutics in pre-clinical and clinical studies and in the fight against SARS-CoV-2, several obstacles, including stability and delivery, need to be overcome.

2.4. Small interfering RNAs (siRNAs)

siRNAs are double-stranded non-coding RNAs, usually 20–25 base pairs in length, that inhibit gene expression by binding complementary mRNA targets (Dana et al., 2017). Although siRNAs share many similarities with miRNAs, including similar physicochemical properties and the ability to gene silencing through a mechanism called RNAi, they are two different classes of RNA with distinct functions and mechanisms of action (Davidson and McCray, 2011). Compared to miRNAs, siRNAs are produced from long dsRNAs and inhibit the expression of one specific mRNA target. Furthermore, siRNAs induce nuclease cleavage of full complementary mRNAs, while miRNAs repress translation, as they usually partially interact with the target mRNA (Lam et al., 2015). As a result, therapeutic applications of siRNAs and miRNAs are different: siRNA, inhibiting the expression of a specific mRNA, are used to produce a gene silencing effect. By contrast, miRNA-based therapy comprises two approaches: miRNA inhibition and miRNA replacement (Hu et al., 2020).

Successful siRNA-based therapy requires the design of siRNA-molecules with high activity and specificity relative to the desired target. Various strategies and chemical modifications are being investigated to improve siRNA-properties, such as stability, efficacy, potency, resistance to nucleases and circulating half-life, and to minimize immunogenic and toxic effects (Watts et al., 2008). In this regard, it has been observed that the presence of two-nucleotide overhangs at the 3′-end (usually TT or UU) promotes recognition by the RNAi machinery (Walton et al., 2010), while increased dsRNA-length enhances its potency (Kim et al., 2005). Moreover, as already observed for other types of therapeutic RNA, substitution of the ribose 2′-OH group with 2′-O-Me, 2′-F or 2′-methoxyethyl (2′-O-MOE) group increases stability and reduces immune system activation (Cekaite et al., 2007). For therapeutic application, the use of siRNA requires the development of safe and effective 'carriers' to deliver it to its site of action. There are two types of vectors, viral and non-viral (such as lipid-based carriers, peptides, polymers, and dendrimers) (Wang et al., 2010). A plausible strategy is bio-conjunction of squalene (SQ) to siRNA to obtain siRNA efficient nanoparticles (Massaad-Massade et al., 2018). Compared to conventional drugs, there are advantages of siRNAs as therapeutics, including the unrestricted choice of targets, safety, and high specificity and efficacy to suppress gene expression (Xu et al., 2019). The efficacy siRNA-based therapy has been evaluated in diverse diseases, including diabetes, hypercholesterolemia, macular degeneration, respiratory diseases, metabolic disorders, rare diseases, hepatitis, virus infections, and cancer. Several siRNA therapeutics have been investigated clinically, mostly for loco-regional treatments such as intravitreal and intranasal administration.

Currently, there are three FDA-approved siRNA drugs (patisiran, givosiran, and lumasiran) and seven siRNAs in late stages of Phase 3 clinical trials (Zhang et al., 2021b). Moreover, encouraging results were obtained using siRNAs in clinical trials involving a wide range of cancer. In this regard, Atu027, a siRNA-based lipid nanoparticle that inhibits the expression of protein kinase N3 (PKN3), has been tested in advanced or metastatic pancreatic cancer (Phase I2completed) and solid tumors (Phase 1), showing good feasibility, safety, and satisfaction, DCR-MYC, a synthetic double-stranded RNA encapsulated within lipid nanoparticles directed against MYC, has been used to treat solid tumors, multiple myeloma, non-Hodgkins lymphoma, and pancreatic neuroendocrine tumors (Phase 2 completed) (Hattab et al., 2021).

Despite the high therapeutic potential of siRNAs, several factors limit its clinical use, such as stability, poor cellular uptake, off-target effects, and immune responses.

2.5. Ribozymes

Ribozymes are RNA molecules with site-specific cleavage activity and catalytic potential. Ribozymes have been used successfully to inhibit gene expression *in vitro* and *in vivo* to treat many diseases including cancer. In this regard, Ad5CRT is a ribozyme that targets human telomerase reverse transcriptase (hTERT)-encoding RNAs in gastrointestinal cancer patients (Lee et al., 2019), and RPI.4610, an anti-VEGFR-1 ribozyme in conjunction with carboplatin and paclitaxel, to treat advanced solid tumor (Morrow et al., 2012). Despite initial success, further investigations are required to improve stability, efficacy, safety, delivery, and long-term expression (Khan, 2006).

2.6. miRNA

MiRNAs are ~22 nt in length, single-strand RNA (ssRNA) molecules, first discovered in 1993 in C. elegans (Lee et al., 1993) and recognised to target mRNA, causing its degradation or translation inhibition by suppressing protein expression (Bartel, 2018). At molecular level, miRNAs suppress protein expression mainly by binding to the 3′-UTR of mRNA targets. Indeed, a perfect pairing complementarity between miRNAs and target mRNAs leads to mRNA degradation, while imperfect matching promotes inhibition of protein translation. Consequently, a specific miRNA sequence may target several mRNAs, thereby affecting related cellular pathways. Therefore, although miRNAs are recognised non-coded transcripts, they orchestrate several biological processes involved in cellular homeostasis and organismal development (Bartel, 2018). Indeed, impaired miRNAs biogenesis results in dysregulation of their level, which has been demonstrated by scientific evidence to be correlated with severe disorders such as cancer (Peng and Croce, 2016).

Thus, it is important to understand the mechanism behind miRNA dysregulation, including chromosomal abnormalities, transcriptional miss-regulation and epigenetic alterations. These negative events represent the hallmarks of cancer pathologies, whereby the communication between tumor and other cellular components forming the stroma takes place via miRNAs distribution throughout the tumor microenvironment (TME) by directing cancer progression stages (Raue et al., 2021). Moreover, miRNAs exert either paracrine or endocrine signalling functions by circulating in blood and other biofluids. For this reason, the detection and evaluation of miRNA levels in extracellular milieu has been largely studied in pre-clinical and clinical trials for the timely diagnosis of several types of cancer (Wang et al., 2018a). If on one hand miRNAs present good biomarkers by identifying tumors in their early stage, on the other hand, circulating miRNAs show low specificity and issues concerning sensitivity, namely differences in their levels in individual patients, and similar miRNA levels in both malignant and benign tumors (Wang et al., 2018a).

Although miRNAs have been widely investigated as cancer biomarkers, there are still no FDA-approved miRNA therapies. As reported above, the ability of miRNAs to target multiple or single components in cellular pathways opens the possibility of exploiting both miRNA mimics and inhibitors (antimiRs) to re-establish altered miRNAs levels in TME. The first are double-stranded synthetic oligonucleotide copies of their specific miRNA counterparts, projected to restore reduced miRNA levels detected in TME. The second are antisense ssRNA molecules that have been designed on the basis of ASOs, developed to hybridize specific miRNA sequences by preventing their pairing with target mRNAs (Rupaimoole and Slack, 2017) (Fig. 1). Below, we elucidate the ongoing status of miRNA in clinical application as potential cancer therapeutics.

3. MiRNA-based therapies in clinic trials

Since their discovery, miRNAs have triggered interest as biomarkers and, over time, the scientific community started to understand their potential in clinical applications. Even though, great advances have been accomplished in miRNA molecules manipulation and their loading on delivery systems, miRNA-based therapies have not yet reached the phase 3 clinical trial. Indeed, diagnostic products specialised on miRNAs detection are already available in the pharmaceutical market (Ciarletto et al., 2021). By contrast, oncological miRNA drugs struggle to pass from bench to bedside. Nonetheless, investments towards miRNA-based therapies by pharmaceutical companies are increasing year by year (Chakraborty et al., 2020), indicating that this biotechnology is not far from entering the therapeutic market. In this paragraph we describe cancer therapeutics based on miRNA mimics and antimiRs currently in clinical trials listed in Table 1.

3.1. MiRNA mimics

An important step for miRNA mimics in entering clinical trials was accomplished with MRX34. MRX34 (Mirna Therapeutic Inc.) is formulated by an ionizable liposome identified as NOV340 (SMARTICLES®, Marina Biotech, Bothell, WA; Mirna Therapeutics Inc., 2011) loaded with a miR-34 mimic, recognised as a tumor suppressor miRNA. MiR-34 targets various oncogenes, whereby inhibiting tumor growth. Given that TME is characterised by a low pH, when NOV340 is near the tumor, it acquires positive charge resulting in its association with cancer cells (Bader, 2012). Following promising results in tumor suppression in a lung cancer mouse model (Wiggins et al., 2010; Trang et al., 2011), MRX34 was tested in a phase 1 clinical trial in which the complex was infused intravenously in patients with primary liver cancer, small cell lung cancer, lymphoma, melanoma, multiple myeloma, or renal cell carcinoma. Of 66 patients, 16 had stable disease (SD) for ≥4 cycles with a median duration of 19 weeks, while 3 patients had partial response (PR) and 31 had progressive disease (PD). Despite the majority of reported adverse events (AEs) being of grade 1 and 2, the test was suspended due to immune-related AEs that caused death in 4 patients (NCT01829971; NCT02862145) (Beg et al., 2017; Hong et al., 2020). Whether the serious immune response was triggered by the liposome carrier, GC-rich seed sequence of miR-34 (Gao et al., 2018) or by the impact of miR-34 on immune cells signalling (Hart et al., 2020), is still not clear.

Little progress has been achieved by a collaboration between EnGeneIC and the Asbestos Diseases Research Institute (Sydney, NSW, Australia), in which an miRNA mimic based on miR-16, a downregulated

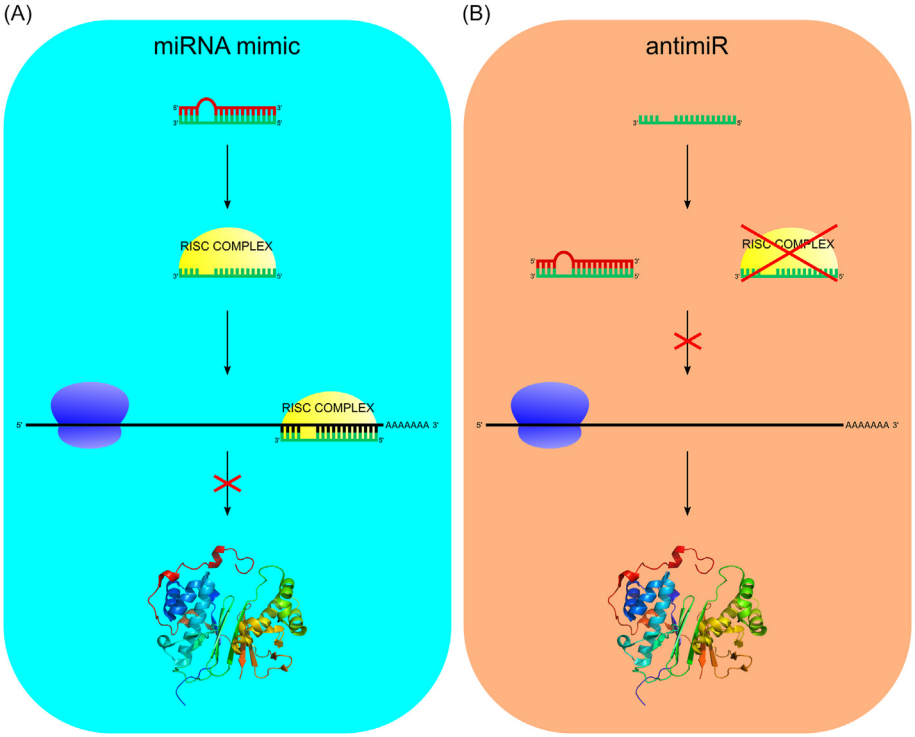

Fig. 1. MiRNA-based therapy. Double strand miRNA mimic molecules introduced into the target cell undergoes the passenger strand degradation. Afterwards the mimic leading strand integrated with RISC complex binds the 3′-UTR of mRNA target by inhibiting its translational activity (**A**). After cell internalization, the single strand antimiR oligonucleotide hybridizes with a specific miRNA sequence by preventing its pairing with mRNA target and protein translation is active (**B**). Protein AASDHPPT PDB 2byd image is licensed under the Creative Commons Attribution-Share Alike 3.0 Unported license. Protein AASDHPPT PDB 2byd image source: https://commons.wikimedia.org/wiki/File:Protein_AASDHPPT_PDB_2byd.png.

Table 1
MiRNA therapeutics - clinical trials.

MiRNA drug	Company	MiRNA	Delivery system	Cancer disease	Clinical trial phase	Clinical outcome	Adverse effects	Clinical trial	Ref.
MRX34	Mirna Therapeutic inc.	miR-34a mimic	Ionizable liposome	Solid tumors	Phase I - suspended	3 patients had PRs; 4 patients had SD $\geq$ 4 cycles (median, 19 weeks, range, 11–55).	G3 AEs in all 85 enrolled patients; 4 deaths due to immune-mediated AEs;	NCT01829971 NCT02862145	Hong et al., 2020
MesomiR 1	EnGeneIC + ADRI	miR-16 mimic	Bacterial minicell	MPM, NSCLC	Phase I - completed	1/22 patient had OR of 32 weeks; SD in 15 patients;	2 patients: infusion-related inflammatory symptoms and coronary ischaemia; 2 patients: anaphylaxis and cardiomyopathy; 1 patient: non-cardiac pain;	NCT02369198	van Zandwijk et al., 2017
Cobomarsen/ MRG-106	MiRagen Therapeutics inc.	miR-155 antimiR	Naked LNA	Mycosis fungoides	Phase I – completed; Phase II – terminated (business problems);	29 out of 32 (91%) had improvement in mSWAt score; 11 receiving 6 doses, achieved 50% reduction in mSWAT score;	No serious AEs; 2 AEs were deemed dose limiting toxicities: Grade 3 worsening ruritus and Grade 3 tumor flare.	NCT02580552 NCT03713320	Querfeld et al., 2018

ADRI = Asbestos Diseases Research Institute; MPM = Malignant pleural mesothelioma; NSCLC = Non-small-cell lung carcinoma; PR = Partial response; SD = Stable disease; OR = Objective response; mSWAT = Modified Severity-Weighted Assessment Tool; AE = Adverse effect.

miRNA in malignant pleural mesothelioma (MPM), has been loaded in a minicell conjugated with anti-EGFR antibody named targomiR. This targeted minicell containing miR-16, dubbed MesomiR-1, reached a phase 1 clinical trial (NCT02369198). Of 27 recruited patients, 22 patients showed an objective response, and 15 patients showed SD. Based on pre-clinical data, the authors suggested a phase 2 clinical trial to test MesomiR-1 in combination with chemotherapeutics and immune checkpoint inhibitors. However, several AEs have been described in the study, such as lymphopenia, inflammatory symptoms, and cardiac-circulatory problems (van Zandwijk et al., 2017).

3.2. AntimiRs

The unique antimiR used for cancer treatment and currently in clinical trial phase is represented by Cobomarsen (MRG-106), developed by MiRagen Therapeutics Inc. Cobomarsen is an LNA and inhibits miR-155. Up-regulation of miR-155 has been associated with uncontrolled immune cell proliferation and survival in cutaneous T-cell lymphoma (CTCL) and mycosis fungoides (MF). Due to a modified chemical structure, Cobomarsen is specially taken up by $CD4^+$ T and mycosis fungoides cells. *In vitro* studies on human lymphotropic virus type 1 (HTLV-1+) CTCL and MF cell lines shows that Cobomarsen inhibits cellular proliferation and induced apoptosis (Seto et al., 2018). A phase 1 clinical trial (NCT02580552) showed that 38 enrolled patients who received Cobomarsen reported no serious AEs and 29 of them registered an improvement of the Modified Severity-Weighted Assessment Tool (mSWAT) score. Duration of the study was 22 months and it investigated CTCL population, in a phase 2 clinical trial that was terminated due to economic problems (NCT03713320).

Of notes, an anti-miR-10b designed by Regulus Therapeutics Inc. (Carlsbad, CA, USA) and identified as RGLS5579 has been reported as a good candidate for the treatment of glioblastoma multiforme (GBM) in a clinical trial. When combined with temozolomide, this miR-10b inhibitor exhibited a remarkable increase of the median survival from 27% to 159% in a xenograft mouse GBM model (Wang et al., 2018b). However, this promising AntimiR is currently still in a pre-clinical phase.

4. MiRNA-based therapy strategies for cancer treatment

One of the strategies in the application of miRNA therapies is their administration in the form of naked nucleic acids. However, several challenges in terms of pharmacokinetics need to be addressed, namely their degradation by cellular and extracellular RNases, endosomal escape, cellular uptake, immunogenicity, and specificity in targeting tissues (Winkle et al., 2021). To face these issues, researchers elaborated two main solutions that comprise stabilization of miRNA drugs by means of chemical modification (Rupaimoole and Slack, 2017) or their loading using carriers (Roberts et al., 2020). Chemical modifications (ASO described previously) made miRNA mimics and antimiRs more stable, resistant to degradation and safer, however, the negative charges and hydrophilic nature of nucleic acids contributes to problems with cellular uptake resulting in higher dose administration that did not translate to high efficiency, mostly in the treatment of leukemia and metastatic cancers (Raue et al., 2021). For these reasons, an efficient delivery system for miRNA-based therapies is needed. Concerning this aim, great advances have been accomplished by the scientific community in developing different oligonucleotide carriers, namely viral or bacterial vectors, lipid- or polymer-based envelopes and oligonucleotide bio-conjugation (Roberts et al., 2020).

Viral and bacterial vectors appear to be plausible nanocarriers in terms of targeting and cellular uptake, but the risk of AE induction is still high (van Zandwijk et al., 2017; Monahan et al., 2021). Among non-viral vectors, lipid-based nanocarriers are the most standardized entities in terms of production and clinical use (Hou et al., 2021). Despite their wide use in the vaccination campaign against SARS CoV-2 virus variants, LPNs-PEG component has been reported to cause the so-called complement activation related pseudo-allergy (CARPA) and IgM and IgG production, thereby potentially provoking at first, an anaphylactic shock (Zhou et al., 2021) and immune reaction in patient administered with LNPs therapeutics and already exposed to PEG-containing products should be monitored and studied. Regarding polymer-based nanocarriers, considerable advances have been accomplished in terms of efficient uptake and safety: use of biodegradable chitosan cationic polymers as carriers exhibited less toxicity compared to previously used polymers *in vitro* and *in vivo* (Raue et al., 2021). However, long-term safety of polymers is still unclear as well as that of bio-conjugated oligonucleotides (Ha et al., 2016). A more promising option is represented by bio-conjugation, which consists of covalent conjugation of oligonucleotides with biomolecules such as lipids, peptides and sugars, in order to exploit their receptor-mediated endocytosis. However, the receptor

saturation effects should be investigated (Raue et al., 2021). The above-described miRNA delivery systems highlight favourable advantages, but several drawbacks remain unsolved (Table 1). In particular, their immunogenicity and accumulation in certain organs represent the major concern for viral and bacterial-derived vectors (Fu et al., 2019), while lipid- and polymer-based vehicles present low delivery efficacy, and their long-term safety is still unclear, similar as is the case of bio-conjugated oligonucleotides (Raue et al., 2021; Ha et al., 2016). Considering the listed hurdles of nanocarriers, a potential solution has been identified in exosomes. In the next sub-paragraph, we provide an overview of this promising miRNA transfer vehicle.

5. Exosomes as miRNA delivery systems

Exosomes, as natural carriers of miRNAs, have been proposed as a delivery system. Exosomes were discovered in 1981, known to be naturally produced by all cell types including procaryotes, and found in most biological fluids (Trams et al., 1981). Exosomes have the diameter of 30–160 nm and are classified as extracellular vehicles (EVs), a group of nano- and micro-particles characterised by a double phospholipid bilayer structure (Trams et al., 1981). Most EVs, such as microvesicles (MVs), micro-particles and large vesicles, originate from cell membrane via a budding mechanism, and they are released in the extracellular milieu. Conversely, exosomes derive from multivesicular bodies (MVBs), which are specialised endosomes containing intraluminal vesicles (ILVs) that are subsequently released out of the cell as exosomes once MVBs fuse with the cell membrane (Kalluri and LeBleu, 2020). Since their discovery, research on exosomes has shed light on their intriguing characteristics and important biological role (Fig. 2-upper panel). Raposo et al. (1996) described their involvement in immune responses, while Valadi et al. (2007) reported exosomes as natural miRNA carriers involved in intercellular communication (Simons and Raposo, 2009). As important carriers of signaling molecules (proteins, lipids, nucleic acids), exosomes are being developed as potential therapeutic agents or prognostic biomarkers in multiple disease models (Pegtel and Gould, 2019). Besides, as the most recent findings on exosomes and miRNAs has ready shown (Fig. 2), many aspects of exosome biology can reveal natural attributes as MBTs carriers, namely biogenesis, uptake, miRNA loading, safety profile and efficient delivery ability.

5.1. Exosome biogenesis and release

As reported, exosomes are formed as ILVs by a process that involves the endosomal system and are secreted upon fusion of endosomal MVBs with the plasma membrane (Tomasetti et al., 2017). Consequently, exosomes have the same membrane topology as donor cells, even though they often lose transmembrane lipid asymmetry and have phosphatidylserine (PHS) residues externalized in the outer leaflet of the vesicle. Currently, pathways involved in exosome biogenesis include the endosomal sorting complex required for transport (ESCRT)-dependent and -independent mechanisms. ESCRT machinery comprises five complexes, i.e. ESCRT-0, –I, –II, –III, and Vps4. These complexes are composed by multiple subunits and orchestrate ILVs formation together with cargo sorting (Gurung et al., 2021). In parallel, ESCRT-independent pathway relies on sphingolipid (SL) ceramide domains present in the MVBs membrane, and it was proposed that biogenesis of exosomes occurs via formation of SL-enriched ceramide microdomains and their fusion into larger domains, by promoting inward vesicle budding (Trajkovic et al., 2008) (Fig. 3).

Ceramide and sphingosine-1-phosphate are the main SLs that act as signaling molecules, controlling a vast number of cellular processes, such as their growth, adhesion, migration, senescence, and cell death (Hannun and Obeid, 2008). The role of ceramide in the biogenesis and release of exosomes was studied by treating cells with GW4869, a specific inhibitor of neutral sphingomyelinase (nSMase) 1/2, and by two structurally unrelated nSMase blockers, spiroepoxide and glutathione (Dinkins et al., 2014). Exosome release was markedly reduced after treatment of the cells with all nSMase inhibitors, which was confirmed by nSMase-2 siRNA-mediated depletion (Trajkovic et al., 2008). Exogenous cell-permeable C6 ceramide dose-dependently increased the number of exosomes released from multiple myeloma cells (Cheng et al., 2018). However, exosomes are a heterogeneous population of vesicles and blocking of nSMases does not block the release of all exosomes or impair exosome biogenesis in all cells (Colombo et al., 2013). Of note, tetraspanin membrane proteins have been also described to affect exosome biogenesis (Trajkovic et al., 2008).

The nSMase and Ras-associated binding (Rab) proteins are canonical regulators of exosome secretion (Fig. 4). Studies have demonstrated that Rab11 is involved in the docking of MVBs to the plasma membrane, and upon induction of autophagy, Rab11 co-localizes with the autophagic marker LC3, which is associated with decreased exosome release (Savina et al., 2002). Silencing of Rab27A and Rab27B reduced exosome secretion (Ostrowski et al., 2010).

5.2. Role of exosomes in cancer

It has been reported that tumor-bearing patients have increased exosomes in their circulation compared to healthy subjects (Kharaziha et al., 2012). Tumor-derived exosomes (TDEs) are actively produced and released by tumor cells and carry messages from tumor cells to healthy cells or abnormal cells, and they participate in the metastatic disease (Bai et al., 2022a). Overexpression of EGFR in glioma cells increased secretion of exosomes that can be taken up by other glioma cells lacking EGFR, resulting in 'transmission' of oncogenic activity (Al-Nedawi et al., 2008). Also, expression of oncogenic RAS in non-tumorigenic epithelial cells increased secretion of exosomes carrying HRAS DNA, RNA and proteins,

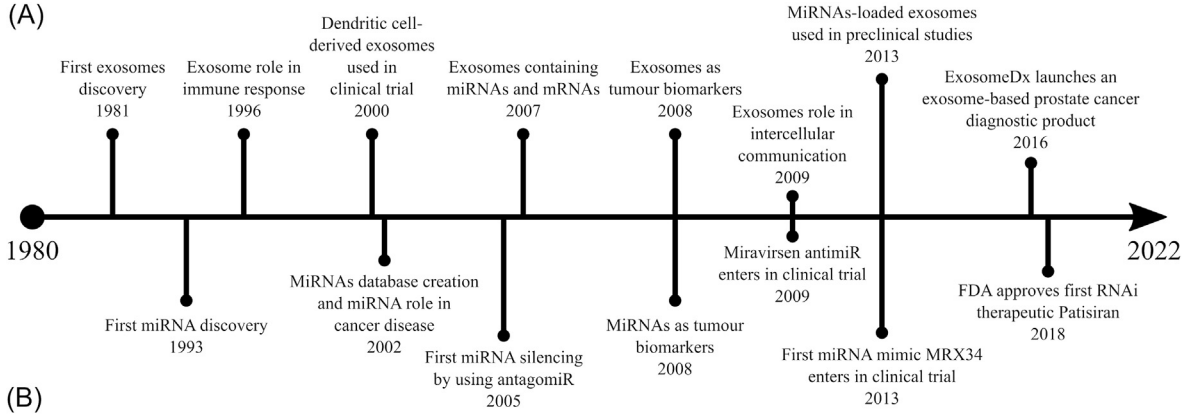

Fig. 2. Discovery timeline. MiRNA (upper) and Exosomes (down) discoveries timeline.

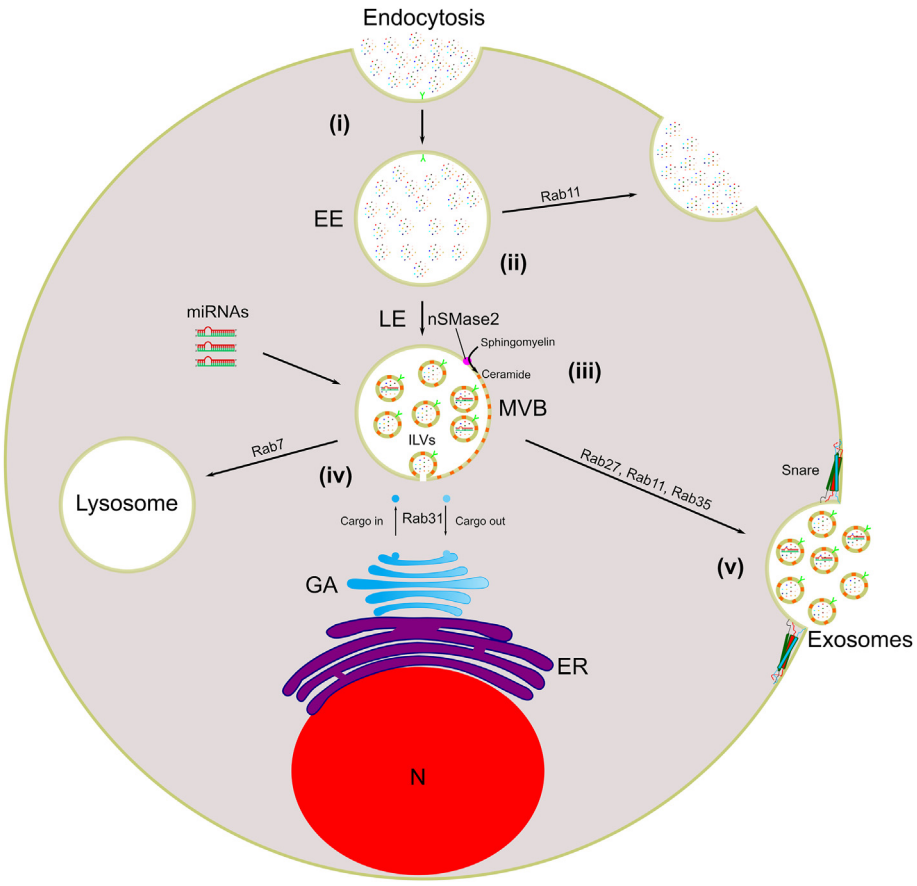

Fig. 3. Exosomes biogenesis. Following internalization, the extracellular cargo is sorted into early endosome (EE) compartment (***i***). The EE may back-fuse with plasma membrane by releasing its content or it may continue a maturation process resulting in late endosomes (LE) formation (***ii***). On the LE membrane the nSmase2 enzyme regulates the hydrolysis of sphingomyelin into phosphorylcoline and ceramide which self-organizes in lipid raft by determining the initial step for intraluminar vesicles (ILVs)-forming inward buddings which internalize the LE content and LE matures into multi vesicular body (MVB) (***iii***). Given the plasma membrane origin of endosome, ILVs share surface proteins and lipid composition with cell membrane. In addition, contents from cytosol and vesicles from Golgi potentially compose ILVs cargo. At this maturation stage, the MVB may be targeted towards lysosome vesicle with consequent ILVs cargo degradation (***iv***). Otherwise, the interplay between Rab-GTPase (Rab11, Rab27 and Rab35) and SNARE proteins regulates docking and subsequent fusion of MVB with plasma membrane and ILVs are released as exosomes (***v***).

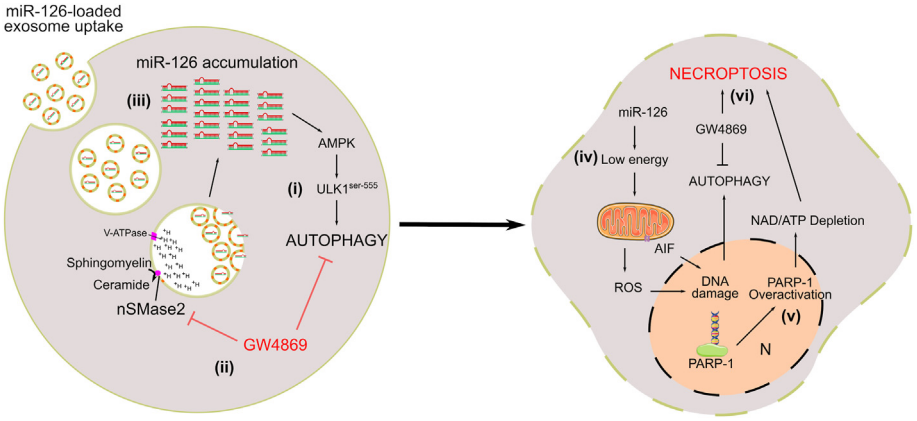

Fig. 4. Mechanism of the MiR-126 and GW4869 combination. Exosome-mediated introduction of miR-126 promotes autophagy through AMPK-ULK1ser-555 pathway (***i***), while GW4869 inhibits autophagy and multi vesicular body (MVB) formation and release (***ii***). Thus, the prevented exosome release leads to miR-126 accumulation within the cell by enhancing the opposite effect to GW4869 (***iii***). MiR-126 disrupts insulin signaling pathway, resulting in lower glucose uptake (***iv***). This stress state induces mitochondria to produce ROS with consequent DNA damage and PARP-1 activation (**v**). Autophagy inhibition by GW4869 leads to a feedback loop in which apoptosis-inducing factor (AIF) is released from mitochondria and PARP-1 is overactivated. When overactivated, PARP-1 sequestrates NAD + to mitochondria, thus inducing ATP depletion and subsequent necroptosis (**vi**).

which can be transferred to recipient cells (Lee et al., 2014). Inhibition of RAS signaling with a farnesyl transferase inhibitor (tipifarnib) or manumycin A decreased exosome secretion in prostate cancer (Datta et al., 2017, 2018). Conversely, restored expression of liver kinase B1 (LKB1/STK11), a tumor suppressor frequently mutated or lost in lung cancer, increased exosome secretion (Zhang et al., 2018).

5.3. Uptake of exosomes

In general, there are many aspects of tumor progression regulated by cancer cells and tumor environment that can impact the exchange of exosomes. Various mechanisms for exosome uptake have been proposed, including clathrin- and caveolae-mediated endocytosis, phagocytosis, macro-pinocytosis and plasma or endosomal membrane fusion (Mulcahy et al., 2014), with exosome internalization being an active process.

Donor cells affect the composition of released exosomes, and lipid rafts and proteins present on the surface of exosomes have been reported to affect the exosome uptake rate into recipient cells (Escrevente et al., 2011). PHS is critical in the uptake of exosomes. It has been found that blockage of PHS sites on hypoxia-induced mesenchymal stem cells (MSC)-MVs with Anx-V greatly reduces their incorporation into HUVECs. The results demonstrate that PHS on hypoxia-induced MSC-MVs is a key molecule responsible for internalization, and other surface molecules may also play a role in the process (Wei et al., 2016).

Therefore, one may assume that different exosomal membrane phospholipids and protein expression could play a role in tissue-selective

exosomal uptake. By contrast, it has been reported that exosome from A549 (lung cancer), HCT116 and COLO205 (colon cancer) cells are incorporated into both donor and recipient cells, and irrespectively of the donor cells, the exosome uptake level was the greatest in HCT116 cells. This suggests that exosomal uptake capability is not dependent on the expression of exosome marker proteins but on the recipient cells (Horibe et al., 2018a). Indeed, exosomes from HUVECs were efficiently internalized by the donor cells themselves and by other cellular components of the tumor environment, such as mesothelial cells, fibroblasts, and malignant mesothelioma cells (Monaco et al., 2019). Receptor-mediated endocytosis (RME) is another proposed mechanism of uptake. While RME traditionally is associated with clathrin-mediated endocytosis, the receptor/ligand interaction facilitating uptake has also been linked to several other endocytosis categories. Some of the well described receptor-ligand complexes include low-density lipoprotein (LDL) and its receptor (LDLR), or transferrin (Tf) and transferrin receptor (TfR). The LDL/LDLR complex is endocytosed and ends up in the lysosome, which allows for LDL degradation into free cholesterol for cellular function. TfR, on the other hand, releases its iron cargo within the endosome and is then recycled back to the cell surface with Tf and TfR intact. The receptor and ligand fates differ based on the receptor and the mechanism of endocytosis.

Whether or not exosomal uptake is a cell type–specific process han not been resolved. Some studies showed that exosomes can be taken up by virtually every cell type tested (Svensson et al., 2013), while others suggest that vesicular uptake is a highly specific process which can only occur if cell and exosomes share the right combination of ligand and receptor (Zech et al., 2012).

5.4. Exosome RNA loading

Certain miRNAs rather than others can be packed into exosomes via a selective process. Indeed, various sorting mechanisms affect the exosome content depending on the cell of origin and its physiological state. A key role in this scenario is played by RNA-binding proteins (hnRNPA2B1, Ago2, YBX-1, MEX3C, MVP, La protein) and membranous proteins (caveolin-1 and nSMase2), which are responsible for selective sorting during exosome biogenesis. The consequent miRNA regulatory functions or dysregulation involved in pathogenesis depends on these mechanisms (Groot and Lee, 2020). For example, a group studying myocardial fibrosis reported YBX-1-mediated sorting of miR-133 into endothelial progenitor cell-derived exosome that increased miR-133 levels in cardiac fibroblasts by promoting mesenchymal-to-endothelial transition (Lin et al., 2019). Another interesting work describes the tumorigenic effect and maturation of pre-miRNAs within breast cancer cell-derived exosomes containing the RISC-complex components that in contrast were not present in exosomes derived from normal breast cells (Melo et al., 2014). Recently, Ago2 selective packaging into colon cancer cell-derived exosomes was shown to be controlled by the KRAS-MEK signalling pathway (McKenzie et al., 2016). Taken together, this information suggests the possibility to enrich exosomes with specific miRNAs by exploiting donor cell selective sorting machinery, which may also influence miRNA biological activity in target cells. Nevertheless, the proper regulation of miRNA sorting is still unclear.

Despite many benefits, loading of RNAs into exosomes represents one of their limits for *in vivo* application. There are several approaches to incorporate RNA into exosomes for therapeutic applications (Amiri et al., 2022). One approach includes pre-loading of RNA by transfecting donor cells. In this case, parental cells are transfected with siRNA/miRNA and exosomes collected. It was found that adipose-derived stem cell (ADSC)-derived exosomes enriched with miR-126 showed a protective effect on acute myocardial infarction (Luo et al., 2017). Transfection of HUVECs with miR-126 mimics enhanced the miR-126 content in exosomes (300-fold increment), and miR-126-enriched exosomes treatment inhibited angiogenesis and induced cell death and *in vivo* tumor growth arrest in MPM (Monaco et al., 2019, 2022). In another study, exosome from HEK293T cells transfected with anti-miR-214 reversed cisplatin resistance of gastric cancer (Wang et al., 2018c). Moreover, miR-34a-loaded exosomes, after transfection of MSCs, efficiently inhibited breast cancer cell proliferation (Vakhshiteh et al., 2021). Although this approach seems simple and feasible, the method is limited by cytotoxicity, poor specificity, and inefficient packaging (Liu and Su, 2019). These problems can be solved by using appropriated donor cells; exosomes produced by donor cells that naturally package the miRNA of interest may overcome the miRNA-induced cytotoxicity and the inefficiency in miRNA packaging. As reported by Monaco et al., miR-126 can be easily and efficiently loaded in HUVECs that naturally produce and package miR-126 into exosomes (Monaco et al., 2019, 2022).

Another method to reduce the pre-loading limits is the cellular-nanoporation procedure. This approach was established for large-scale production of exosomes with therapeutic mRNA molecules (Zarovni et al., 2015). The cells transfected with plasmids were provoked with focal/transient electrical stimulations, which induce secretion of exosome (55-fold more vesicles) harboring transcribed mRNA (1000-fold increment).

The post-loading of RNA procedure includes electroporation of exosomes, exosome transfection with specific reagents, and production of exosome-liposome hybrids. Electroporation has been widely applied for loading siRNA into exosomes, while this is not adequate for miRNAs, small hairpin RNAs and mRNA insertion (Ohno et al., 2013). An alternative post-loading RNA approach is transfection of exosomes with specific reagents, such as lipofectamine, or Exosome-Fect, enabling insertion of small molecules, DNAs or RNAs into isolated exosomes. However, the main disadvantage of this method is that exosomes cannot be separated from the transfection reagent (Li et al., 2018). The best performance was achieved by fusing exosomes with liposomes. The exosome-liposomes hybrid can efficiently package the CRISPR-Cas9 expression vector as a large plasmid (Lin et al., 2018).

6. Clinical aspects of exosomes

In the previous paragraph we described exosomal features that show their potential as natural MBT transfer vehicles. Here we discuss whether and how exosomal features suit their clinical application.

As potential MBTs carrier for cancer treatment, exosomes must meet specific requirements, namely high-yield production, safety, target specificity, cargo internalization and release. In this paragraph we describe how exosomes meet these requirements, and we also suggest possible solutions when these needs are not satisfied. Furthermore, we depict a new and promising interesting strategy to treat cancer, which involves the use of miRNA-loaded exosomes and which exploits regulation of exosome biogenesis within the cell.

6.1. Exosome production

Acquisition of exosomes involves a culture of the donor cells, harvesting from the conditioned medium, and separation or purification. The cells mainly used in the production of exosomes include MSCs, ADSCs, dendritic cells, and HEK293 cells. Exosomes secreted by MSCs are rather well studied in the context of the treatment of a range of therapies.

In addition to the appropriate selection of donor cells for exosome production, exosome isolation is the key factor for their potential clinical applications for exosome-based therapies. Exosome isolation methods include ultracentrifugation and density-gradient centrifugation. The most widely used method for exosome isolation is ultracentrifugation, which consists of multiple centrifugation steps with increasing centrifugal strength to sequentially pellet cells (300 g), cell debris (10,000 g) and exosomes (100.000 g). In addition to this method, easy-to-use precipitation solutions such as ExoQuick and Total exosome Isolation have been commercialized in the last few years with no need for expensive equipment or skillful techniques. Moreover, methods using these kits have an advantage in that they can purify exosomes from smaller volumes of cell

culture media and blood than the ultracentrifugation method. Although their mode of action has not been disclosed, these kits are commonly used. However, exosome isolated by precipitation methods showed no or low expression of exosome marker proteins such as CD63, CD9, CD81 and HSP70 (Horibe et al., 2018b).

Nevertheless, exosome production yielding needs proper starting material and needs to be improved. A focus in this sense regards the upregulation of proteins involved in exosome biogenesis such as STEAP3, SDC4 and NadB (Kojima et al., 2018). Another approach involves increase in exosome production by regulating the exosome release pathway. The treatment of donor cells with monensin increased intracellular calcium concentration, which in turn increased exosome secretion (Savina et al., 2003). Therefore, by acting both on exosome biogenesis and their release, exosomes can be produced on a large scale for clinic application (Qu et al., 2023).

6.2. Exosome safety

In terms of safety, each exosomal therapeutic employment must be evaluated to maintain the safety profile confirmed by *in vivo* experiments. Indeed, immunocompetent mice showed no cytotoxic effect and no immune response when administered intravenously (IV) or intraperitoneally (IP) with 10^{10} engineered and wild type HEK293-derived exosomes for 22 days (Zhu et al., 2017). MSC-derived exosomes (MSC-EXOS) were regularly administered in patients without side effects (Kordelas et al., 2014). Furthermore, tumor and dendritic cells-derived exosomes have been tested in clinical trials to activate anti-cancer immune responses and, except one study with 10% of patients showing grade 3 or 4 toxicity (Besse et al., 2015), no toxicity higher than grade 2 was reported (Dai et al., 2008; Escudier et al., 2005; Morse et al., 2005). A pilot study evaluated the safety of placental MSC-EXOS in patients affected by malignant middle cerebral artery infarct (mMCAI). Intraparenchymal implantation of MSC-EXOS showed no post-interventional AEs in five ischemic stroke patients. Local injection of exosomes for treatment of mMCAI can be safe and in future, it may be applied as a supportive, restorative, and preventive treatment in patients who suffer from acute ischemic stroke and post ischemic disability (Dehghani et al., 2022). All these studies confirm reasonable exosomal safety profile; however, attention must be paid with exosomes obtained from tumor cells due to their potential oncogenic cargo (Bai et al., 2022b). Tumor cells actively produce, release, and utilize exosomes to promote tumor growth (Whiteside, 2016).

6.3. Target specificity, cargo internalization and release

The route of administration can influence the tissue distribution of MBTs-loaded exosome *in vivo*. Intravenous administration of exosomes is an appropriate delivery route for malignancies. After intravenous injection, the pharmacokinetic profile of exosomes showed a half-life of around 2 min in systemic circulation, with minimal presence observed after 4 h. Although intravenous administration allows exosomes to reach the target site, their short half-life index in circulation is one of the major limitations of this route of administration. Another route for cancer types is represented by intratumoral injection of exosomes loaded with a therapeutic agent. The advantage of this approach is that direct injection of exosomes to tumors allows specific delivery of the therapeutics (Takahashi et al., 2013). Exosomes were detected in the liver, lungs, kidneys, and spleen 1 h after intraperitoneal injection, while exosomes were found distributed in brain and intestines after intranasal administration. However, they were rapidly cleared from the systemic circulation by the liver (Sun et al., 2010).

It has been stated that in case more nanocarriers are present in circulation, there is a higher probability that they reach a specific target. Therefore, whether the natural ability in crossing physiological barriers (Alvarez-Erviti et al., 2011) confers longer-term half-life of exosomes or faster clearance is still debated (Smyth et al., 2015; Lai et al., 2014; Kooijmans et al., 2016). Because of this, considerable effort has been taken in exosome bioengineering to increase their concentration in target sites, circulation time, and half-life in the body. Exosomes should be engineered to preferably target cell types relevant to disease pathogenesis, e.g., rabies virus glycoprotein is used to engineer exosomes specifically for central nervous system (CNS) disease (Ferrantelli et al., 2020). Exosomes harboring the GE11 peptide on their surface efficiently deliveres miRNA to EGFR-expressing cancer tissues (Ohno et al., 2013). However, exosomes with the surface protein CD47 exhibit prevention of phagocytosis by monocytes and macrophages by ensuring a low clearance rate and extended half-life (Kamerkar et al., 2017). Moreover, to understand exosome targeting ability, the concept of exosome heterogeneity needs to be addressed. As previously mentioned, depending on cell of origin, exosomes express specific surface proteins affecting their targeting capacity concerning the recipient cell as well as their internalization pathways and therapeutic effects (Kalluri and LeBleu, 2020). For instance, major histocompatibility complex class II molecules are enriched in exosomes from B lymphocytes, dendritic cells, mast cells, and intestinal epithelial cells, whereas higher levels of growth factors and their receptors are found in exosomes released from cancer cells (Simpson et al., 2008).

For allogeneic exosomal therapy, the presence of major histocompatibility complex proteins is problematic owing to potential immune responses. Exosome heterogeneity allows to select cell-producing exosomes to target the desired tissue type by obtaining the desired therapeutic effect depending on the way of entrance (Ohno et al., 2013). An efficient delivery system must accomplish a proper cargo release at intracellular level dependent on the endosomal escape capacity of the nanocarrier. Currently, the mechanisms of the cargo exosome delivery are poorly understood, however it was found that during the pH drop in the endo-lysosomal compartment, the exosome membrane undergoes destabilization and fuses with the endo-lysosome membrane by releasing its content in the cytoplasm through a protein-mediated mechanism (Bonsergent and Lavieu, 2019; Joshi et al., 2020). A strategy suggested to avoid the endosomal pathway and increase the cytosolic miRNA drug delivery is presented by the fusion of exosome membranes with fusion proteins such as SYCY1, SYCY2 and EFF-1 (Prada and Meldolesi, 2016).

6.4. Combination of MiRNA-exosomes and exosome-released inhibitors as new strategy

Irrespective of the route of administration, it was observed that the miRNA-cargo delivered by exosomes into cancer cells was immediately released by the cell themselves in the tumor micro-environment through exosomes themselves (Monaco et al., 2019). Exosomes have been used by cells as disposal mechanisms for unnecessary or unwanted miRNAs (Yu et al., 2016). Iinhibition of exosome release by an inhibitor of nSMase2 (GW4869) caused accumulation of the miRNA within the cell in a dose- and time-dependent manner to induce cell death (Monaco et al., 2022). This therapeutic approach may overcome the clearance problems, thus allowing the use of low amount of exosomes in a single dose. Tumor-suppressive miRNAs are involved in regulating cancer cell survival, and secretion of these miRNAs has been documented in malignancies (Kanlikilicer et al., 2016; Munson et al., 2019). It was previously reported that over-expression of miR-29b induces apoptosis and caspase-3 activation in multiple myeloma (MM) cells (Zhang et al., 2011) and that miR-15a/16 can promote MM apoptosis by suppressing expression of the anti-apoptosis protein Bcl-2 (Li et al., 2016). MPM significantly secrets higher levels of miR-16–5p, blocking of exosome release using GW4869, leading to significant reductions in exosomal miR-16–5p and significant increase cytoplasmic miR-16–5p (Munson et al., 2019).

Adopting a new approach, Monaco et al. combined the effects of miR-126-loaded exosomes and GW4869, and obtained promising results using an MPM-derived spheroid model. MiR-126 induces autophagy and autophagosome formation via the AMPK-ULK1ser-555 pathway

activation; in contrast GW4869 inhibits autophagy. This short circuit of autophagy induction and inhibition leads to necroptosis cell death associated with PARP1 activation. As previously said, GW4869 prevents exosome release with consequent amplified effect of miR-126 and accumulation within the target cell (Monaco et al., 2022) (Fig. 4). In this scenario, ceramide biosynthesis seems to be the meeting point between exosome biogenesis and autophagy (Mathieu et al., 2019). These two pathways encounter each other when MVB and autophagosome vesicles are not degraded into the lysosome and fuse together by originating amphistome, which is responsible for exosome secretion (Salimi et al., 2020). Considering this, nSMase2 inhibition by GW4869 ceases the biosynthesis of ceramide, consequently, ILV biogenesis and MVB formation are repressed as well as exosome releasing via the amphisome-forming pathway. In summary, both routes of exosome release, by means of MVB and autophagy, are inhibited. Therefore, after uptake of the miR-126-loaded exosome by cell targets, re-packaging and secretion of internalized miR-126 is repressed by interfering with the crosstalk between exosome formation and autophagy induction, and maximum therapeutic effect of miR-126 is obtained (Fig. 4).

6.5. *nSMase2 inhibitors*

Given the unique role for nSMase2 in the control of ceramide-dependent exosome release (Verderio et al., 2018), several nSMase2 inhibitors have gained increasing attention as a strategy to regulate ceramide levels in various disease conditions, including cancer (Hwang et al., 2015). The drug GW4869 is the most widely used in research but has poor solubility due to its highly hydrophobic nature (practically insoluble in water with poor solubility in organic solvents such as DMSO), which limits its clinical potential. Compared to GW4869, cambinol was thought to be a more attractive candidate due to its improved solubility; however, it was found to have a poor *in vivo* pharmacokinetic profile. Scyphostatin and manumycin A are known for their inhibitory activity towards nSMase2. However, lack of information related to specificity, potency, and physicochemical properties, limit the use of these inhibitors in the clinic (Kumar and Kumar, 2021). A strategy to enhance their solubility, stability, and bioavailability is that these hydrophobic drugs are loaded in exosomes; both the drug and the exosomes can be incubated together so that the drug diffuses into exosomes along the concentration gradient. The drug loading efficacy using this method is related to the hydrophobicity of the drug because of the potential of hydrophobic drugs to interact with lipid bilayer membranes (Butreddy et al., 2021).

The farnesyl transferase inhibitor tipifarnib also showed an exosome inhibitory effect (Datta et al., 2018). Janssen commenced phase 2 clinical trials in patients with advanced non-small cell lung cancer and, phase 3 trials for potential treatment of pancreatic cancer and leukemia, and finally phase 2 trials for RAS-dependent solid tumors. Credit Lyonnais Securities predicted NDA filings in 2002 and 2003 for pancreatic cancer and other cancers, respectively (Norman, 2002).

Recently, by screening 365,000 compounds from the Molecular Libraries Small Molecule Repository (MLSMR) and 2816 compounds from the NCGC pharmaceutical collection (NPC) library for human nSMase2 inhibitors, DPTIP (2,6-dimethoxy-4-(5-phenyl-4-thiophen-2-yl-1H-imidazol-2-yl)-phenol) was identified as the most promising compound, based on its selectively, potency and chemical optimization feasibility (Rojas et al., 2018). Ketoconazole (KTZ) is an FDA approved anti-fungal medication, which has been shown to suppress exosome biogenesis and secretion (Greenberg et al., 2021).

7. Conclusion and future perspectives

Among the RNA therapies, miRNA-based therapy has demonstrated clinical potential for cancer treatment. Despite that the onco-suppressor role of miRNAs is well established, a safe, effective, and targeted drug vehicle to protect them from degradation and facilitate their targeted delivery *in vivo* is lacking. The recent introduction of exosomes, with their natural compartmentalization properties, have successfully 'beaten' the traditional vehicles to provide strong support for miRNA therapy. A new strategy would be to introduce the miRNA in cancer cells by means of exosomes and accumulate it within the cells themselves by inhibiting nSMase, an enzyme involved in exosome release. This therapeutic approach allows the miRNA to accumulate in cancer cells in a dose and within time periods to induce cancer cell death. In addition to its role in inhibiting exosome release, nSMase is also involved in autophagy. Concomitant inhibition of exosome release and the autophagic process concur to induce cell death, which was higher in cancer cells that use autophagy for survival, such as in the case of cancer stem cells.

MiRNAs and exosomes are both used in clinical trials and are nearing their approval by the FDA; treatment of cancer by an inhibitor of exosome release already FDA-approved will further increase the onco-suppressive performance of miRNA-based therapy.

Declaration of competing interest

The authors declare no conflict of interests.

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