Review

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Trials and tribulations of microRNA therapeutics

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Abstract: Non-coding RNAs, specifically microRNAs (miRNAs), exhibit altered expression and contribute significantly to the pathological processes observed in numerous diseases, encompassing various types of human cancers. Recent discoveries regarding miRNAs underscore their crucial roles in tumor pathogenesis and their responses to diverse therapeutic interventions. miRNAs are a subset of non-coding RNAs that regulate the expression of a multitude of genes post-transcriptionally and thus are potential diagnostic, prognostic, and predictive biomarkers and have also emerged as potential therapeutics. Because miRNAs are involved in the post-transcriptional regulation of their target mRNAs via repressing gene expression, defects in the miRNA biogenesis pathway and miRNA expression perturb the expression of a multitude of oncogenic or tumor-suppressive target genes that are involved in the pathogenesis of various cancers. As such, numerous miRNAs have been identified to be downregulated or upregulated in many types of cancers functioning as either oncomiRs or oncosuppressor miRs. Moreover, dysregulation of miRNA biogenesis pathways can also change miRNA expression and function in cancer. Profiling deregulated miRNAs in many cancer types has been shown to correlate with disease diagnosis, indicate optimal treatment options, and predict response to a specific therapy. Specific miRNA signatures can track all stages of disease including many cancer types and hold potential as biomarkers and therapeutic targets as well as therapeutics as miRNA mimics and inhibitors (antagomirs). As such, identifying specific miRNAs and mRNAs they regulate in many types of cancer along with downstream pathways can be used as potential therapeutic targets. Because a single miRNA can regulate a pool of targets involved in similar cellular processes and pathways, thereby amplifying the cellular response, this potentially makes miRNAs powerful therapeutics to restore cell functions that are altered as part of a disease phenotype. However, because a single miRNA regulates multiple gene expressions their cellular effects are numerous leading to potential off-target effects. Besides their intended targeting issues, there is a need to reduce the immunogenic reactions and determine the minimal dosing to achieve the desired effect while minimizing side effects. As such, a careful risk evaluation of miRNA therapeutics is needed to minimize off-target effects and to avoid overdosing of miRNA drugs. Furthermore, a limited understanding and validation of the specific roles of miRNAs limits their clinical application. In addition, there are many challenges concerning the delivery, sensitivity, specificity, toxicity, and applicability of the potential utility of miRNAs as therapeutic targets or therapeutics. Thus, future work will warrant if miRNAs can be used as cancer biomarkers as well as therapeutics for clinical application.

Keywords: microRNAs, miRNAs; post-transcriptional gene regulation; miRNA therapeutics; miRNA mimics; antimirs; antagomirs

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1. Introduction

Although the majority of oncology research is still focused on the dynamic variations of proteins and protein coding RNAs (their corresponding coding sequences only account for ~2% of the genome), the role of non-coding RNAs (ncRNAs) transcribed from the remaining 98% of the genome including microRNAs (miRNAs) plays key roles in a multitude of biological processes in normal physiological states but also the development of various types of diseases including cancer underscoring the importance of miRNAs and other ncRNAs in tumor initiation and progression. While most of the research in oncology predominantly centers around the ever-changing aspects of proteins and the RNA molecules responsible for coding those proteins, it's important to note that these coding sequences account for only about 2% of the genome (https://www.genomicseducation.hee.nhs.uk/genotes/knowledge-hub/non-coding-dna/) [1-3]. However, the remaining 98% of the genome, which includes non-coding RNAs (ncRNAs) such as miRNAs, plays pivotal roles in numerous biological processes during typical physiological conditions, as well as in the onset and advancement of different diseases, including cancer [4]. This emphasizes the significance of miRNAs and other non-coding RNAs in the initiation and progression of tumors.

In addition, miRNAs play key roles in the regulation of gene expression at the transcriptional [5-7] and post-transcriptional [8-11] levels, and exhibit tissue-specific [12, 13] and developmental expression patterns [14-16] and play key functional roles in a broad range of biological processes within cells and organisms. Altered expression of miRNAs has emerged as an additional molecular mechanism implicated in the pathogenesis of numerous diseases [17-19], spanning innate immunity [20], autoimmunity and autoimmune diseases [21], viral infections [22-25], acute hepatitis [26], depression [27], anxiety [28], Alzheimer's disease [29], Huntington's disease [30], metabolic and cardiovascular diseases [31-34], diabetes [8, 33-38] and a many types of cancers [12, 39-69]. Consequently, these miRNAs can serve as indicators for the presence of a pathological condition, as well as provide insights into its stage, progression, or genetic associations.

More recently, there is an emerging evidence suggesting that diet-derived exogenous miRNAs (or "xenomiRs") can enter the circulatory system and tissues, potentially influencing gene expression and biological functions [70-75]. Uptake of miRNAs by gastric and intestinal cells as well as their potential effects on the gut microbiota by orally delivered miRNAs and their potential immunomodulatory properties indicate the possibility of cross-species or cross-kingdom communication through miRNAs [75]. Because of these observations, one potential method of administering miRNAs is orally. MiRNAs are often associated with extracellular vesicles (EVs), RNA-binding proteins, lipoproteins, or lipid derivatives, along with nanoparticles. These protective elements shield miRNAs from the adverse gastrointestinal environment, including salivary and pancreatic RNases, the stomach's low pH, digestive enzymes, peristaltic activity, and microbial enzymes. Such protection likely facilitates the absorption of miRNAs from the digestive tract [75]. However, there is ongoing debate surrounding the absorption, stability, and physiological impact of these food-derived miRNAs and there are contrary findings regarding the bioavailability and the in-human functionality of miRNAs contained in plant food [76, 77].

Ongoing research continues to unravel new insights into the molecular mechanisms underpinning the dysregulation of miRNA biogenesis and expression in cancer. For example, it is widely acknowledged that genetic deletions or amplifications, epigenetic methylation of miRNA genomic loci, and modifications influencing the regulation of primary miRNAs (pri-miRNA) by transcription factors, as well as components involved in the miRNA biogenesis pathway frequently alter miRNA expression and function in many cancers [56, 78, 79]. Furthermore, additional factors, such as oncogenic drivers like mutations in the KRAS gene, can also affect global miRNA biogenesis and effector function, contributing to the broader dysregulation of miRNAs [80]. Consequently, miRNAs and their dysregulation have garnered significant interest from both academia and industry as a focal area of research for both understanding of disease biology and explore their

applications as potential diagnostic, prognostic, and predictive biomarkers [68] as well as well as their roles as drug targets or therapeutic agents [81].

It is well-established that miRNAs are powerful genetic regulators of diverse biological and developmental processes, in addition to playing a pivotal role in the pathogenesis of various diseases. This is due to the ability of a single miRNA to regulate entire cellular pathways by interacting with numerous target genes [77]. Because of this, miRNAs have emerged as a novel class of therapeutic agents with the potential to restore disrupted cellular functions, particularly in various malignancies, including cancer. However, the very potency of miRNAs can be a double-edged sword. Their far-reaching effects, while beneficial, can also lead to off-target effects in non-targeted tissues, a concern documented in recent clinical trials [82-84]. Managing these off-target effects represents a significant challenge to be addressed. Take, for instance, MRX34, a miR-34a mimic encapsulated within a liposome-formulated nanoparticle (NOV40) was evaluated in a first-in-human, Phase 1 study in patients with advanced solid tumors, including melanoma NSCLC, hepatocellular carcinoma, renal carcinoma.

Although MRX34 exhibited significant efficacy, with three patients achieving prolonged confirmed partial responses and 14 patients maintaining stable disease (median duration, 136 days) [85]; however, the clinical trial was terminated due to the occurrence of serious immune-mediated adverse events which resulted in the deaths of four patients (NCT01829971) [82-84]. Despite the setback, dose-dependent modulation of pertinent target genes provides proof-of-concept for miRNA-based cancer therapy.

This review discusses the dysregulation of miRNA expression in cancer and their potential as therapeutics and further discusses the main challenges and strategies to address the problems that must be overcome to fully harness the therapeutic potential of miRNAs.

2. miRNAs

Following the discovery of lin-4, the first miRNA in 1993 in Caenorhabditis elegans [86, 87] [79,80], it became evident that miRNAs are widespread in the animal and plant kingdoms, some of which exhibit high levels of conservation across species [88-90].

MiRNAs, which are short non-coding RNA molecules typically composed of approximately 22 nucleotides, are naturally encoded in the genomes of various species [88-91] and play vital roles in regulating gene expression at both transcriptional [5-7] and post-transcriptional [8-11, 92] levels of their target mRNAs [8, 10] by and via the modulation of the stability and translation of mRNA [93] in a broad range of biological processes [94], impacting activities such as cell differentiation, proliferation, angiogenesis, and apoptosis. Furthermore, miRNAs display specific patterns of expression in different tissues [12, 13] and during various stages of development [14-16].

There are currently estimated to be more than 2588 mature human miRNAs present in human cells [95], each with a unique temporal and tissue-dependent expression pattern. These miRNAs are estimated to control over 60% of human gene expression, showcasing their significant regulatory roles in diverse physiological processes. Because a single microRNA can regulate multiple genes, many miRNAs can contribute to the development of many human diseases when they become dysfunctional [2, 8, 18, 20-26, 28, 30-35, 37, 66, 96-98] including many types of cancer [39, 41-44, 47, 50, 51, 53, 55-67, 69, 99-104].

However, determining the precise relevance of individual miRNAs has been challenging, despite their evident significance as regulatory molecules [105]. Studies investigating miRNA functions by either overexpressing or silencing specific miRNAs have generated data that sometimes conflict with findings from loss-of-function models [105]. For example, studies in Caenorhabditis elegans involving systematic miRNA deletions suggest that fewer than 10% of the miRNAs are individually essential for normal development or viability [106] and this trend appears consistent in mice as well [97].

As illustrated in **Figure 1** and discussed in detail in the literature, miRNAs are primarily transcribed from DNA sequences into primary miRNAs (pri-miRNAs), which

undergo initial processing by DROSHA in the nucleus to generate precursor miRNAs (pre-miRNAs) [8, 68, 107]. It's worth noting that as many as 40% of miRNA genes may be located within the introns or exons of other genes [108]. Pre-miRNAs are subsequently transported from the nucleus to the cytoplasm by exportin 5 (XPO5), where they are further processed by DICER, resulting in small RNA duplexes with specific 3' overhangs of 2 nucleotides. These double-stranded RNA duplexes are loaded onto the Argonaute (AGO) protein, which retains one mature miRNA strand while discarding the other [10]. The AGO-miRNA complex, along with co-factors like GW182 (TNRC6A), forms the RNA-induced silencing complex (RISC) [92], responsible for mRNA transcript degradation and translational inhibition through interaction with complementary mRNA target sequences, typically located within the 3'-untranslated region (3'-UTR) of mRNAs (**Figure 1**) [109-112]. The interaction between miRNA and target mRNA typically takes place at the 5′ end of the miRNA, known as the 'seed' region. Yet, recent evidence points to a unique group of target mRNAs that bind the miRNA not just through the seed but also via a complementary region at the 3′ end of miRNAs. This extended complementarity displaces the miRNA from Ago2, rendering it vulnerable to enzymatic degradation. This process is referred to as the target-directed miRNA degradation mechanism (TDMD) [113, 114].

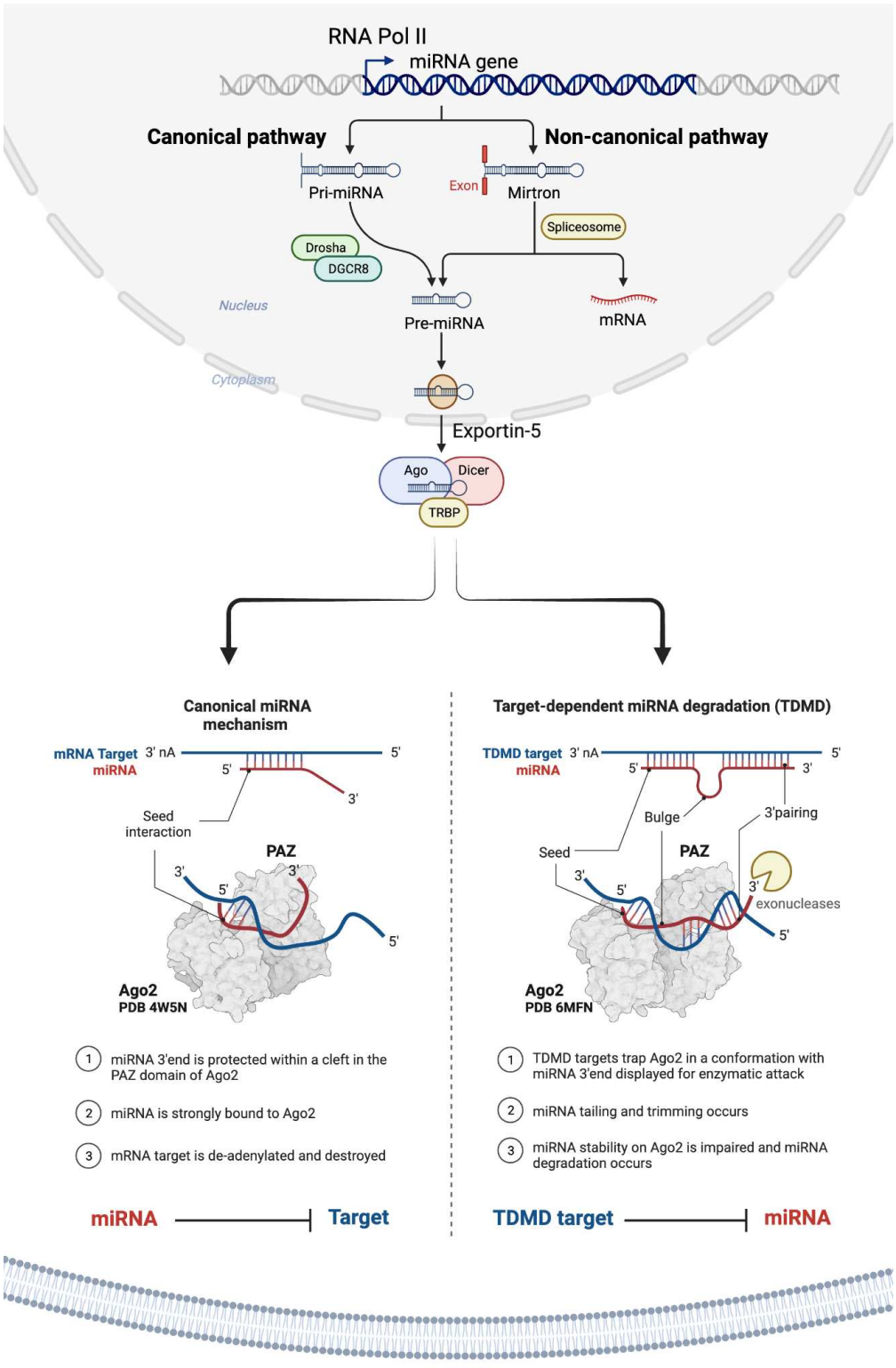

Figure 1. Illustration of miRNA biogenesis, processing, and target RNA translational suppression or degradation via various mechanisms. Created with BioRender.com. miRNAs are a class of small, single–stranded non-coding RNAs that function as a guide molecule in RNA silencing and hence modulate the expression of most mRNAs. The miRNA:target mRNA interaction usually occurs at the 5′ end of the miRNA (i.e., 'seed' region). However, recent evidence suggests that there is a special class of target mRNAs which bind the miRNA not only through the 'seed' region, but also through a second region of complementarity at the 3′ end of the miRNA. The extended complementarity

forces the miRNA out of Ago2, where it becomes accessible to enzymatic degradation. This phenomenon is referred to as target-directed miRNA degradation mechanism (TDMD) [113, 114].

miRNAs are regarded as master regulators of the genome due to their ability to bind to and alter the expression of many protein-coding RNAs [115]. Because of this, a single miRNA can potentially regulate distinct mRNAs (anywhere from 10 to 100 protein-coding RNAs) due to their ability to bind to target mRNAs even when the pairing is not perfect [55, 116]. As a result, a single miRNA can regulate a pool of targets involved in similar cellular processes and pathways, thereby amplifying the cellular response and potentially making miRNAs powerful therapeutics to restore cell functions that are altered as part of a disease phenotype. Conversely, a specific messenger RNA can become the target of many miRNAs, whether concurrently or in a context-dependent manner [117], leading to a collaborative repression effect [118, 119]. Bioinformatic analyses indicate that a single miRNA can potentially bind to as many as 200 distinct gene targets with various functions, such as transcription factors, receptors, and more (https://bitesizebio.com/24926/mysterious-mirna-identifying-mirnas-and-their-targets/).

3. miRNAs role in cancer

Cancer is a complex and heterogeneous disease characterized by a series of genetic and genomic aberrations that promote tumorigenesis [120]. These changes within the genome, which affect gene function, often arise from genomic aberrations like chromosomal translocations, insertions, deletions, amplifications, single-nucleotide mutations, or in the epigenome. These genetic and epigenetic aberrations frequently lead to the activation of oncogenes and the suppression of tumor suppressor genes [121]. In addition, miRNAs have been identified as additional genomic regulators that also play a crucial role in various aspects of organismal development, normal physiological processes, and the development of disease, including many types of cancers [68]. It has been demonstrated that miRNAs play a pivotal role in all the known processes involved in cancer including proliferation, survival, metastasis, and apoptosis [115]. Data suggest that dysregulation of miRNA function, either through its loss or gain, contributes to cancer development by either upregulating or silencing specific target genes. As a consequence, utilizing miRNAs either as miRNA mimics or antagomirs could present a potent therapeutic strategy to interfere with key molecular pathways associated with cancer as such miRNAs have the capacity to regulate all the recognized hallmarks of cancer, either acting as tumor suppressors or promoting oncogenic processes. Several of these cancer hallmarks influenced by miRNAs are discussed in detail in literature [65, 66].

It is generally acknowledged that various changes in miRNA genes and their expression, such as genetic deletions or amplifications, epigenetic methylation of miRNA gene locations, and modifications that influence the regulation of primary miRNA (pri-miRNA) through transcription factors, as well as factors involved in miRNA biogenesis process frequently alter miRNA expression and function in a wide range of cancer types [66].

In addition, changes in the miRNA biogenesis process can also impact the availability of target mRNAs, including those associated with the development of cancer (**Figure 3**) [122]. When miRNAs or the machinery involved in miRNA processing are altered or dysregulated this often leads to the loss of normal homeostatic state, leading to malignant transformation, including various types of cancer [51, 52, 56, 65-67, 123].

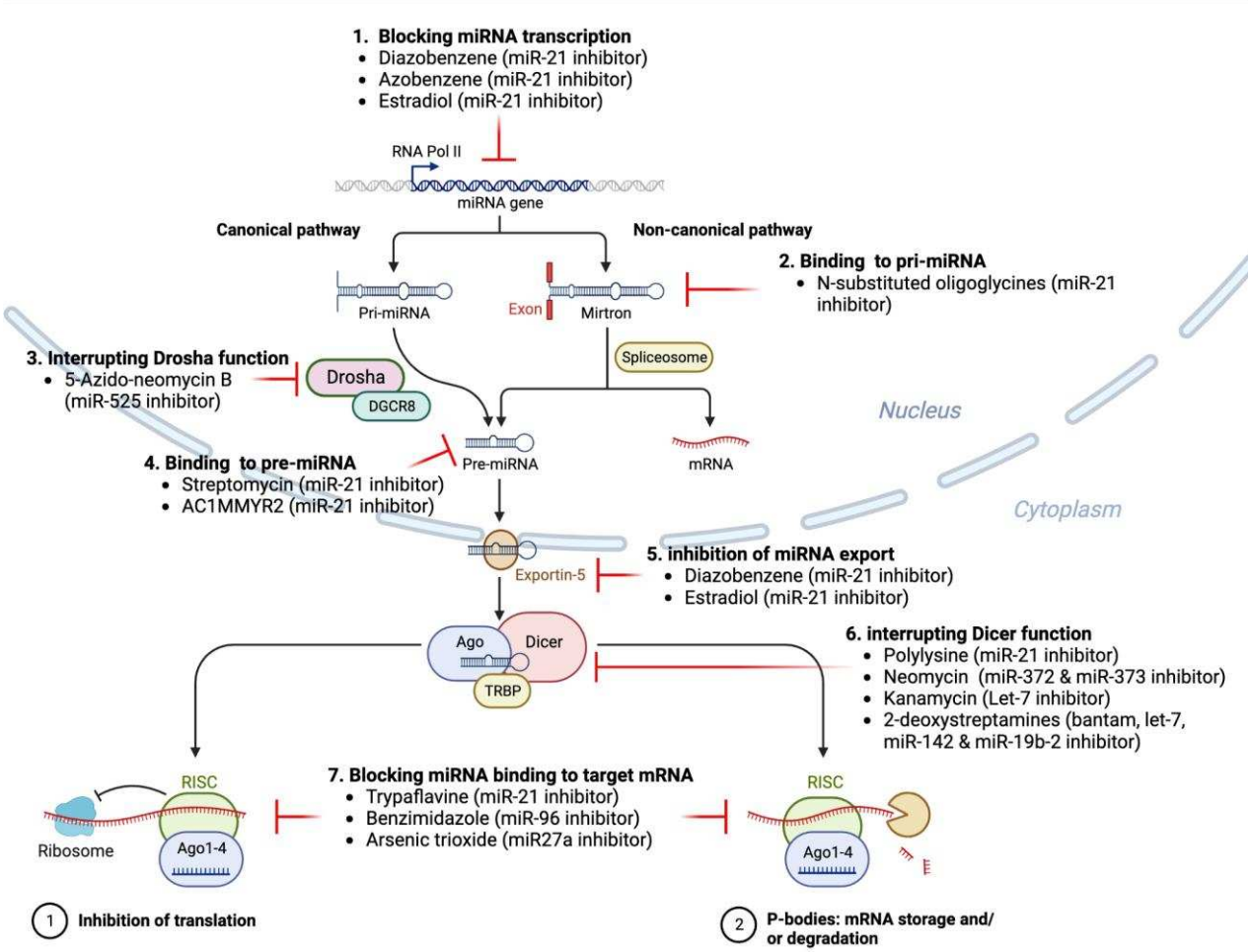

Figure 3. Schematic illustration of different inhibition mechanisms of miRNA specific small molecule inhibitors. Created with BioRender.com.

Because of miRNAs key role in controlling the expression of numerous genes that are involved in cellular responses to environmental stressors such as DNA damage, hypoxia, oxidative stress, and nutrient deprivation, they can function either as oncogenes (oncomiRs), or as tumor suppressors (onco-suppressor miRs). This is supported by recent findings that have identified miRNAs with oncogenic and tumor-suppressing roles in a range of neoplastic malignancies, and the dysregulation of miRNA expression is closely linked to the initiation, progression, and metastasis of cancer [43, 45, 104].

Furthermore, dysregulated circulating miRNAs have been shown to be associated with the origin, progression, treatment response, and the survival of patients with the disease [124, 125]. For instance, the unique tissue-specificity of miRNAs [13], which is essential for maintaining normal cells and tissues [40], renders them promising candidates as potential biomarkers for diagnosing cancers of unknown primary [126, 127]. Furthermore, given the frequent genetic and epigenetic alterations observed in specific miRNAs and components of the miRNA biogenesis process in different cancer types, the oncogenic and tumor suppressor miRNAs have now emerged as promising candidates for miRNA-based therapeutics and diagnostic applications.

4. RNA therapeutics

As discussed in detail in literature, in the recent years, more than 50 siRNA-based drugs have entered clinical trials (phase I, II, and III) [128, 129]. Among those, approximately 15 phase I-, II-, and III-programs based on siRNA therapeutics are being explored

for the treatment of various cancer types [129]. Two siRNA-based drugs Patisiran and Givosiran (Alnylam Pharmaceuticals) were approved by the Food and Drug Administration (FDA) in 2018 and 2019 for hereditary transthyretin-mediated amyloidosis and acute hepatic porphyria, respectively [130, 131]. The first example of an FDA approved RNA-based drug, a siRNA-based therapy developed by Alnylam Pharmaceuticals is Patisiran, sold under the brand name Onpattro™ for the treatment of polyneuropathy of hereditary transthyretin-mediated amyloidosis in adults. Based on the completion of a successful Phase III APOLLO trial, Onpattro™ was approved by the US FDA in August 2018. Onpattro™ contains patisaran, which comprises a siRNA targeting transthyretin (TTR) mRNA conjugated with a lipid complex which leads to decrease in TTR protein levels in the liver thus resulting in a reduction in amyloid deposits. Patisiran specifically targets and binds to a genetically conserved sequence in the 3' untranslated region (3'UTR) of mutant and wild-type TTR mRNA [132]. Findings from the APOLLO trial, a placebo (77 patients) controlled Phase III trial which enrolled 225 patients showed that 51% of patients receiving Onpattro™ (148 patients, once every three weeks (0.3 mg/ kg body weight)) exhibited an improved quality of life (measured using the Norfolk Quality of Life Diabetic Neuropathy (QoL-DN)), as compared to only 10% in the control arm of the patients that received a placebo drug [132, 133].

With the onset of the COVID-19 pandemic, mRNA technology emerged as a pivotal force, serving as the cornerstone for remarkably effective mRNA-based vaccines that have played a crucial role in mitigating the spread of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). The groundbreaking science behind mRNA vaccines earned Katalin Karikó and Drew Weissman the prestigious 2023 Nobel Prize in Physiology or Medicine for their pioneering work on nucleoside base modifications, enabling the development of these impactful COVID-19 vaccines.

The evolution of cap analogs has vastly improved mRNA translation, while advancements in purification, packaging, and delivery methods have revolutionized medicine. Visionaries like Katalin Karikó, Drew Weissman, Edward Darzynkiewicz, Robert Rhodes, Ugur Sahin, and Ozlem Tureci made pivotal early contributions to mRNA research, deserving recognition for their pioneering efforts. This mRNA narrative charts a remarkable journey of breakthroughs in a field holding immense promise for the future of medicine.

The success of mRNA vaccines has paved the way for mRNA-based technology in personalized neoantigen vaccines, seamlessly integrating them into standard oncological workflow [134, 135]. These mRNA-based vaccines can be tailored and manufactured as individualized vaccines with multiple neoantigens [136], and can effectively stimulate antigen-presenting cells [137-140] and be delivered using clinical-stage delivery formulations [141]. The studies and insights from the mRNA-based COVID-19 vaccines highlight the promise of RNA therapeutics as an innovative class of treatments.

However, the effectiveness of miRNA and other nucleic acid-based therapies hinges on a reliable delivery method with minimal adverse events and drug or treatment related toxicity. Delivering miRNA treatments into cells presents challenges due to the need for precise targeting of diseased cells while avoiding healthy ones. Unlike mRNA COVID-19 vaccines, which are taken up by scavenging immune cells, miRNA therapeutics must evade immune recognition to reach their target cells without triggering an immune response.

5. miRNA therapeutics

The discovery of the link between miRNAs and human diseases in 2002 sparked a strong interest in their potential as a new class of therapies. Consequently, interdisciplinary fields encompassing biology, chemistry, and medical science have made significant investments in the development of miRNA-based therapies.

As illustrated in **Figure 2** and **Table 3**, and discussed in literature in detail [77, 122, 128], there are only a few miRNA therapeutics that have entered clinical trials with none of them entering Phase III or being approved by the FDA and several of them were

terminated due to toxicity. Despite the significant progress made in preclinical research, the progress in the field of miRNA-based diagnostic [68] and therapeutic applications remain at an early stage and only a few of these miRNA-based therapies have advanced to clinical development. Given this situation, several efforts in the biotechnology and pharmaceutical industry have integrated miRNAs into their development pipelines focusing on the development of two categories of miRNA drugs, miRNA mimics and inhibitors (antagomirs or antimirs) (**Figure 2, Table 2** and **3**) [77, 142]. As a consequence, several of miRNA-based therapeutics that are being tested in clinical trials are continuously growing for the treatments of a variety of genetic, metabolic, and oncological conditions [143-145].

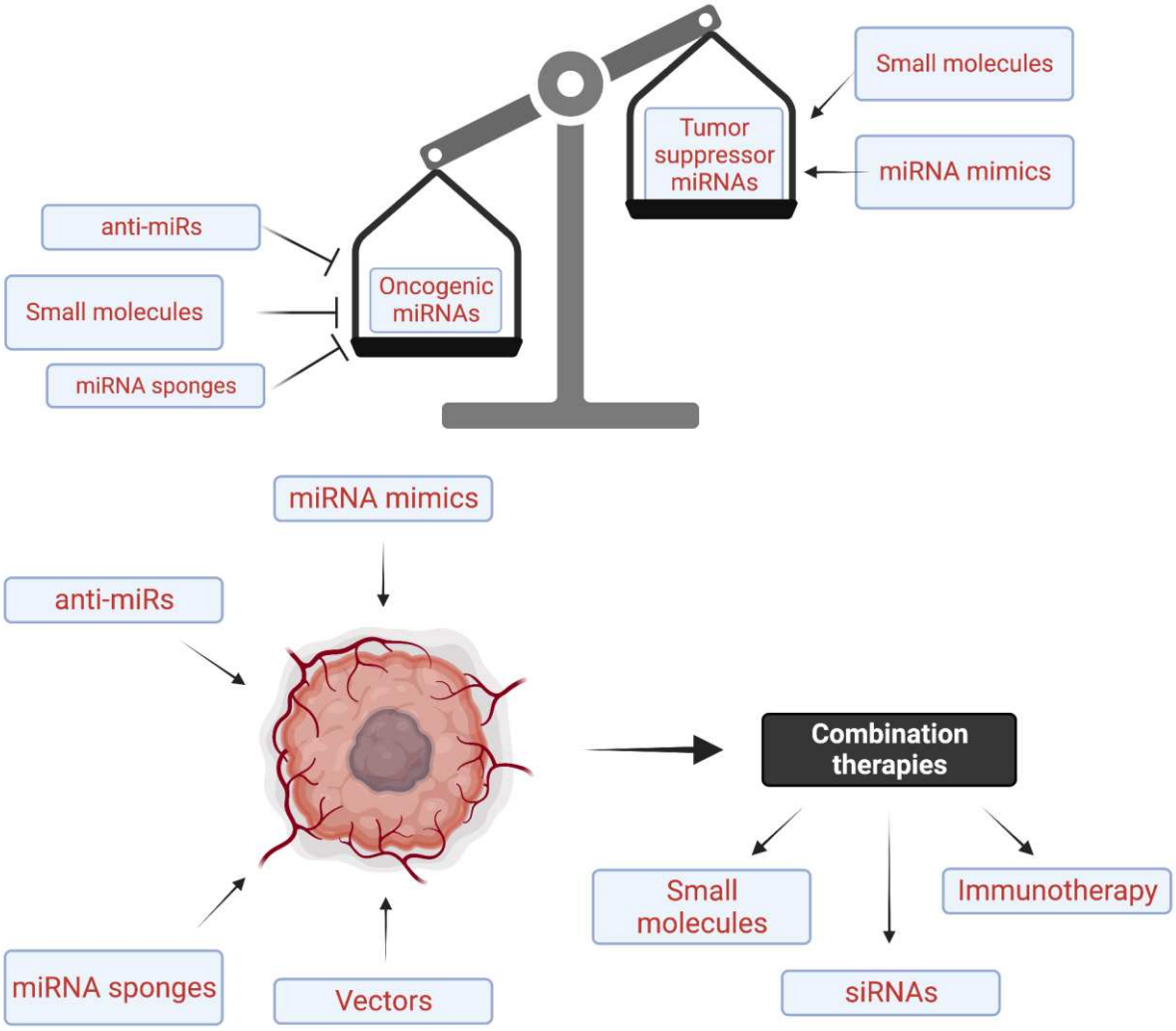

Figure 2. Schematic overview of miRNA therapeutic strategies to regulate the function of oncogenic and tumor suppressor miRNAs involved in cancer. Top panel: The strategy of miRNA therapeutics is based on the strategy of restoring the balance of oncogenic miRNAs and tumor suppressor miRNAs by downregulation of oncomiRNAs or the restoring of tumor suppressor miRNAs. Bottom panel: Therapeutic miRNA manipulations can target the expression or function of pathologically relevant miRNAs via miRNA inhibitors (anti-miRs) mediating degradation or functional blocking of endogenous miRNAs, synthetic miRNA mimics imitating endogenous miRNA double-strands, viral vector expressed miRNAs, small molecules inhibitors interfering with miRNA biogenesis, or miRNA sponges causing functional inhibition by diverting endogenous miRNAs from their mRNA targets. In addition, combining miRNAs with chemotherapies, immunotherapies and other conventional drugs or siRNAs is another strategy to overcome drug resistance. Created with BioRender.com.

Table 2. Major miRNA based therapeutics in various stages of the preclinical development phase for human malignancies.

Therapeutic molecule	Target miRNA	Disease	Biopharmaceutical company	Stage of development
RG-012	miRNA-21	Alport nephropathy	Regulus therapeutics (with the strategic alliance with Genzyme)	Preclinical stage
MGN-1374	miRNA-15 and miR-195	Post-myocardial infarction	miRagen therapeutics	Preclinical stage
MGN-2677	miR-143/145	Vascular disease	miRagen therapeutics	Preclinical stage
MGN-4220	miR-29	Cardiac fibrosis	miRagen therapeutics	Preclinical stage
MGN-4893	miR-451	For the treatment of disorders like abnormal red blood cell production	miRagen therapeutics.	Preclinical stage
MGN-5804	miR-378	Cardiometabolic disease	miRagen therapeutics	Preclinical stage
MGN-6114	miR-92	Peripheral arterial disease	miRagen therapeutics	Preclinical stage
MGN-9103	miR-208	Chronic heart failure	miRagen therapeutics	Preclinical stage
MRG-107	miR-155	Amyotrophic lateral sclerosis (ALS)	miRagen therapeutics	Completed preclinical stage

Table 3. Clinical trials with miRNA therapeutics in various diseases. NCT numbered trials are registered at ClinicalTrials.gov; EudraCT numbered trials are registered at EU Clinical Trials Register (clinicaltrialsregister.eu).

miRNA drug name	Targeted miRNA	Study Title	Mode of action	Disease/condition	Mode of delivery	Phase	Status	Clinical trial number(s)	References
miR-10b	miR-10b	Evaluating the Expression Levels of MicroRNA-10b in Patients With Gliomas	miR-10b as diagnostic and in vitro testing of anti-mir-10b as therapeutic	Astrocytoma Oligodendroglioma Oligoastrocytoma Anaplastic Astrocytoma Anaplastic Oligodendroglioma Anaplastic Oligoastrocytoma Glioblastoma Brain Tumors Brain Cancer		Observational	Recruiting	NCT01849952	
INT-1B3	miR-193a-3p mimic	First-in-Human Study of INT-1B3 in Patients With Advanced Solid Tumors	miRNA mimic	Advanced solid tumors		Phase I	Recruiting	NCT04675996	NA
AMT-130	Artificial miRNA	Safety and Proof-of-Concept (POC) Study With AMT-130 in Adults With Early Manifest Huntington's Disease	A miRNA expression	Huntington disease	Stereotaxic infusion/viral transfer (adeno-associated vector)	Phase I	Ongoing	NCT04120493	[195-197] [23–25]
RG-012/lademirsen/SAR339375	miR-21	A Study of RG-012 in Subjects With Alport Syndrome	Anti-miR-21 Lademirsen—also known as RG-012, RG456070 or (SAR339375)	Alport syndrome	Subcutaneous injection/chemical modification (phosphorothioate)	Phase II	Completed	NCT03373786	[209, 222, 253, 254]

RG-012/lademirsen/SAR339375	miR-21	Study of Lademirsen (SAR339375) in Patients With Alport Syndrome	Anti-miR-21 Lademirsen—also known as RG-012, RG456070 or (SAR339375)	Alport syndrome	Subcutaneous injection/chemical modification (phosphorothioate)	Phase II	Terminated	NCT02855268	[209, 222, 253, 254]
RGLS4326	miR-17	A Study of RGLS4326 in Patients With Autosomal Dominant Polycystic Kidney Disease	Anti-miR-17	Autosomal dominant polycystic kidney disease	Administered via subcutaneous injection	Phase I	Completed	NCT04536688	
RG-125/AZD4076	miR-103/107	A Study to Assess the Safety and Tolerability of Single Doses of AZD4076 in Healthy Male Subjects	Anti-miR	Non-alcoholic Steatohepatitis (NASH)	Subcutaneous injection/biomolecule conjugation (GalNAc)	Phase I	Active, not recruiting	NCT02612662, NCT02826525	[207-209]
RG-125/AZD4076	miR-103/107	AZD4076 in Type 2 Diabetic Subjects With Non-Alcoholic Fatty Liver Disease	Anti-miR	T2DM With NAFLD	Subcutaneous injection/biomolecule conjugation (GalNAc)	Phase I	Completed	NCT02826525	[207-209]
MRG-110	miR-92a	Safety, Tolerability, Pharmacokinetics, and Pharmacodynamics of MRG-110 Following Intradermal Injection in Healthy Volunteers	Anti-miR	Healthy Volunteer	Skin injection/chemical modification (LNA)	Phase I	Completed	NCT03603431	[159, 255]
MesomiR 1	miR-16	MesomiR 1: A Phase I Study of TargomiRs as 2nd or 3rd Line Treatment for Patients With	miRNA mimic	Malignant pleural mesothelioma, non–small cell lung cancer	Intravenously/vehicle transfer (nonliving bacterial nanocells	Phase I	Completed	NCT02369198	[162, 200, 256]

		Recurrent MPM and NSCLC			(EDVs or Targo-miRs)				
CDR132L	miR-132	Clinical Study to Assess Safety, PK and PD Parameters of CDR132L	Anti-miR	Heart failure	Intravenously/chemical modification (LNA)	Phase I	Completed	NCT04045405	[257, 258]
Remlarsen/MRG-201	miR-29	Efficacy, Safety, and Tolerability of Remlarsen (MRG-201) Following Intradermal Injection in Subjects With a History of Keloids	miRNA mimic	Keloid disorder	Skin injection/biomolecule conjugation (cholesterol)	Phase II	Completed	NCT03601052	[160, 259, 260]
Miravirsen/SPC3649	miR-122	Long-Term Extension Study of Miravirsen Among Participants With Genotype 1 Chronic Hepatitis C (CHC) Who Have Not Responded to Pegylated-Interferon Alpha Plus Ribavirin	Anti-miR	Chronic hepatitis C virus	Subcutaneous injection/chemical modification (LNA)	Phase II Phase II Phase II Phase II Phase II Phase I	Completed Completed Completed Completed Unknown Unknown Completed	NCT02508090 NCT02508090, NCT02452814, NCT01200420, NCT01872936, NCT01727934, NCT01646489	[152, 261-264]
Miravirsen/SPC3649	miR-122	Long Term Extension Study is Designed to Monitor Long-Term Efficacy and Safety of Miravirsen Sodium in Combination With Telaprevir and Ribavirin in Subjects With Chronic Hepatitis C Virus Genotype 1 Infection	Anti-miR	Chronic hepatitis C virus	Subcutaneous injection/chemical modification (LNA)	Phase II	Completed	NCT02452814	[152, 261-264]

Miravirsen/ SPC3649	miR-122	Multiple Ascending Dose Study of Miravirsen in Treatment-Naïve Chronic Hepatitis C Subjects	Anti-miR	Chronic hepatitis C virus	Subcutaneous injection/chemical modification (LNA)	Phase II Phase II Phase II Phase I	Completed Unknown Unknown Completed	NCT01200420	[152, 261-264]
Miravirsen/ SPC3649	miR-122	Miravirsen in Combination With Telaprevir and Ribavirin in Null Responder to Pegylated-Interferon Alpha Plus Ribavirin Subjects With Chronic Hepatitis C Virus Infection	Anti-miR	Chronic hepatitis C virus	Subcutaneous injection/chemical modification (LNA)	Phase II Phase II Phase I	Unknown	NCT01872936	[152, 261-264]
Miravirsen/ SPC3649	miR-122	Miravirsen Study in Null Responder to Pegylated Interferon Alpha Plus Ribavirin Subjects With Chronic Hepatitis C	Anti-miR	Chronic hepatitis C virus	Subcutaneous injection/chemical modification (LNA)	Phase II Phase II Phase I	Unknown	NCT01727934	[152, 261-264]
Miravirsen/ SPC3649	miR-122	Drug Interaction Study to Assess the Effect of Co-Administered Miravirsen and Telaprevir in Healthy Subjects	Anti-miR	Chronic hepatitis C virus	Subcutaneous injection/chemical modification (LNA)	Phase II Phase II Phase I	Completed	NCT01646489	[152, 261-264]
RG-101	miR-122	A Randomized, Multi-Center, Phase 2 Study to Evaluate Safety and Efficacy of Subcutaneous Injections of RG-101 in Combination with Oral	Anti-miR	Chronic hepatitis C virus	Subcutaneous injection/biomolecule conjugation (GalNAc)	Phase II		EudraCT numbers **2015-001535-21,** **2015-004702-42,**	[156, 209, 210]

		Agents in Treatment Naïve, Genotype 1 and 4, Chronic Hepatitis.						**2016-002069-77**	
RG-101	miR-122	A Multi-Center, Parallel Group, Open-Label, Phase 2 Study to Evaluate the Efficacy and Safety of a Single Subcutaneous Injection of RG-101 Combined with Oral GSK2878175	Anti-miR	Chronic hepatitis C virus	Subcutaneous injection/biomolecule conjugation (GalNAc)	Phase II		**EudraCT numbers** **2015-004702-42**	[156, 209, 210]
RG-101	miR-122	An Observational Long-Term Safety and Efficacy Follow-Up Study of Subjects Who Have Previously Received RG-101	Anti-miR	Chronic hepatitis C virus	Subcutaneous injection/biomolecule conjugation (GalNAc)	Observational	Unknown	**EudraCT numbers** **2016-002069-77**	[156, 209, 210]
MRX34	miR-34a	A Multicenter Phase I Study of MRX34, MicroRNA miR-RX34 Liposomal Injection	miRNA mimic	Primary Liver Cancer SCLC Lymphoma Melanoma Multiple Myeloma Renal Cell Carcinoma NSCLC	Intravenously/vehicle transfer (liposomal)	Phase I	Terminated (5 immune related serious adverse events)	NCT01829971	[29,32, 133] [65, 82, 83]
MRX34	miR-34a	Pharmacodynamics Study of MRX34, MicroRNA Liposomal Injection in Melanoma Patients With Biopsy Accessible Lesions	miRNA mimic	Solid tumors (e.g., hepatocellular carcinoma, melanoma, SCLC, NSCLC, lymphoma, multiple myeloma, renal cell carcinoma	Intravenously/vehicle transfer (liposomal)	Phase I Phase II	Withdrawn (5 immune related serious adverse events in Phase I)	NCT02862145	[29,32, 133] [65, 82, 83]

				Mycosis fungoides (MF)					
Cobomarsen/MRG-106	miR-155		Anti-miR	Cutaneous T-cell Lymphoma (CTCL) Chronic Lymphocytic Leukemia (CLL) Diffuse Large B-Cell Lymphoma (DLBCL), ABC Subtype Adult T-Cell Leukemia/Lymphoma (ATLL)	Intravenously/chemical modification (LNA)	Phase I Phase II Phase II	Completed Terminated Terminated	NCT02580552, NCT03713320, NCT03837457	[157, 265, 266]
Serum MicroRNA-25	miR-25		Serum miR-25 as diagnostic	Pancreatic cancer	Serum miR-25	Observational	Not yet recruiting	NCT03432624	
Patisiran (ALN-TTR02),			RNAi therapeutic	Transthyretin (TTR)-Mediated Amyloidosis	ALN-TTR02 administered by intravenous infusion	Phase III	Completed	NCT01960348	[267]
miR-10	miR-10	Evaluating the Expression Levels of MicroRNA-10b in Patients With Gliomas	anti-miR-10	Glioma	Evaluating the expression levels of microRNA-10b in patients with gliomas	Observational	Recruiting	NCT01849952	

MiRNA therapeutics are a type of RNAi-based therapeutic that targets and modulates the activity of specific endogenous miRNAs in the body. Because miRNAs play a crucial role in regulating gene expression in normal health and disease states, by targeting and manipulating specific miRNAs, miRNA therapeutics aim to treat various diseases by restoring (miRNA mimics) or correcting miRNA expression patterns (antagomir). MiRNA-based therapeutic programs for cancer are predominantly conducted by a few biopharmaceutical companies, including Santaris Pharma, Roche Pharmaceuticals, Regulus therapeutics (San Diego, CA, USA), Mirna Therapeutics Inc. (Carlsbad, CA, USA), miRagen Therapeutics (Boulder, CO, USA), and EnGeneIC (Sydney, Australia).

Immune evasion and chemotherapy resistance is a challenge in cancer therapy and this resistance can be mediated by various factors including miRNAs induced by tumor microenvironment stimuli, like hypoxia or cell–cell communication [146].

Hypoxia has been shown to influence microRNA expression in cancer and stromal cells in the tumor microenvironment (TME) via downregulation of factors involved in miRNA biogenesis machinery or regulation of transcription factors that control miRNA expression. Accordingly, many hypoxia-regulated miRNAs and their role in tumor progression have been reported. These hypoxia-regulated miRNAs including miR-26a, miR-181b, miR-210, miR301-a, miR-424, and miR-519c have also been associated with chemo- or radiotherapy response in different cancers [147-150]. Therefore, therapeutic targeting of these miRNAs could be a strategy to re-sensitize hypoxic tumors to chemo and other therapies. For example, in the hypoxic pancreatic cancer microenvironment, HIF-1α induces gemcitabine resistance. A recent study showed that delivery of miR-519c, which is downregulated in pancreatic cancer, could inhibit HIF1-α in gemcitabine-resistant pancreatic cancer cells under hypoxia [151]. Furthermore, a redox-sensitive nanoplatform used to co-deliver miR-159c and gemcitabine was demonstrated to inhibit the expression of HIF-1α and genes responsible for glucose uptake and cancer cell metabolism, thus inhibiting orthotopic desmoplastic pancreatic cancer growth in NSG mice and reversing hypoxia-induced chemotherapy resistance [151]. Similarly, tumor suppressor miR-34a has been shown to downregulate the expression of more than 30 oncogenes across multiple oncogenic pathways, as well as genes implicated in tumor immune evasion, but is lost or under-expressed in many malignancies [82]. However, if miRNAs are to be used for the treatment of a cancer, miRNAs must be delivered to the target tissue, not trigger an immune response and be economically feasible so that wide-spread adoption of these nano therapies can be realized. Although there has been some success in clinical development, several clinical trials have been terminated mostly due to various serious adverse events, indicating that there are still several challenges to overcome before the clinical application of RNAi-based therapies becomes widespread.

5.1. Examples of miRNA therapeutics in clinical trials

As illustrated in **Figure 2**, and **Table 2** and **Table 3**, and discussed in detail in the literature [85, 145], there are several miRNA-based therapeutics being tested both in preclinical studies (**Table 2**) or in human clinical trials (**Table 3**).

Miravirsen: The first miRNA-based therapeutic entering clinical trials was Miravirsen (SPC3649), a miR-122 15-mer LNA-PS-modified modified ASO antagomir of miR-122, as a therapy against Hepatitis C Virus (HCV) infections developed by Santaris Pharma, Roche Pharmaceuticals. miR-122 was shown to play a role in HCV replication.[152]. Phase II clinical trials were conducted to evaluate the safety and antiviral efficacy of Miravirsen in patients with chronic HCV infection. Miravirsen showed strong efficacy by reducing viremia in patients with HCV [155,156,157] and underwent multiple phase II clinical trials (NCT01200420, NCT01872936, NCT02031133, NCT02508090). However, due to severe side effects the trial was halted [145, 153].

RG-012: RG012 is an anti-miR-21 therapy developed by Regulus Therapeutics for the management of Alport syndrome. miR-21 has been shown to be up-regulated in fibrotic kidney disease. Preclinical studies have shown that treatment with an anti-miR-21

significantly reduced kidney failure by reducing the rate of progression of renal fibrosis. RG-012 has been granted orphan drug status in the US and Europe. However, some sequence-independent side effects have been reported in context with phosphorothioate-modified oligonucleotides [[154].

RG-101: RG-101, an antagomir of miR-122 developed by Regulus Therapeutics is an N-acetyl-d-galactosamine(GalNAc)-conjugated synthetic RNA oligonucleotide that targets and inhibits miR-122, which is involved in HCV replication for patients with HCV. in addition to its essential role for HCV replication, miR-122, a liver-specific miRNA, has relevant functions in liver metabolism [155] [20]. This miRNA is also an essential host factor for HCV.

Clinical trials were conducted to evaluate RG-101's safety and efficacy as a potential treatment for chronic HCV infection. Although RG-101 showed considerable efficacy and showed a significant reduction in viral loads in chronic HCV subjects [156]; however, the trial was terminated due to some serious adverse events of severe hyperbilirubinemia [156].

MRG-201: MRG-201 is a synthetic RNA oligonucleotide that targets and activates miR-29, which has been shown to inhibit fibrosis. Clinical trials were conducted to assess MRG-201's safety and efficacy in treating fibrotic disorders such as hypertrophic scars and idiopathic pulmonary fibrosis.

MRX34: MRX34, developed by miRNA Therapeutics Inc. is a synthetic miRNA mimic designed to mimic the activity of a tumor suppressor miR-34a encapsulated into a liposome-formulated nanoparticle (NOV40) for the treatment of advanced solid tumors including melanoma, NSCLC, hepatocellular carcinoma, and renal carcinoma. miR-34a is a naturally occurring tumor-suppressor miRNA expressed at reduced levels in many tumor types. MRX34, is considered to be a first-in-class miRNA mimic for the treatment of many cancers, such as non-small cell lung cancer, hepatocellular carcinoma, colon cancer, ovarian cancer, cervical cancer, and others. The formulation was tested in a phase I clinical trial [82].

Although MRX34 displayed strong activity, at the end of the trial, only three patients achieved prolonged confirmed partial responses, and 14 patients presented with stable disease (median duration- 136 days) [85]. However, the trial was terminated due to serious immune-mediated adverse events that resulted in four patient deaths (NCT01829971) [82-84]. After this, MiRNA Therapeutics ceased operations in 2017 and agreed to merge with Synlogic Inc.

Cobomarsen (MRG-106): MRG-106 (Cobomarsen), an LNA-based antagomir of miR-155 was developed by Miragen Therapeutics (Viridian Therapeutics Inc) that aimed to inhibit the activity of miR-155 [157, 158] in several lymphoma subtypes, as well as in diffuse large B-cell lymphoma [102] [83] where miR-155 is up-regulated. Phase II clinical trials are being conducted to assess its effectiveness in treating certain cancers and immune disorders including cutaneous T-cell lymphoma (CTCL), chronic lymphocytic leukemia, diffuse large B-cell lymphoma, and mycosis fungoides (NCT03837457), and adult T-cell leukemia/lymphoma (NCT02580552, NCT03713320). While Phase I trial was completed, two of the Phase II studies were terminated. The study was terminated early for business reasons, and not due to concerns regarding safety or lack of efficacy. (https://classic.clinicaltrials.gov/ct2/show/NCT03713320).

MRG-107: MRG-107 an antagomir of miR-155 was developed by Miragen Therapeutics (Viridian Therapeutics Inc) that aimed to inhibit the activity of miR-155. miR-155 plays relevant functions in the immune mechanisms and inflammation processes in amyotrophic lateral sclerosis (ALS) and miR-155 is elevated in the spinal cords of ALS patients. In preclinical models of ALS, inhibition of miR-155 has reduced the ALS symptoms and extended survival [145].

MRG-110: MRG-110 is a synthetic antagomir of miRNA-92a developed by MiRagen Therapeutics in collaboration with Servier to treat ischemic conditions such as heart failure [159]. MRG-110 is developed to promote the growth of new blood vessels by inhibiting

miR-92a. MRG-110 is being studied to determine if it can accelerate healing of wounds by improving blood flow into the wound area. A phase I clinical trial that tested safety, tolerability, pharmacokinetics, and pharmacodynamics of MRG-110 following intradermal Injection in healthy Volunteers was recently completed (NCT03603431).

Remlarsen (MRG-201): Remlarsen (MRG-201) is an LNA RNA mimic of miR-29 developed by MiRagen Therapeutics for keloid disorder and decreases the expression of collagen and other proteins that are involved in scar formation and has been shown to inhibit fibrosis [160]. miR-29 family members are typically downregulated in fibrotic diseases [161]. Clinical trials is being conducted to assess Remlarsen's safety and efficacy in treating fibrotic disorders such as hypertrophic scars and idiopathic pulmonary fibrosis when administered by intradermal injection at the site of an excisional wound [160] 90]. The phase II clinical trial is currently underway to determine if it can limit the formation of fibrous scar tissue in certain diseases (NCT03601052).

TargomiRs: The MesomiR 1 trial (NCT02369198) tested the safety and efficacy of miR-15/16 encapsulated in bacterial minicells (TangomiRs) in patients with recurrent malignant pleural mesothelioma (MPM. TangomiRs developed by EnGeneIC to deliver miR16 mimics encapsulated in TargomiRs composed of nonliving bacterial nanocells with anti-EGFR bispecific antibody to target EGFR-expressing cancer cells were tested as 2nd or 3rd Line Treatment for patients with recurrent malignant pleural mesothelioma (MPM) and non-small cell lung cancer (NSCLC) (NCT02369198) [162]. More specifically, mir-16 mimic was encapsulated in EnGeneIC's bacterially-derived EDV™ (EDV)TM nanocells (a 400 nm particle of bacterial origin able to carry a drug cargo) and targeted with EGFR antibodies (TargomiRs). miR-15/16 are implicated as tumor suppressors in MPM. Although variable response rates were observed with 5% of the patients showing partial response, 68% showing stable disease and 27% showing progressive disease after low dose systemic administration of TargomiRs; however, dose-dependent toxicities were observed (i.e., anaphylaxis, inflammation as well as cardiac events)[128, 162].

MGN-1374: MGN-1374, an 8-mer LNA ASO developed by miRAgen Therapeutics, is designed to specifically target the seed region of the miR-15 family and is currently in the preclinical phase for the control of postmyocardial infarction remodeling.

RGLS4326: RGLS4326 is a single-stranded, chemically modified, 9-mer ASO with full complementarity to the seed sequence of miR-17. RGLS4326 is designed to inhibit the pathologic functions of the miR-17 family in Polycystic kidney disease (ADPKD) [163] [87], an autosomal dominant disease, one of the most frequent monogenic disorders, caused by mutations in the PKD1 or PKD2 gene and therapeutic options for the treatment of ADPKD are limited. A phase I clinical trial of RGLS4326 was recently completed (NCT04536688).

Additionally, **Table 2** highlights several miRNA-based drugs currently under preclinical investigation, targeting various diseases such as Peripheral Arterial Disease, Chronic Heart Failure, Amyotrophic Lateral Sclerosis (ALS), among others.

Moreover, miRNA therapeutics in combination with chemotherapeutic agents have also been explored to overcome cancer therapy resistance [142]. Studies indicate that combining therapeutic miRNAs with chemotherapy can decrease the required drug doses for cancer treatment [164, 165]. For example, miR-3622b-5p, when paired with cisplatin, not only enhances apoptosis but also sensitizes ovarian tumor organoids to cisplatin [166], suggesting the potential of miRNAs in combination with chemotherapy to address cancer treatment and counteract drug resistance.

5.2. Small molecule modulators of miRNA expression

Because altered levels of miRNA expression is associated with many cancers, restoring the function of tumor suppressor miRNAs by overexpressing or introducing of miRNA mimics to restore to their relatively normal physiological levels or function or by inhibiting overexpressed oncogenic miRNAs by miRNA inhibitors (antagomirs), or

miRNA sponges in cancer represents two major strategies for miRNA therapeutics in cancer [122, 167] (**Figure 3**).

The function of repressed miRNAs can also be restored to their relatively normal physiological levels and function by using some small molecules that can transcriptionally activate the expression of miRNA genes leading to expression of endogenous miRNAs and restoring the expression of tumor suppressive miRNAs. Conversely, overexpressed oncogenic miRNAs can also be suppressed by small-molecule inhibitors.

As illustrated in **Figure 3** and **Table 1** and discussed in literature [122], because nucleic acid-based therapeutics have poor cell-permeability for drug delivery, in recent years small-molecule drugs in the regulation of miRNA expression have been explored since they can cross the cell membrane by free diffusion and can modulate the expression of miRNAs and also traditional drug development can be applied for the development of novel miRNA inhibitors (or activators) [168] (Wu, 2020b).

Table 1. Representative examples of small-molecule miRNA inhibitors.

Small molecule inhibitors of miRNAs	Target miRNAs	Mechanism of action	References
Trypaflavine	miR-21	Blocking the assembly of miR-21 with Ago2	[175]
Streptomycin	miR-21/ miR-27a	Blocking the cleavage of pre-miR-21 by Dicer	[242]
AC1MMYR2	miR-21	Blocking the cleavage of pre-miR-21 to produce mature miR-21	[243]
Diazobenzene	miR-21	Inhibition the transcription of miR-21 gene	[244]
Azobenzene	miR-21	Inhibition the transcription of miR-21 gene	[244]
Estradiol	miR-21	Inhibition the transcription of miR-21 gene	[245]
Polylysine	miR-21	Blocking the formation of mature of pre-miR-21 by the inhibition of Dicer	[175]
4-benzoylamino-N-(prop-2-yn- 1-yl)benzamides	miR-21	Up-regulation of PDCD4, the function target of miR-21	[246]
Arylamide derivatives	miR-21	Blocking the mature of pre-miR-21	[247]
Kanamycin A	Let-7/ miR-27a	Binding to pre-let-7 and blocking the function of Dicer	[176]
2-DOS Compound 1	Let-7	Binding to pre-let-7 and blocking the function of Dicer	[176]
2-DOS Compound 2	Let-7	Binding to pre-let-7 and blocking the function of Dicer	[176]
2-DOS Compound 3	Let-7	Binding to pre-let-7 and blocking the function of Dicer	[176]
2-DOS Compound 4	Let-7	Binding to pre-let-7 and blocking the function of Dicer	[176]
2-DOS Compound 5	Let-7	Binding to pre-let-7 and blocking the function of Dicer	[176]
2-DOS Compound 6	Let-7	Binding to pre-let-7 and blocking the function of Dicer	[176]
2-DOS Compound 7	Bantam	Binding to pre-bantam and blocking the function of Dicer	[176]
2-DOS Compound 8	miR-142	Binding to pre-miR-142 and blocking the function of Dicer	[176]
2-DOS Compound 9	miR-19b-2	Binding to pre-miR-19b-2 and blocking the function of Dicer	[176]
NSC 158959	miR-122	Inhibition of the transcription of miR-122	[177]
NSC 5476	miR-122	Inhibition of the transcription of miR-122	[177])
Benzimidazole	miR-96	Up-regulation of FOXO1, the function target of miR-21	[248]
2-methoxy-1,4-naphthalenequin	miR-1	Down-regulation the expression level of miR-1	[183]
Arsenic trioxide	miR-27a	Down-regulation the expression level of miR-27a	[249]
Neomycin	miR-27a	Blocking the mature of miR-27a by the inhibition of Dicer	[250]

Amikacin	miR-27a	Blocking the mature of miR-27a by the inhibition of Dicer	[250]
Tobramycin	miR-27a	Blocking the mature of miR-27a by the inhibition of Dicer	[250]
5″-azido-neomycin B	miR-525	Binding to the processing site of Drosha to block the generation of pre- miR-525	[251]
N-substituted oligoglycines	miR-21	A specific ligand binding with pri-miR-21.	[252]

Various small molecule inhibitors of various miRNAs with various chemical structures and different mechanisms of action **(Table 1**) have been described with different target sites of inhibition and interference across the whole process of miRNA biogenesis such as processing, maturation, and function (Figure X) [122].

For example, as discussed in literature [122], reduced expression of tumor suppressor miRNAs can be reactivated to their normal physiological levels by some small molecule compounds, such as hypomethylating agents [169].

Decitabine or 5-azacytidine are two drugs for the treatment of myelodysplastic syndrome and were shown to upregulate the expression of several miRNAs [106].

Similarly, enoxacin was shown to activate the expression of several miRNAs in vitro [170] and to suppress tumor growth by upregulating the expression of 24 miRNAs in vivo in mice xenograft models [170] suggesting that small molecule compounds can potentially restore miRNA expression and function to a more physiological setting.

For example, miR-21 is one of the tumor-associated miRNAs (oncomiR) has been shown to be upregulated in a variety of tumor cells, including breast cancer, ovarian cancer, colon cancer, pancreatic cancer, thyroid cancer, and others and its high expression in cancer which is closely associated with tumorigenesis [171-174].

Trypaflavine (TPF), a small molecule inhibitor of miR-21 was shown to suppress the expression of miR-21 [175].

Trypaflavine was shown to inhibit the formation of RISC by blocking the assembly of miR-21 and AGO2 protein, leading to the suppression of the expression level of miR-21.

Similarly, Kanamycin A was shown to inhibit the expression of let-7 by binding to pre-let-7 and interfering with Dicer [176].

Furthermore, small-molecule inhibitors of miR-122, NSC 158959 and NSC 5476 [177] which may be involved in the modulation of transcription of miR-122 gene to pri-miR-122.

Importantly, mir-122 is a liver specific miRNA, accounting for about 72% of the total miRNA in the adult liver and is one of the earliest miRNAs with tissue-specific and high abundance expression [178].

miR-122 plays a key role in the regulation of cholesterol and fatty-acid metabolism in the adult liver suggesting that miR-122 may be an attractive therapeutic target for metabolic disease [179].

Moreover, it was also shown that miR-122 also plays a key role in the development of various types of liver diseases including acute and chronic liver injury,, liver tumor and hepatitis C virus (HCV) infection, liver cirrhosis, and alcoholic hepatitis [180].

miR-1 which is abundantly expressed in skeletal muscle cells plays a role in the regulation of the formation of skeletal muscle cells and the development of muscle and is closely associated with the development of the heart [181, 182].

Various small molecular Inhibitors of miR-1 have been identified from photocycloadducts of acetylenes with 2-methoxy-1,4-naphthalenequinone as the basic skeleton by photocyclization reaction [183]. For example, the small molecule 2-methoxy-1,4-naphthalenequinone was demonstrated to exert specific inhibitory effects on miR-1 and was shown to significantly inhibit the expression of mature miR-1 in cells. However, the specific mechanism of action of 2-methoxy-1,4-naphthalenequin for its inhibitory function on miR-21 remains to be elucidated.

6. Advances in delivery of miRNA therapeutics

Although a few phase 1 and 2 clinical trials involving miRNA-targeting or miRNA-based therapeutics, there are no miRNA-based therapeutics in phase III trials so far. This is partly due to the challenge of targeted delivery of miRNAs to specific cell types, tissues, and organs. Although targeting of miRNAs to a cell of interest by using various methods (e.g. antibodies, nanoparticles, or ligands) have been reported to improve the efficacy of miRNAs and reduce off-target effects (e.g., immunotoxicity [184], there are still limitations and challenges for miRNA therapeutics warranting more research in this field.=

As illustrated in **Figure 4** and discussed in detail in the literature in detail [77, 84, 185, 186], there are various strategies being explored as a mechanism to deliver miRNA therapeutics (mimics and antagomirs) to the indented tissue and to improve the pharmacokinetic mechanisms, and avoid off-target effects. These include vector-based and non-vector-based methods including lipid-based nanoparticles, polymeric vectors/dendrimer-based vectors, cell-derived membrane vesicles, 3D scaffold-based delivery systems, and other nanoparticles derived from polymers and metals that are biodegradable and biocompatible.

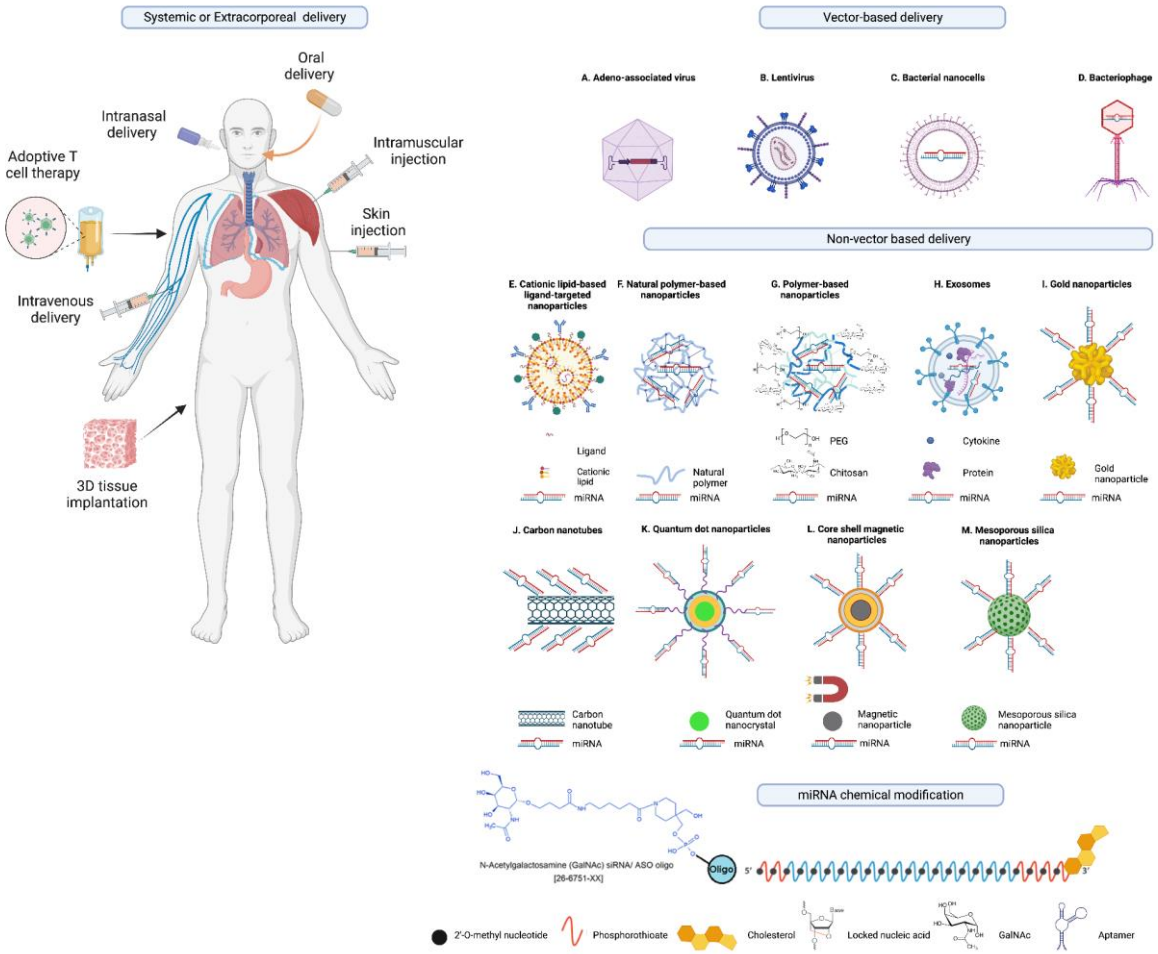

Figure 4. Examples of miRNA delivery systems. MiRNA therapeutics can be administered orally or intranasally or through venous (intravenously) or muscle (intramuscularly) or skin (subcutaneously) injections, or via cell-/tissue-directed approaches, or adoptive cell transfer, or the implantation of 3D matrices that release miRNA therapeutics, or other extracorporeal miRNA delivery strategies [77]. Other modes of delivery of miRNA therapeutics include vector based and non-vector-based delivery systems including adeno-associated virus. (**A**), lentivirus (**B**), bacterial nanocells (**C**), bacteriophages (**D**) liposomes, including monovalent and multivalent lipids such as cationic lipid-based ligand-targeted nanoparticles (**E**), natural polymer-based nanoparticles (**F**), polymer based nanoparticles (natural, green and synthetic, blue) conjugated with polyethylene glycol (PEG) (**G**), extracellular vesicles or exosomes (**H**), gold nanoparticles [268] (**I**) carbon nanotubes (**J**), quantum

dot nanoparticles (**K**), core-shell magnetic nanoparticles (**L**), and others such as polymeric micelles, and mesoporous silica nanoparticles are the examples of nanocarriers as drug-delivery systems [145]. Moreover, there have been efforts to improve the serum stability, pharmacokinetics, and tissue specificity by targeted delivery of miRNA mimics, miRNA inhibitors and other nucleic acid therapeutics by incorporation of various chemical modifications and/or conjugation of these RNA and nucleic acid therapeutics to biomolecules to facilitate receptor-mediated uptake such as N-acetylgalactosamine (GalNAc), 2′-O-methyl nucleotide, phosphorothioate, cholesterol, locked nucleic acid (LNA), and aptamer moieties are also shown as examples [77, 84, 186, 208, 269]. **Created with BioRender.com.**

As such, there is a need for an efficient delivery system for developing miRNA-based therapeutics.

As discussed in detail in literature [187], several different strategies have been explored to efficiently deliver RNA-based therapeutics (**Figure 4**) including vector-based and non-vector-based delivery systems including adeno-associated virus, lentivirus, bacterial nanocells [188], bacteriophages, cationic lipid-based liposomes, including monovalent and multivalent lipids, natural polymer-based nanoparticles, polymer based nanoparticles conjugated with polyethylene glycol (PEG), extracellular vesicles (EVs) or exosomes, nanocomplex-forming functionalized metals such as gold nanoparticles, and carbon nanotubes and many others that are engineered to contain biomolecule conjugates for improved stability and pharmacokinetics and target delivery to the intended cell or tissue type [84, 186, 187, 189, 190]. A recent report demonstrated enhanced antitumor potency of STING agonists after covalent conjugation of cyclic dinucleotides (CDN) to polymer nanoparticles (poly(β-amino ester) formulation for the intravenous delivery [191].

Non pathogenic recombinant viral vectors including retroviruses and lentiviruses (which bears a risk for genomic integration), adenovirus, and adeno-associated viruses (only remains transiently stable in a episomal form in the host cell's nucleus [192, 193] encoding the intended RNA are also being explored for intracellular delivery of miRNA-based therapeutics and hence become a major focus of attention [194]. A Phase II trial is currently testing an adeno-associated viral vector for the delivery of the miRNA drug AMT-130 for the treatment of Huntington's disease (ClinicalTrials.gov identifier NCT04120493) [195-197]. Despite their potential for the delivery and expression of miRNAs, there are various side effects with the use of viral vectors such as immunogenicity and transgene-related immune responses [198].

Packaging of the negatively charged nucleic acids in liposome nanoparticles mask their negative charge and also protects against serum nuclease degradation [190, 199]. Delivery of miRNAs using liposome nanoparticles has already been applied in several clinical studies, such as MRX34 (NCT01829971, NCT02862145) [82, 83].

Similarly, miRNA-loaded bacterial minicells were used for the delivery of miR-16 mimics in a phase 1 trial in patients with recurrent malignant pleural mesothelioma (MesomiR 1, NCT02369198) [162, 200]. However, the study also reported several side effects including dose-limiting toxicities, decreased lymphocyte counts, or cardiac events [162].

Extracellular vesicles (and exosomes) that can be loaded with a desired cargo to permit in-body cellular transfer are also being explored as drug delivery systems [201]. EVs derived from mesenchymal stromal cells from human adipose tissue were engineered to package miR-125b which consequently was shown to inhibit the proliferation of human hepatocarcinoma cells [202].

In addition, different modalities of drug delivery systems have been explored for the delivery of miRNA-based drugs such as core-shell magnetic nanoparticles, quantum dot nanocrystals, polymeric micelles, and mesoporous silica nanoparticles are among the other examples of nanocarriers as drug-delivery systems to improve the therapeutic effectiveness and specificity, and tissue targeting of miRNA and other nucleic acid therapeutics [145].

For example, covalent conjugation of miRNAs and other nucleic acid-based drugs and biomolecules to lipids, peptides, or sugars that work via receptor-mediated endocytosis mechanisms is another promising strategy [190].

Similarly, lipophilic cholesterol conjugate was used for a cell type–independent delivery of miR-29–based mimic (remlarsen/MRG-201) to human skin fibroblasts by skin injection in a Phase II trial on keloid disorder to repress extracellular matrix expression and fibroplasia in the skin (NCT02603224, NCT03601052) [160].

Aptamer conjugates coupled to corresponding miRNA therapeutics by simple sticky-end annealing [203] is yet another strategy for the delivery of miRNAs to intended cell type. Aptamers are single-stranded nucleic acids that are developed as high-affinity ligands specific to a cell surface receptors to facilitate the delivery of therapeutic cargo including miRNAs through receptor-mediated transport [190, 203]. Aptamer-conjugated miRNAs, including the Aptamer-miR-34c conjugate (GL21.T-miR-34c) are currently being explored in preclinical studies in non-small-cell lung cancer cells [204].

Other biomolecule conjugates including N-acetylgalactosamine (GalNAc) which enables a targeted delivery of nucleic acid therapeutics via endocytosis by the stimulation of the liver cell–specific asialoglycoprotein receptors have also been explored in clinical trials [205, 206]. GalNAc conjugated to a miR-122 inhibitor (RG-101) and to an miR-103/107 inhibitor (RG-125/AZD4076) were explored in clinical trials for chronic HCV [EU Clinical Trials Register (clinicaltrialsregister.eu) EudraCT numbers 2015-001535-21, 2015-004702-42, 2016-002069-77] and steatohepatitis (NCT02612662, NCT02826525), respectively [206-208]. However, because of some side effects (i.e., jaundice cases), the RG-101 clinical trial was halted and the cause of which is still under investigation [156, 209, 210].

Other examples of the use of GalNAc-Conjugated LNA, anti-miR-122 antisense oligonucleotides, or nano-carrier vehicles in combination with cell type-specific biomolecule conjugates or miR-155 inhibitors by gold nanoparticles functionalized with antagomir and AS1411 aptamer have been explored in recent preclinical studies [211, 212].

In addition, the 3D matrices for delivering nucleic acid–based therapeutics and conventional drugs are currently undergoing optimization with diverse design features. This encompasses various application routes, such as edible or injectable carriers [77, 213-215]. One potential method of administering miRNAs is orally [75]. MiRNAs are often associated with extracellular vesicles (EVs), RNA-binding proteins, lipoproteins, or lipid derivatives, along with nanoparticles which shield miRNAs from the adverse gastrointestinal environment, including salivary and pancreatic RNases, the stomach's low pH, digestive enzymes, peristaltic activity, and microbial enzymes. Such protection likely facilitates the absorption of miRNAs from the digestive tract [75]. However, there is ongoing debate surrounding the absorption, stability, and physiological impact of these food-derived miRNAs.

7. Progress in chemical modifications of miRNAs for improved stability and cellular uptake

As illustrated in **Figure 4** and discussed in the literature [77], a combination of chemical modifications, biomolecule conjugation, or the use of carriers improves site-directed and efficient cell targeting of miRNA and other nucleic acid therapeutics. In addition, various types of chemical modifications of nucleobases, ribose sugar, or the phosphate backbone can mask the negative charge of the miRNAs and other nucleic acids and increase their adhesion to the cell surface, thereby facilitating cellular uptake [190, 216] and also improving their stability.

Among the commonly used nucleic acid modifications, locked nucleic acid (LNA) bases [216] are characterized by the introduction of methylene bridges which reduce the flexibility of the ribose ring, resulting in a locked conformation of the modified nucleotides [217, 218].

LNA-modified RNA-based therapeutics are more resistant to ribonucleases and exhibit improved cellular uptake, primarily through an endocytosis mechanism that

remains not fully understood [219]. The locked conformation enhances the capability of RNA-based therapeutics to establish stable duplexes by binding to and inhibiting the function of the target miRNA.

Consequently, LNA-modified RNAs are frequently employed in single-stranded antagomirs, such as antisense oligonucleotides (ASOs) and this locked conformation enhances the capability of ASOs to establish stable duplexes by binding to and inhibiting the function of the target miRNA [216, 219]. Due to these benefits, LNA-modified oligonucleotides have emerged as one of the primary approaches for inhibitory therapeutics targeting both miRNA and mRNA.

Phosphorothioate modifications, involving the introduction of a sulfur atom into the oligonucleotide's phosphodiester backbone, have emerged as a promising method for improving oligonucleotide stability and promoting endosomal uptake, particularly through stabilin receptors found on cell surfaces (e.g., in kidney cells) [220, 221]. The latter approach has been utilized for the targeted renal delivery of synthetic miR-21–anti-miR (RG-012/lademirsen/SAR339375) in a clinical investigation involving Alport syndrome (ClinicalTrials.gov identifiers NCT03373786, NCT02855268) [222]. However, certain sequence-independent effects have been documented in association with phosphorothioate-modified oligonucleotides [154]. A recent investigation has described a fully modified form of miR-34a (FM-miR-34a) that effectively addresses issues related to miR-34a stability, non-specific delivery, and delivery-related toxicity [69]. FM-miR-34a demonstrated a potent inhibition of proliferation and invasion, leading to sustained suppression of its target genes for over 120 hours following the in vivo delivery of FM-miR-34a conjugated to folate (FM-FolamiR-34a). This treatment resulted in the inhibition of tumor growth, leading to complete cures in some mice [69]. No significant changes observed in the body weight of mice throughout the study indicating the safety of FM-FolamiR-34. These results have the ability to revitalize miR-34a as an anti-cancer agent, providing a strong rationale for clinical testing.

8. Progress in predicting and validating miRNA targets

As discussed in the literature [85] the mechanistic functions of candidate miRNAs can be evaluated through bioinformatic analysis and/or in vitro experiments before progressing to testing in preclinical animal models. In addition. multiple databases and algorithms have been developed and are available for predicting their targets associated with each miRNA [109, 223]. To enhance predictive accuracy of miRNA target prediction, it is a common practice to employ multiple distinct algorithms simultaneously to predict miRNA binding sites in protein-coding genes and relevant biological pathways and networks. An example algorithms is TargetScan [109], which predicts miRNA targets based on seed regions that are essential for mRNA binding. TargetScan encompasses nearly all miRNA sequences documented in miRBase to date.

Likewise, advances in high-throughput screens and bioinformatic tools for target predictions have significantly facilitated the study of miRNAs and prediction of their putative targets and biological pathways. For example, bioinformatics tools such as KEGG and Ingenuity Pathway Analysis not only predict potential biological pathways but, in some instances, also identify disease states that may be influenced by miRNAs.

Furthermore, various computational tools can be used for calculating the free energy between the RNA sequences of interest [224]. For example, a lower free energy, typically around -20 or less, is indicative of a more robust binding [224]. Consequently, the integration of clinical research databases with miRNA bioinformatics platforms could further improve the identification and assessing potential therapeutic candidates.

As for the preclinical models, various human cell lines and induced-pluripotent stem (IPS) cells have been used to investigate the mechanisms, toxicity, and potential therapeutic efficacy of miRNA candidates as well as epigenetic manipulation of target transcripts [85]. For example, use of IPS cells enable modulation of biological pathways along distinct stem cell lineages from readily available skin tissue source [225, 226]. Furthermore, the

availability of various animal models through academic laboratories or commercially facilitated efficient validation of findings from in vitro miRNA studies. Other animal models including nonhuman primate models have also been successfully used for the preclinical safety and toxicology testing of miRNA therapeutics supporting the initiation of several human miRNA therapeutic clinical trials.

Like other drug classes, the development of miRNA-based drugs must go through a sequence of developmental stages, spanning from discovery to preclinical studies, toxicology assessment, pre-IND, and multiple phases of human clinical trials prior to approval by regulatory agencies before market entry (**Figure 5**) [142].

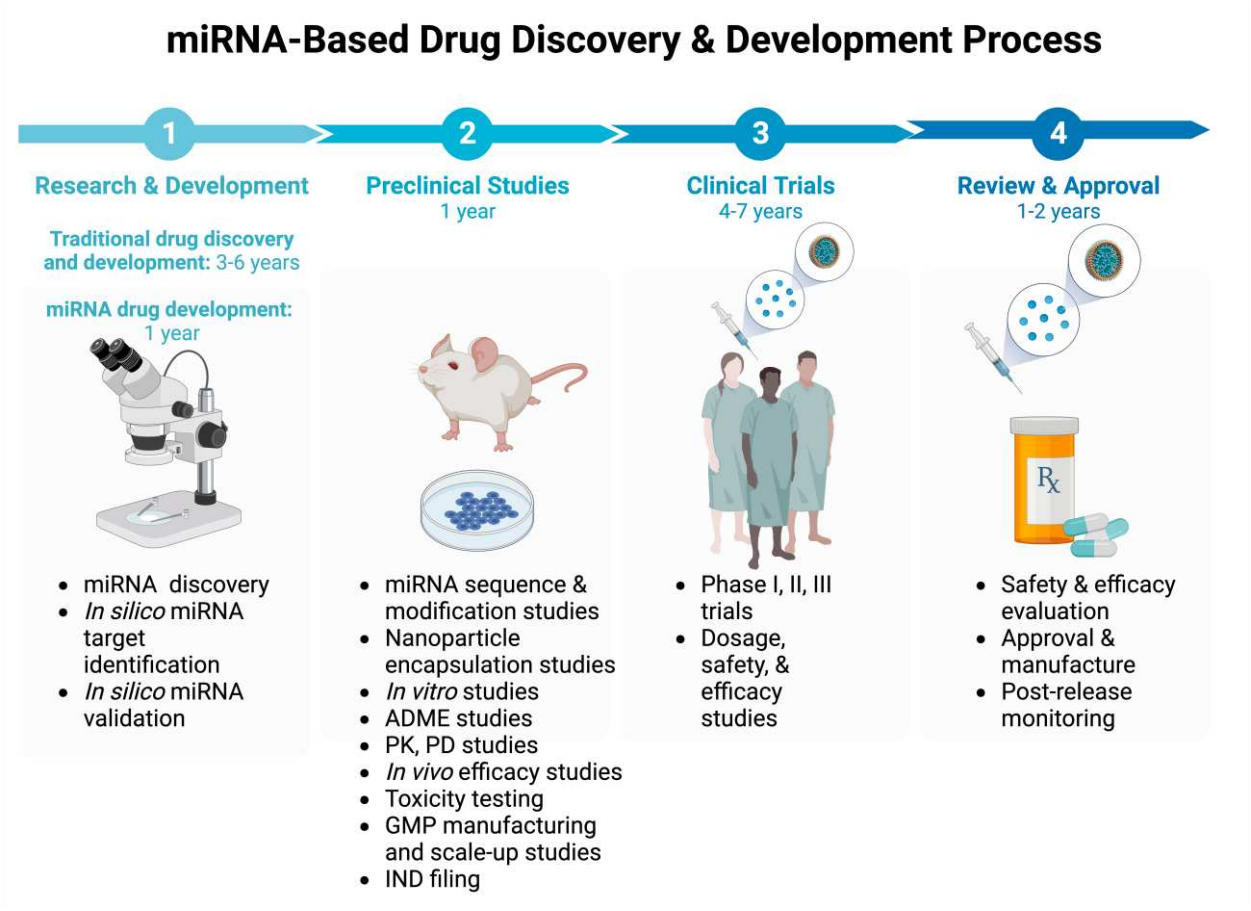

Figure 5. Illustration of the miRNA drugs discovery and development process beginning from the target identification and miRNA discovery to FDA approved miRNA therapeutics in the market. In the traditional drug development process, the timeline from target, drug discovery to phase 1-3 human clinical trials and, ultimately, FDA approval, followed by Phase 4 studies can go on for several years. Conversely, RNA-based and more specifically, miRNA-based drug development can accelerate miRNA-based drug discovery and development, potentially mitigating attrition rates, time constraints, and costs. **Created with BioRender.com.**

In the traditional drug development process, the timeline from identifying a drug target to drug discovery and lead development through preclinical and phase 1-3 human clinical trials, FDA approval, and subsequent Phase 4 studies often spans several years. However, the escalating costs and time required in this process have become unsustainable, urging the imperative to hasten drug discovery and development while curbing associated expenses and timeframes.

Conversely, leveraging RNA-based methodologies, particularly miRNA-based approaches, holds promise in expediting both the discovery and development of drugs, potentially mitigating attrition rates, reducing time constraints, and cutting costs.

9. Progress in preclinical validation of miRNA therapeutics

As discussed in the literature [77, 85], single miRNAs not only can regulate an entire pathway and multitude of mRNA targets but also each mRNA may also be targeted by multiple miRNAs [227]. It has been suggested that miRNAs can regulate a diverse range of RNAs stems from their ability to bind to target mRNAs even when the pairing is not perfect. As a result, a single miRNA can regulate a pool of targets involved in similar cellular processes, thereby amplifying the cellular response Although a single miRNA can inhibit hundreds of genes, the effect on each gene is generally mild [116] and multiple miRNAs can regulate the same gene [117, 227] thereby amplifying the cellular response.

Because a single miRNA has the potential to bind to as many as 200 target mRNAs, each with diverse functions, including transcription factors, receptors and many others, consequently, entire signaling pathways can be regulated by individual miRNAs [117] or miRNA clusters [228].

The pivotal role of miRNAs in the regulation of multiple genes and their far-reaching effects within regulatory networks presents significant challenges for miRNA therapeutics [229][85]. Therefore, the successful development of miRNA-based therapeutics requires a comprehensive functional characterization and validation of the molecular effects of each miRNA prior to their application as therapeutics [77, 85]. Toward the functional characterization of each miRNA, many steps must be undertaken, ranging from the confirmation of the authenticity of an miRNA as a true miRNA, the enhancement of miRNA target prediction algorithms, and the experimental validation of their functional effect on their intended targets in relevant preclinical models [77].

10. Off-target effects of miRNA therapeutics

Among the challenges inherent in miRNA-based therapeutics, off-target effects and associated toxicities emerge due to the capacity of each miRNA to regulate the expression of multiple genes and are one of the main challenges associated with miRNA therapeutics. Thus, further research is essential for the development of miRNAs as effective cancer therapeutics. Additionally, numerous miRNAs are dysregulated not only in cancer cells but also in other cells within the tumor microenvironment (TME), where they might have opposing functions.

Furthermore, depending on the route of administration and the way to enable an intracellular delivery, the miRNA therapeutics are not necessarily restricted to the intended tissue or cells but can also lead to systemic side effects [77]. For example, MRX34, a synthetic miR-34a mimic as a tumor suppressor [230] systemically administered by a liposomal amphoteric (i.e., pH-dependent) delivery, which takes advantage of the low-pH environment of tumorous tissues [231] used for the treatment of various solid tumors and hematologic malignancies (NCT01829971) was terminated prematurely because of severe immune-related side effects leading to the death of four patients [82, 83]. Previous animal studies, however, demonstrated that a miR-34a mimic was not only taken up by tumor tissue but also by bone marrow and spleen [232, 233] which are involved in the generation and preservation of immune cells. Supporting these preclinical observations, the clinical testing of miR-34a mimic demonstrated a dose-dependent change in several target genes in white blood cells [83]. Consequently, miR-34a mimic not only functions as a tumor suppressor but also impacts the immune cells by modulating calcium or chemokine signaling such as CXCL10/CXCL11/CXCR3-axis in CD4+, CD8+ T cells, and M1 macrophages [234, 235].

Despite the lack of the direct causative link between the patient death, the miR-34a function in immune cells has not been yet established. However, the serious and deadly adverse effects of miR-34a mimic MRX34 underscores the need for a priori risk assessment of miRNA therapeutics, specifically their potential off-target effects in other unintended tissues highlighting the need for the development of more precise tissue target delivery systems.

11. Challenges and future perspectives

In addition to their potential utility as biomarkers [68], miRNA mimics, and inhibitors provide a significant potential as therapeutics as many miRNAs function as oncomirs or tumor suppressors, and restoring deregulated miRNA levels to that of healthy tissue levels could potentially help in maintaining the endogenous anti-tumor regulatory mechanisms. While many new immunotherapeutic such as antibodies, recombinant proteins, cell therapies, and small molecules have shown their success in the treatment of various types of cancers [236], because of the global success of mRNA vaccines as modulators of immune stimulation for tackling COVID-19 pandemic, there has been a resurgence of RNA-based cancer immunotherapies [187]. Moreover, further improvements in RNA chemistry and delivery are opening new opportunities for RNA-based immunotherapy. Although, drug development, including RNA-based drugs, often requires many years and substantial costs before approval by the FDA or other regulatory agencies (**Figure 5**), the global threat of the COVID-19 pandemic has catalyzed the extremely rapid development of a new class of mRNA-based vaccines. These mRNA vaccines whether developed by BioNtech in partnership with Pfizer or Moderna have shown to deliver on their promise i.e., they can be developed extremely fast, can be manufactured under GMP-compliant manufacturing processes, and can be scaled for rapid availability of large numbers of doses, were safe and active at a relatively low dose range [141, 187, 237, 238]. Because of this, many companies are now leveraging the experience gained from the COVID-19 vaccine development to develop RNA–based cancer therapies and potentially for other diseases. This of course have reignited the potential of miRNA as both diagnostics and therapeutics. The approval of several antisense, small interfering RNA (siRNA), and mRNA-based drugs and vaccines have validated their potential and opened the door for expansion into new indications. Research surrounding RNA-based therapies comprises a broad ecosystem, ranging from RNA engineering, and RNA chemistry including various modifications to improve pharmacokinetics and reduce non-specific undesirable side effects to delivery technologies. Furthermore, novel RNA constructs including those self-amplifying RNA, circular RNA, siRNA, as well as gene editing (Cas9 mRNA, single guide RNA [sgRNA]) all hold promise for next-generation cancer immunotherapy.

However, despite this potential over many years, there are several challenges including sensitivity, specificity, toxicity immunogenicity, delivery among many others which are a significant barrier to exploiting the full potential of miRNAs as therapeutics.

In addition, the development of novel targeted delivery systems would be vital for the delivery of miRNAs. The delivery method must be target-specific, and be able to deliver the miRNA drugs to the targeted cells or tissues [142]. As illustrated in **Figure 3**, the future lies in the targeted delivery vehicles, including lipid and polymer nanoparticles, cell or extracellular vesicle-based packaging, and hybrid systems, as well as viral vectors that will likely increase the therapeutic potency of various RNA-based therapies while decreasing side effects.

In addition, there are other challenges that need to be addressed with regard to the sensitivity, specificity, toxicity, and applicability of potential utility of miRNAs as therapeutics and therapeutic targets. Because each miRNA regulates more than one gene, sometimes a single miRNA can regulate entire cellular pathways via interacting with multiple target genes. Likewise, each mRNA is regulated by more than one miRNA [77]. This phenomenon is referred to as “too many targets for miRNA effect” (TMTME) [129]. Although this characteristics of miRNAs make them a powerful new class of therapeutic, it also represents a major challenge in terms of controlling adverse effects that have been observed in clinical trials [77]. Because of this, Zhang et al. [129] proposed that adverse events observed in terminated clinical trials involving miRNA therapeutics could be attributed to the broad-ranging effects of miRNAs.

In addition, the type of miRNAs might change during the course and stage of cancer which further complicates the target prediction, however, can also be beneficial for assigning a specific miRNA or several miRNAs to a specific stage of cancer. Therefore, more

innovative approaches for predicting miRNA targets might be required to validate the predicted targets.

Additional issues that need to be resolved include immunogenic reactions. Although viral delivery systems undoubtedly enhance cellular uptake and expression of miRNAs, they are associated with various side effects, including immunogenicity [198]. To address this issue, there needs to be a better understanding on the prevalence of immunogenic reactions resulting from viral transfer systems.

In addition, further research is needed to investigate whether severe immunogenic reactions are also possible due to modifications in miRNAs, such as LNA miRNAs and artificial miRNAs (amiRNAs), or through miRNA interfering molecules like small cell-permeable molecules, application systems including biodegradable 3D matrices, carriers like functionalized metals, viral transfer systems, or biomolecule conjugates such as aptamers? Additionally, is it feasible to diminish immunogenic reactions by concealing or masking reactive components?

Additionally, as detailed, as summarized in **Table 4** and discussed in literature, [77] there are many other key outstanding issues that must be overcome before miRNA therapeutics can become widely adopted as novel therapeutics in the clinic.

Table 4. Key questions to address before miRNA therapeutics become clinically impactful.

1	What methods can be used to effectively guide therapeutic miRNAs/miRNA inhibitors to their intended target tissue and cells in vivo?
2	How can the design of miRNA/miRNA-based drugs and delivery vehicles be optimized to reduce or, ideally, eliminate unintended impacts on non-targeted cells?
3	What other strategies can be used to improve more accurate targeting for miRNA/miRNA inhibitor therapeutics?
4	Is there a risk of incompatibilities when using diverse carrier materials for advanced miRNA/miRNA inhibitor-based drug delivery, which may lead to undesired interactions between the materials and miRNA therapeutics?
5	Is there a risk of incompatibilities when using miRNA/miRNA inhibitor therapeutics in combination with traditional drugs pose the risk of incompatibilities?
6	Do modifications of miRNA/miRNA inhibitors such as LNA miRNA/miRNA inhibitors and other synthetic miRNAs, as well as agents such cell-permeable molecules, delivery methods such as biodegradable 3D matrices, carriers like functionalized metals, viral transfer systems, or biomolecule combinations such as aptamers invoke immunogenic responses? If so, can the activation of immunogenic responses be ameliorated through the masking of reactive components or moieties?
7	What is the level of risk associated with genomic integrations of viral transduction constructs that carry miRNA or miRNA inhibitors?
8	What is the impact of the expression of endogenous miRNAs and mRNAs on exogenously delivered therapeutic miRNAs and miRNA inhibitors which may be also affected by factors like cell type, cell cycle, and the cellular environment?
9	What is the necessary dosage for particular administration techniques for miRNA/miRNA inhibitors, such as skin injection, infusion, or inhalation, and for carrier-based methods like biodegradable 3D matrices?
10	How can the administration of miRNA/miRNA inhibitor therapeutic doses be regulated along intricate in vivo delivery pathways?
11	Is it possible to achieve consistent and sustainable rates of cellular uptake of miRNA/miRNA inhibitor therapeutics under varying in vivo conditions?
12	In what ways can dosing of miRNA mimics and inhibitors support the desired gene targeting outcome?

These findings emphasize the need for further investigation in developing miRNAs as both novel therapeutics and therapeutic targets for cancer.

12. Conclusions

Because many miRNAs is abnormally expressed or mutated in many cancers acting as oncogenes or a tumor suppressors [99, 239-241], they have emerged as potential biomarkers [68], therapeutic targets, and therapeutics [81]. However, there are many outstanding issues and challenges with respect to specificity and associated off-targeting effects of miRNA-based therapeutics since each miRNA appears to regulate more than target and each target is regulated more than one miRNA leading to undesired toxicity hence limiting their use as therapeutics.

Currently, most of the miRNA therapeutics are still in early phases of human clinical trials; as such, it is awaited to see how other miRNA therapeutics perform in human clinical trials in terms of toxicity or side effects. Notably, a few recent clinical trials using miRNA therapeutics have reported some serious adverse events. For example, MRX34, a microRNA liposomal injection developed by Mirna Therapeutics, Inc. evaluated in a Phase 1 clinical trial for its efficacy against melanoma was withdrawn (NCT02862145) or terminated (NCT01829971) [82-84] due to serious adverse events. As a result, numerous challenges must be addressed to bring therapeutic miRNAs into clinical practice. These include establishing miRNA specificity to their intended targets, reducing immunogenic reactions and adverse events, determining optimal dosing for the desired therapeutic effect while minimizing side effects [77], and developing improved methods for targeted delivery.

Despite these significant challenges, the potential of miRNAs as a therapeutic approach for various diseases is clear. Further research will be necessary to establish whether miRNAs can effectively serve as therapeutics or therapeutic targets for clinical applications.

Abbreviations and acronyms:

AGO	Argonaute
AGO2	Argonaute RISC Catalytic Component 2
ALS	Amyotrophic lateral sclerosis
ASO	Antisense oligonucleotides
CLL	Chronic Lymphocytic Leukemia
CTCL	Cutaneous T-cell Lymphoma
ATLL	Adult T-Cell Leukemia/Lymphoma
COVID-19	coronavirus disease 2019
DICER1	Dicer 1, Ribonuclease III
DLBCL	Diffuse Large B-Cell Lymphoma
EGFR	Epidermal growth factor receptor
DGCR8	DiGeorge Syndrome Critical Region 8
DROSHA	Drosha Ribonuclease III
EVs	Extracellular vesicles
GalNAc	N-acetylgalactosamine
HCV	Hepatitis C virus
KEGG	Kyoto Encyclopedia of Genes and Genomes
LNA	Locked nucleic acid (LNA)
MF	Mycosis fungoides
mRNA	messenger RNA
miRNA	microRNA
ncRNAs	non-coding RNAs
P-bodies	Processing bodies
pre-miRNA	precursor microRNA
pri-miRNA	primary microRNA
RNA Pol II	RNA polymerase II

SARS-CoV-2	Severe acute respiratory syndrome coronavirus 2
sgRNA	Single guide RNA
siRNA	Small interfering RNA
TDMD	Target-directed miRNA degradation mechanism
TME	Tumor microenvironment
TRBP	The TAR RNA-binding protein
TTR	Transthyretin
UTR	Untranslated region
XenomiRs	Exogenous miRNAs
XPO5	Exportin 5

Review criteria: Publicly available information such as PubMed and Internet were used for the literature review. We focused on identifying articles published on the use of miRNAs or miRNA-targeting as therapeutics. The research was restricted to the most recent studies in this field and all research was limited to human studies published in English.

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Data Availability Statement: The data presented in this study are openly available in https://seer.cancer.gov, https://pubmed.ncbi.nlm.nih.gov/, https://clinicaltrials.gov/, and https://www.genomicseducation.hee.nhs.uk/genotes/knowledge-hub/non-coding-dna, all accessed on 26 July 2023.

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