Clinical Applications of Short Non-Coding RNA-Based Therapies

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RNA therapies have demonstrated clinical potential for both the treatment of cancer and other pathologies. Therapeutic delivery and resulting adverse events remain significant roadblocks in implementing many of these drugs into clinical practice, but the FDA approval of three Alnylam Pharmaceuticals' small interfering RNAs (siRNAs) therapies has been a milestone in developing therapies tailored to disease-driving target genes. While it seems that RNAs can be administered "naked" in closed-compartment organs such as eyes and lungs, more research is needed for systemic administration. Lipid nanoparticles represent a promising delivery method, but some challenges remain because of their potential to elicit an immune response, relatively low circulation times, and relatively large size. The use of GalNAc for the delivery and targeting of siRNAs has made significant progress, but delivery systems targeted to organs other than the liver would broaden the range of diseases that could be treated with RNA therapies.

RNA interferences microRNA siRNA drug delivery precision oncology nanomedicine nanoparticle

1. Unconjugated Short Non-Coding RNAs (sncRNAs)

1.1. Diabetic Macular Edema and Age-Related Macular Degeneration

The first clinical trials that utilized siRNA targeting vascular endothelial growth factor (VEGF) in humans were performed in patients with macular degeneration [1]. Exudative, or "wet" age-related macular degeneration (AMD), is the leading cause of severe vision impairment for Americans over 65 years old [2]. When drusen accumulate on the retina during dry AMD, the resulting pressure on the retinal pigment epithelium causes an inflammatory response that upregulates VEGF, leading to choroidal neovascularization [2]. Diabetic macular edema (DME is) the leading cause of blindness occurring between the ages of 20 and 74; it can occur when upregulated VEGF causes increased permeability in the blood-retinal barrier, which leads to excess fluid in the eye, resulting in edema [3]. Prior clinical studies have established VEGF as an effective target for therapies, most commonly antibodies, to decrease vision loss that results from DME or AMD. The standard of treatment is anti-VEGF antibodies such as ranibizumab, the intravitreal injections of which pose the risk of lens injury, intravitreal bleeding, endophthalmitis, and retinal tears due to the frequency of their administration every 4 to 6 weeks [2]. Bevasiranib (also known as Cand5) is a siRNA that targets VEGF mRNA [3].

The advantage of siRNA-based therapies rather than antibody-based therapies is that the upregulation of VEGF is due to mRNA stabilization rather than increased translation, and the use of siRNA theoretically allows downregulation rather than blocking the action [2]. Bevasiranib does show a definite anti-angiogenic effect, which takes about 6 weeks to develop from the start of treatment because existing VEGF mRNA is not fully eliminated. In this case, combination treatment with the anti-VEGF antibody could be most effective [2]. A phase 3 clinical trial was designed to address the benefits of this combination treatment but was never started (NCT00557791). Multiple studies have indicated that bevasiranib's main mechanism of action in the eye is not eliciting an RNAi response, but instead the RNA-mediated activation of the cell surface toll-like receptor 3 (TLR3), which suppresses CNV via intracellular signaling [1]. These siRNAs were not specifically designed for cell permeation, and the amounts of these small interfering RNAs (siRNAs) that reach their cognate target may have been lower than anticipated [1]. In 2009, a phase 3 clinical trial for bevasiranib was terminated because preliminary data suggested that the possibility of reaching the primary endpoint was very low (NCT00499590).

1.2. Respiratory Syncytial Infection

Respiratory syncytial virus (RSV) is the leading cause of infant hospitalization in the US, partly because there is no vaccine, and very few therapeutic options are available against this infection [4]. For lung transplant patients, RSV infection is the most common community-acquired respiratory virus and is associated with bronchiolitis obliterans syndrome, which is a serious roadblock to patient and graft survival [5]. ALN-RSV01 is a siRNA developed by Alnylam Pharmaceuticals that targets the mRNA encoding the nucleocapsid protein, which is critical for RSV replication [4][6]. As is the case with lung-targeted siRNAs, delivery without a carrier works well as it can be applied directly to the mucosa and degraded by the nucleases if it enters the systemic circulation [7]. Two safety and tolerability studies with 101 healthy adults showed intranasal administration to be safe and well-tolerated, with doses of 150 mg either once or five times [6]. After 88 healthy adults were experimentally challenged with RSV, 71.4% of those who received the placebo were infected compared to only 44.2% of those who received ALN-RSV01 [4]. In Phase 2a trials with transplant patients naturally infected with RSV, ALN-RSV01 was found, in combination with standard of care, to reduce the incidence of new or progressive bronchiolitis obliterans syndrome (BOS). However, it did not meet the primary endpoint of reduced day 180 BOS and failed to progress to a phase 3 trial [5].

1.3. Pachyonychia Congenita

Pachyonychia congenita (PC) is a dominant genetic condition characterized by thickened nails, leukokeratosis, keratoderma, and painful blistering primarily located on the soles of the feet [8]. Over 50% of patients are not able to walk without the aid of an ambulatory device [9]. Current treatments for PC are limited to inadequately effective symptom management through mechanical callus removal, topical keratolytics, and oral retinoids [9]. This condition results from mutations in keratins *K6a*, *K6b*, *K16*, or *K17*. A siRNA therapeutic, TD101, targets *K6a* mRNA, which is the most commonly mutated gene [8][9]. Quantitative reverse transcription PCR (qRT-PCR) was used to measure in vivo mRNA levels to verify the effectiveness of intradermal injection of TD101 in reducing the expression of mutant

K6a. PC-10 cells and collected patient callus samples both expressed equal amounts of mutant and wild-type *K6a*. However, the mutant *K6a* expression was reduced by about 98% when TD101 was administered [8].

1.4. Hepatitis C

miR-122, the most abundant hepatocellular miRNA, promotes hepatitis C virus (HCV) propagation. By binding to the 5′ end of HCV RNA, miR-122 protects this RNA from nuclease attack and masks an RNA motif that may elicit an innate immune response [10]. Chronic HCV can lead to cirrhosis and eventually hepatocellular carcinoma [11]. Miravirsen, which is an anti-miR-122 ASO (**Figure 1**A, **Table 1**) composed of locked nucleic acid (LNA) ribonucleotides that hybridize to mature miR-122 and block its interaction with HCV RNA, is currently in clinical trials [12]. LNAs have the second oxygen molecule linked to the 4′ carbon in the ribonucleotide (**Figure 1**B). This modification protects the oligonucleotide from nuclease degradation and can increase target affinity [11][13]. Treatment with miravirsen has been found to cause a dose-dependent decrease in viral load in chronic HCV patients in clinical trials, with no significant effects on the plasma levels of other miRNAs [10]. A placebo-controlled study of 5 weekly doses of miravirsen reduced plasma levels of miR-122 from 3.9×10^3/4 to 3.1×10^1/4 µL one week after the first dose in the experimental group and maintained these levels for the entirety of the study period in the highest-dose group [10]. In comparison, the mean plasma levels in the placebo group were 1.3×10^4/4 µL at baseline and 1.1×10^4/4 µL after one week of treatment. All dosed patients responded to therapy, with some having undetectable levels of miR-122 after treatment. Although the viral load decreased in dosed patients, there was no correlation between the decrease in miR-122 plasma levels and HCV viral load. Many of the patients with virological relapse after taking miravirsen had a C3U nucleotide change in the 5′UTR region of their HCV RNA, which is hypothesized to render this process miR-122-independent and therefore resistant to miravirsen [14][15]. miR-122 may also act as a tumor suppressor [16], which has raised some concerns that anti-miR-122 treatment could lead to an increased risk of hepatocellular carcinoma [16]. However, in preclinical studies, mice that were fed miravirsen for 5 weeks did not develop tumors. Still, this concern warrants further safety studies to evaluate the risk [12], given that mir-122-knockout mouse models do develop hepatocellular carcinoma [14][16].

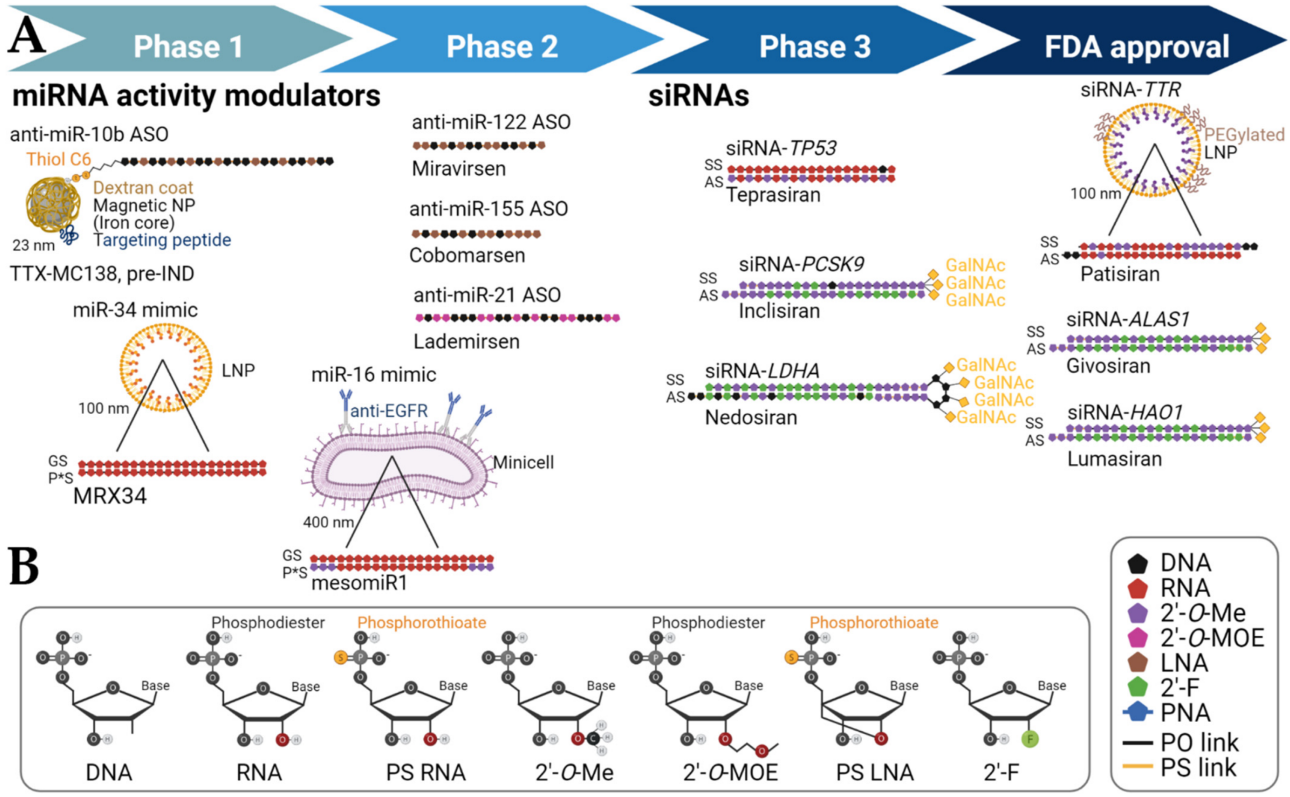

Figure 1. Chemical modifications and specific sequence patterns of these modifications facilitate the clinical application of RNA therapeutics. (**A**) Stage of clinical development of representative RNA therapies and chemical formulations behind their therapeutic effect. Pattern and location of chemical modifications are approximations (in some cases, the exact sequence is not disclosed). For siRNAs, the top strand (5′-end on the left) is the sense strand (SS), and the bottom strand (5′-end on the right) is the active antisense (AS) strand. For miRNA mimics, the top strand is the active mature miRNA guide (GS), and the bottom strand is the passenger strand (P*S). Nucleotides and other molecules are not drawn to scale. (**B**) Chemical structure of common sugar and backbone modifications of RNA therapeutics in clinical trials are depicted (inset). Abbreviations: 2′-F = 2′-deoxy-2′- fluoro; 2′-*O*-Me = 2′-*O*-methyl; 2′-*O*-MOE = 2′-*O*-methoxyethyl; *ALAS1* = delta-aminolaevulinic acid-synthase; ASO = antisense oligonucleotide; EGFR = epidermal growth factor receptor; GalNAc = *N*-acetylgalactosamine; *HAO1* = hydroxyacid oxidase 1; IND = investigational new drug; *LDHA* = hepatic lactate dehydrogenase A; LPN = lipid nanoparticle; NP = nanoparticle; *PCSK9* = proprotein convertase subtilisin/kexin type 9; PEG = polyethylene glycol; PS = phosphorothioate; PO = phosphodiester; *TP53* = tumor protein p53; *TTR* = transthyretin.

Table 1. miRNA-based therapies in clinical trials.

miRNA Modulation	Drug Name	Chemistry	Platform	Delivery	Disease (Organ Site)	Sponsor	Clinical Status	References
miR-10b inhibition	RGLS5579	ASO (2′-O-MOE, partial PS backbone)	-	Intravenous or intracranial	Glioblastoma (Brain)	Regulus Therapeutics (San Diego, CA, USA)	Pre-IND filing	[17]

miRNA Modulation	Drug Name	Chemistry	Platform	Delivery	Disease (Organ Site)	Sponsor	Clinical Status	References
miR-10b inhibition	TTX-MC138	ASO (partial LNA, partial PS backbone)	Dextran-coated iron oxide magnetic nanoparticle	Intravenous	Metastatic breast cancer (Lung, other organs)	Transcode Therapeutics (Boston, MA, USA)	Pre-IND filing, scheduled 2022	[18]
miR-16 restoration	mesomiR1 (TargomiR)	dsRNA mimic (2′-O-Me on passenger strand only)	Bacterial minicells with anti-EGFR bispecific antibody	Intravenous	Recurrent malignant pleural mesothelioma and non-small cell lung cancer (Lung)	Asbestos Diseases Research Foundation (New South Wales, Australia), EnGeneIC Limited (Lane Cave West, Australia)	Phase 1	NCT02369198, Competed
miR-21 inhibition	Lademirsen (SAR339375; previously known as RG-012 [Regulus])	ASO (sugar 2′ position modifications, PS backbone)	Unconjugated	Subcutaneous, 1.5 mg/kg	Alport syndrome (Kidney)	Genzyme, a Sanofi Company (Cambridge, MA, USA)	Phase 1	NCT02855268, Completed
miR-21 inhibition	Lademirsen (SAR339375; previously known as RG-012 [Regulus])	ASO (sugar 2′ position modifications, PS backbone)	Unconjugated	Subcutaneous	Alport syndrome (Kidney)	Genzyme, a Sanofi Company (Cambridge, MA, USA)	Phase 2	NCT02855268, Recruiting
miR-34a restoration	MRX34	dsRNA mimic	Liposome	Intravenous	Primary liver cancer or other selected solid tumors or hematologic malignancies (Liver, other organs)	Mirna Therapeutics (Austin, TX, USA)	Phase 1	NCT01829971, Terminated; NCT02862145, Withdrawn
miR-92a inhibition	MRG-110	ASO (LNA-modified)	-	Intradermal	Wound healing	miRagen Therapeutics, Inc. (Boulder, CO, USA)	Phase 1	NCT03603431, Completed
miR-122 inhibition	Miravirsen (SPC3649)	ASO (partial LNA, PS	Unconjugated	Subcutaneous	HCV chronic infection	Copenhagen, Denmark	Phase 2	NCT01200420, Completed

This list includes all studies that resulted from the search of the keyword “miRNA” as an interventional drug in the US National Library of Medicine (www.clinicaltrials.gov, accessed on 15 March 2022). Additional examples are included when there is strong evidence for clinical evaluation. Abbreviations: 2′-O-Me= 2′-O-methyl; 2′-O-MOE = 2′-O-methoxyethyl; ASO = antisense oligonucleotide; ATLL = adult T-cell leukemia/lymphoma; CLL = chronic

miRNA Modulation	Drug Name	Chemistry	Platform	Delivery	Disease (Organ Site)	Sponsor	Clinical Status	References
		backbone)			(Liver)			
miR-155 inhibition	MRG-106 (Cobomarsen)	ASO (partial LNA)	Unconjugated	Intratumoral and/or intravenous or subcutaneous	Certain lymphomas and leukemias, including CTCL [mycosis fungoides subtype], CLL, DLBCL [activated B-cell (ABC) subtype], and ATLL	miRagen Therapeutics, Inc. (Boulder, CO, USA)	Phase 1 [19][20]	NCT02580552, Completed
miR-155 inhibition	MRG-106 (Cobomarsen) [21]	ASO (partial LNA)	Unconjugated	[21] Intravenous	CTCL [mycosis fungoides subtype]	miRagen Therapeutics, Inc. (Boulder, CO, USA)	Phase 2	NCT03713320 and NCT03837457, Terminated

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1.6. Alport's Disease

miR-21 is a multi-faceted miRNA involved in carcinogenesis, fibrosis, inflammation, and immune response [22][23][24][25]. Alport syndrome is a genetic disorder caused by mutations in the genes encoding several α chains of collagen 4. Altered collagen 4 function compromises the capillary membranes in the kidney and other organs. miR-21 expression is upregulated in patients with Alport syndrome and genetic mouse models of this disease [26][27]. In the *Col4a3*$^{-/-}$ mouse model, subcutaneous injection of 25 mg/kg anti-miR-21 ASO twice a week extended animal survival by 46% [26]. The anti-miR-21 ASO treatment significantly delayed glomerulosclerosis, the formation of glomerular crescents, and periglomerular fibrosis, which are associated with the progression of Alport syndrome [26]. Mechanistically, anti-miR-21 ASO treatment dampens TGF-β induced fibrosis and inflammation and protects PPARα/retinoid X receptor (PPARα/RXR)-dependent mitochondrial activity, which extends kidney function. In phase 1 clinical trials, patients with Alport syndrome received subcutaneous injections of 1.5 mg/kg of anti-miR-21 ASO (RG-021, now known as lademirsen) as a single dose or four doses given every 14 days (NCT03373786). Treatment was well-tolerated, and a phase 2 clinical trial is now actively recruiting patients with Alport syndrome to evaluate the therapeutic efficacy of lademirsen in maintaining kidney function (NCT02855268).

1.7. Cardiovascular Disease

MRG-110, an LNA-modified ASO, targets miR-92a-3p as a therapy for cardiovascular disease and wound healing [13]. miR-92's negative impact on wound healing due to anti-angiogenic effects, caused in part by the downregulation of pro-angiogenic integrin alpha 5, can be modulated by its inhibition, which is shown to improve vascularization after a heart attack, circulation after hind limb ischemia, and wound healing [13][28]. MRG-110 treatment causes the dose-dependent reduction of miR-92a-3p in whole blood. It also increases granulation tissue formation and promotes angiogenesis in experimental porcine and db/db mouse models of acute and chronic excision wounds [28]. These effects were greater in the MRG-110 group than in the positive control groups treated with rhVEGF-165 and rhPDGF-BB, indicating significant clinical promise. No safety concerns were of note. In human trials, it was found that with half-maximal dosing between 0.27 and 0.31 mg/kg, the effectiveness of the

treatment was significant [13]. In the high dose groups, there was more than 95% inhibition after 24–72 h of treatment, and this inhibition remained significant for 2 weeks.

1.8. Leukemias and Lymphomas

MRG-106 (cobomarsen), an LNA-modified ASO, targets miR-155 as a therapy for several hematologic cancers, including cutaneous T-cell lymphoma (CTCL), diffuse large B-cell lymphoma (DLBCL), and chronic lymphocytic leukemia (CLL). Functional studies and clinical data supported an important role of miR-155 in the etiology of mycosis fungoides (MF), the most common subtype of CTCL [29]. The way cobomarsen was formulated favored uptake by MF cells and CD4+ T-cells [29]. Cobomarsen treatment elevated expression of miR-155′s direct targets, BACH1, PICALM, and JARID2, and interfered with the pro-survival activity of miR-155 [29]. BACH1 (BTB and CNC homology 1, basic leucine zipper transcription factor 1) is a mediator of the oxidative stress response, PICALM (phosphatidylinositol binding clathrin assembly protein) is an endocytosis adaptor, and *JARID2* (jumonji and AT-rich interaction domain containing 2) is a negative regulator of leukemia cell proliferation [29]. A phase 1 clinical trial (NCT02580552) demonstrated the safety and low toxicity of cobomarsen in patients with hematological cancers. A phase 2 clinical trial (NCT03713320) was initiated in 2018 to determine the efficacy and safety of cobomarsen treatment in comparison to vorinostat, a histone deacetylase (HDAC) inhibitor, in patients with CTCL of the MF subtype. An appealing aspect of cobomarsen treatment is that it can be administered weekly in contrast to daily dosing of vorinostat; however, cobomarsen is administered intravenously vs. the oral delivery of vorinostat. This clinical trial was terminated after recruiting 37 patients due to business reasons and not any specific concerns with cobomarsen's efficacy [30]. An anticipated crossover phase 2 clinical trial (NCT03837457) was terminated due to the lack of eligible patients. Genetic studies in *Mir-155*-knockout mouse models and successful treatment with anti-miR-155 ASO or similar inhibitors in in vivo animal models [22] and exceptional response in a single patient diagnosed with an aggressive subtype of DLBCL [31] support the further clinical evaluation of cobomarsen.

2. GalNAc-Conjugated sncRNAs

2.1. Porphyria

Acute hepatic porphyria arises from defects in the enzymes essential to the hepatic heme biosynthesis pathways, leading to the accumulation of toxic heme intermediates [32]. In 2019, givosiran (GIVLAARI®, Alnylam Pharmaceuticals) was approved by the FDA as a first-of-its-kind siRNA for the treatment of acute hepatic porphyria [33][34]. Givosiran targets and degrades delta-aminolaevulinic acid-synthase 1 (*ALAS1*) mRNA, ameliorating acute porphyric attacks. Givosiran is a GalNAc-conjugated siRNA that targets *ALAS1* in the liver (**Figure 1**A). In phase 3 trials (**Table 2**), patients dosed subcutaneously with 2.5 mg/kg of givosiran monthly for 6 months had a 74% lower annual rate of porphyria attacks than patients who received the placebo [35]. The rate was 3.2 for the experimental group versus 12.5 for the placebo group. Notably, the experimental group had higher rates of renal and hepatic adverse events than the placebo group.

Table 2. RNAi-based therapies in phase 3 clinical trials.

Target Gene	Drug Name	Chemistry	Platform	Delivery	Treatment (Organ Site)	Sponsor	References
ALAS1	ALN-AS1 (Givosiran) *	siRNA (2′-*O*-Me, 2′F, partial PS backbone)	GalNAc conjugation, 2.5 mg/kg	Subcutaneous	Acute Hepatic Porphyrias (Liver)	Alnylam Pharmaceuticals (Cambridge, MA, USA)	NCT03338816, Completed
AT	Fitusiran ALN-AT3SC (Fitusiran)	siRNA (2′-*O*-Me, 2′-F, partial PS backbone)	GalNAc conjugation	Subcutaneous	Hemophilia A or B (Liver)	Genzyme, a Sanofi Company (Cambridge, MA, USA)	NCT03417102/03417245, Completed; NCT03754790/NCT03549871, Active
CASP2	QPI-1007	siRNA (2′-*O*-Me)	Up to 3 mg	Intraviteal	Acute Nonarteritic Anterior Ischemic Optic Neuropathy (Eye)	Quark Pharmaceuticals (Newark, CA, USA)	NCT02341560, Terminated
HAO1	ALN-GO1 (Lumasiran) *	siRNA (2′-*O*-Me, 2′F, partial PS backbone)	GalNAc conjugation, up to 3 mg/kg	Subcutaneous	Primary Hyperoxaluria Type 1 (Liver)	Alnylam Pharmaceuticals (Cambridge, MA, USA)	NCT03681184, Active; NCT03905694, Active; NCT04152200, Active
LDHA	DCR-PHXC (Nedosiran)	DsiRNA pseudo-hairpin (2′-*O*-Me, 2′F, DNA, partial PS backbone)	GalXC	Subcutaneous	Hyperoxaluria (Liver)	Dicerna Pharmaceuticals (Lexington, MA, USA)	NCT04042402, Enrolling by invitation
PCSK9	Inclisiran	siRNA (2′-*O*-Me, 2′F, internal DNA, partial PS backbone)	GalNAc conjugation, 300 mg	Subcutaneous	Homozygous Familial Hypercholesterolemia (Liver)	Novartis Pharmaceuticals (Basel, Switzerland)	NCT03851705, Active; NCT04659863, Recruiting
PCSK9	Inclisiran	siRNA (2′-*O*-Me, 2′F, internal DNA, partial PS)	GalNAc conjugation, 300 mg	Subcutaneous	Atherosclerotic Cardiovascular Disease (ASCVD) or ASCVD High Risk and Elevated LDL-C (Liver)	Novartis Pharmaceuticals (Basel, Switzerland)	NCT04765657, Recruiting
PCSK9	Inclisiran	siRNA (2′-*O*-Me, 2′F, internal DNA, partial PS backbone)	GalNAc conjugation, 300 mg	Subcutaneous	Prevent Cardiovascular events in Participants with Established Cardiovascular Disease (Liver)	Novartis Pharmaceuticals (Basel, Switzerland)	NCT05030428, Recruiting

Target Gene	Drug Name	Chemistry	Platform	Delivery	Treatment (Organ Site)	Sponsor	References
TP53	QPI-1002 (Teprasiran)	siRNA (2′-*O*-Me)	-	Intravenous	Improved Graft Function after Donor Kidney Transplant (Kidney)	Quark Pharmaceuticals (Newark, CA, USA)	NCT02610296, Completed
TP53	QPI-1002 (Teprasiran)	siRNA (2′-*O*-Me)	-	Intravenous	Prevention of acute kidney injury after cardiac surgery (Kidney)	Quark Pharmaceuticals (Newark, CA, USA)	NCT03510897, Terminated
TRPV1	SYL1001 (Tivanisiran)	siRNA	Ophthalmic solution	Periocular	Sjögren's Syndrome, Dry eye (Eye)	Sylentis, S.A. (Madrid, Spain)	NCT04819269, Recruiting
TRPV1	SYL1001 (Tivanisiran)	siRNA	Ophthalmic solution, 11.25 mg/mL	Periocular	Moderate to Severe Dry Eye Disease (Eye)	Sylentis, S.A. (Madrid, Spain)	NCT03108664, Completed
TTR	ALN-TTR02 (patisiran)*	siRNA (2′-*O*-Me, DNA overhangs)	Lipid nanoparticle	Intravenous	Transthyretin-Mediated Polyneuropathy (Liver)	Alnylam Pharmaceuticals (Cambridge, MA, USA)	NCT01960348, Completed
TTR	ALN-TTR02 (patisiran)	siRNA (2′-*O*-Me, DNA overhangs)	Lipid nanoparticle, 0.3 mg/kg	Intravenous	hATTR amyloidosis with disease progression after liver transplant (Liver)	Alnylam Pharmaceuticals (Cambridge, MA, USA)	NCT03862807, Completed
TTR	ALN-TTR02 (patisiran)	siRNA (2′-*O*-Me, DNA overhangs)	Lipid nanoparticle	Intravenous	ATTR Amyloidosis with Cardiomyopathy (Liver)	Alnylam Pharmaceuticals (Cambridge, MA, USA)	NCT03997383, Active
TTR	ALN-TTRSC (Revusiran)	siRNA (2′-*O*-Me, 2′-F)	GalNAc conjugation	Subcutaneous	Transthyretin-Mediated Familial Amyloidotic Cardiomyopathy (Liver)	Alnylam Pharmaceuticals (Cambridge, MA, USA)	NCT02319005, Completed
TTR	ALN-TTRSC02 (Vutrisiran)	siRNA (2′-*O*-Me, 2′-F, partial PS backbone)	GalNAc conjugation, 25 mg	Subcutaneous	Transthyretin Amyloidosis with Cardiomyopathy (Liver)	Alnylam Pharmaceuticals (Cambridge, MA, USA)	NCT04153149, Active
TTR	ALN-TTRSC02 (Vutrisiran)	siRNA (2′-*O*-Me, 2′-F, partial PS backbone)	GalNAc conjugation	Subcutaneous	hATTR Amyloidosis (Liver)	Alnylam Pharmaceuticals (Cambridge, MA, USA)	NCT03759379, Active

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Alpha-1 antitrypsin deficiency is caused by a mutation in the AAT (SERPINA1), leading to the misfolding and polymerization of the AAT protein that promotes fibrosis and cirrhosis upon accumulation in hepatocytes, leading to pulmonary and liver disease [36]. There are very few available treatments for this condition, which leads to approximately 76 liver transplants every year in the United States [36]. ARC-AAT is a medicine consisting of a cholesterol-conjugated siRNA and a melittin-derived peptide conjugated to GalNAc formulated as the excipient, EX1, and is injected intravenously. With a maximum dose of 4 mg/kg for patients with AATD and healthy volunteers, the reductions of mutant AAT were 78.8% and 76.1%, respectively. Despite phase 1 trials having no SAEs and only minimal adverse events, clinical trials were terminated due to toxicity findings in primates when using the ARC excipient. Trials are now underway to develop the drug for subcutaneous injection, which does not require a delivery excipient.

2.3. Primary Hyperoxaluria

Target Gene	Drug Name	Chemistry	Platform	Delivery	Treatment (Organ Site)	Sponsor	References
VEGF	Bevasiranib	siRNA	Up to 2.5 mg	Intraviteal [37]	Age-Related Macular Degeneration following initiation of anti-VEGF Lucentis® antibody therapy (Eye)	OPKO Health, Inc. (Miami, FL, USA) [38]	NCT00557791, Withdrawn

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hyperoxaluria type 1 (**Figure 1**A). A phase 1/2 placebo-controlled trial of lumasiran achieved normal ranges of urinary oxalate (UOx) in 83% of patients receiving 3 mg/kg monthly, with no discontinuations or drug-related SAEs [39]. The mean maximal reduction was 75%, which remained at 66% 28 days after the last dose. A phase 3 trial dosed patients at baseline and at months 1, 2, 3, and 6 [40]. The primary endpoint was the percent change in 24-h UOx excretion over the 6-month study period, and the experimental group exhibited a 64.5% reduction, with effects seen within one month from the beginning of the treatment. At month 6, in 84% of the patients in the experimental group, UOx output fell within the range of 1.5 times from normal, whereas none of the placebo group patients exhibited any change [41].

2.4. Hemophilia

Fitusiran is a therapeutic siRNA against hemophilia that binds to and degrades the mRNA that encodes antithrombin [42]. Fitusiran, or ALN-AT3, is targeted to the asialoglycoprotein receptor in the liver via GalNAc conjugation. Prophylaxis for hemophilia is effective but invasive, so less than half of adults use it, whereas others use on-demand therapy; 35% of hemophilia A patients and between 3% and 5% of hemophilia B patients develop resistance to factor infusions, which can require less-effective bypass therapy [42][43]. Clinical trials have been able to achieve dose-dependent knockdown of AT with weekly or monthly subcutaneous injections [42]. This has been associated with increased thrombin generation and a reduction in annualized bleed rate for both hemophilia A and B. Of 30 patients with hemophilia, 48% were bleed-free and 67% were free of spontaneous bleeds. Two patients had dental procedures and two had surgeries while being treated, and fitusiran was found to minimize bleeding during those procedures. Although most patients only had mild injection site reactions, one patient had a fatal thrombosis resulting from using high doses of clotting factor VIII against trial guidance. No antibody development was seen. Monthly doses of 80 mg are being administered in ongoing phase 3 trials (**Table 2**).

2.5. Hepatitis B

ARC-520 is a hepatitis B drug comprised of two siRNAs that target HBV's viral mRNA and inhibit protein production [44]. ARC-520 uses a dynamic polyconjugates (DPC) delivery system with GalNAc for hepatocyte targeting and is able to target HBV mRNA transcripts to reduce hepatitis B surface antigen (HBsAg) [44]. The use of two siRNAs rather than one makes it less likely to allow for the selection of resistant mutants. Phase 1 trials used doses ranging from 0.01 to 4 mg/kg of intravenous ARC-520 on 54 healthy volunteers in combination with an antihistamine to avoid drug administration-related histamine release. Another study investigated the reduction of HBsAg in response to dosing with ARC-520 in combination with nucleos(t)ide analogs NUC [45]. A dose-dependent reduction from baseline was demonstrated in HBV e-antigen negative patients. There was a significant reduction of HBsAg in the experimental group compared to the placebo; however, this effect was not observed in the low-dose

experimental group. DCR-HBVS is another therapeutic from Dicerna Pharmaceuticals in phase I trials designed to treat chronic hepatitis B [46].

2.6. Cholesterol Metabolism and Atherosclerotic Cardiovascular Disease

The number one cause of death in the United States is cardiovascular disease (CVD), and atherosclerotic heart disease is the number one cause of CVD morbidity and mortality [47]. After a period of improved CVD health outcomes, United States death rates are on the rise again [47]. One of the most targetable factors in CVD is low-density lipoprotein cholesterol; LDL-C-lowering therapies have demonstrated improved survival and decreased risk of cardiovascular events. A determining factor in the plasma concentration of LDL-C is proprotein convertase subtilisin-kexin type 9 (PCSK9). Monoclonal antibodies that bind to PCSK9 have been found to effectively lower LDL-C levels by blocking protein interactions on the LDL-C receptor, thereby increasing its expression on hepatocytes. Treatments targeting *PCSK9* mRNA can also reduce symptoms of familial hypercholesterolemia [48]. Inclisiran (Novartis) is a *PCSK9*-targeting siRNA that is injected subcutaneously in the abdomen at the dose of 300 mg [49]. It is administered in addition to statins and requires infrequent dosing; most studies have dosing on days 1 and 90, with every 6 months thereafter [50]. The ORION 10 and 11 trials demonstrated 54.1 and 51.9 mg/dL loss of LDL cholesterol levels, respectively, when treated with inclisiran versus placebo. Inclisiran was also found to raise HDL cholesterol and lower total cholesterol, triglycerides, lipoprotein (a), and apolipoprotein B. As renal clearance is the main form of elimination, safety studies have been done on patients with either ASCVD or hypercholesterolemia and varying degrees of renal impairment [50]. Although renal clearance decreased as renal impairment increased, the LDL cholesterol level changes were similar across the groups, with the treatment well tolerated [47]. The total occurrence of cardiovascular events in both the placebo and experimental groups in efficacy trials was too small to draw conclusions about the efficacy in reducing adverse events [50]. Trials are currently underway to determine long term effects: the ORION-8 research will look at long term safety over 990 days and is expected to conclude in 2023, while the ORION-4 research will look at the number of occurrences of major adverse cardiovascular events in patients treated with inclisiran as compared to the placebo group over 5 years and is expected to conclude in 2024 [51][52]. Inclisiran is currently under consideration by the FDA and could become the first siRNA drug with a wide impact in the USA and around the world [53].

2.7. Atypical Hemolytic Uremic Syndrome

Atypical hemolytic uremic syndrome is a rare, life-threatening condition that causes renal impairment, thrombocytopenia, and microangiogenic hemolytic anemia [54]. Cemdisiran is a siRNA-targeting *C5* mRNA that is being developed to replace eclizumab as the standard of care. This siRNA inhibits terminal complement pathway activity and prevents the formation of membrane attack complex on kidney endothelial cells. Phase 1 trials of this drug were discontinued due to lack of funding as a result of the COVID-19 pandemic.

3. Lipid Nanoparticle Therapies

3.1. Transthyretin Amyloidosis

In 2018, patisiran (ONPATTRO®, Alnylam Pharmaceuticals) was approved by the FDA as the first-of-its-kind siRNA therapeutic for the treatment of polyneuropathy caused by hereditary ATTR amyloidosis [55][56][57]. Mutant transthyretin clusters into insoluble fibers, causing amyloidosis in the heart, kidneys, GI tract, and nerves. Transthyretin amyloidosis is a multisystemic disease causing neuropathy, such as sensorimotor difficulties and cardiomyopathy that can be fatal. Currently, the available treatments include liver transplantation and the administration of transthyretin tetramer stabilizers. Patisiran targets and degrades both mutant variant and wild-type transthyretin (*TTR*) mRNA, ameliorating the symptoms of this rare genetic disease [55]. The targeting siRNA is encapsulated in lipid nanoparticles targeted to the liver, which are administered intravenously at 0.3 mg/kg for 80 min every 3 weeks. Earlier trials had successfully demonstrated a dose-dependent reduction of circulating transthyretin and slowed disease progression with the administration of the drug [55]. For phase 3 trials (NCT03997383), 148 patients were given patisiran, while 77 were given a placebo. In the experimental group, the median reduction of transthyretin was 81% at the completion of the trial at 18 months [55]. There was a 56% improvement in the neuropathy impairment score mNIS+7 neurophysiologic test in the experimental group compared to the placebo group's 4% improvement. Every identified secondary endpoint showed significant improvements in the experimental group compared to the placebo group, with no safety concerns.

Another *TTR*-targeting siRNA, revusiran, reached phase 3 trials (NCT02319005), but dosing was discontinued earlier than planned due to the differences in death rates between the placebo and experimental groups. A post hoc safety investigation determined that patients that died while on treatment were older than 75 years old and had a more advanced heart failure at baseline [58]. On-target effects of lowering TTR mRNA were comparable in patients that lived or died after revusiran treatment. There was no evidence that revusiran treatment per se contributed to this increase in death rates [58].

3.2. Liver Fibrosis

Heat shock protein 47 is necessary for the deposition of hepatic collagen and forming collagen fibrils. It works with hepatic stellate cells (HSCs) to form procollagen, which causes fibrosis when it is cleaved into insoluble collagen [59]. BMS-986263 is a lipid nanoparticle injected intravenously, composed of cationic and anionic lipids targeted to HSCs via surface retinoid moieties that contains an anti-HSP47 siRNA. BMS-986263 is designed to treat hepatic fibrosis resulting from hepatitis C, cirrhosis from nonalcoholic steatohepatitis, or other forms of liver impairment [60][61]. The drug has been tested for immunogenicity and safety, with no negative effects found to date.

3.3. Hepatocarcinoma and Liver Metastases

MRX34 is a 23 nucleotide-long double-stranded miR-34 mimic in a 110 nm liposomal nanoparticle, developed to treat patients with advanced solid tumors (**Figure 1**A, [62][63]). miR-34 is a tumor suppressor that can downregulate over 30 oncogenes (e.g., MET, MYC, PDGFR-α). miR-34 transcription is regulated by p53 and serves as an important mediator of p53-dependent tumor suppression [62][63]. In 2016, a phase 1 clinical trial (NCT01829971) found the maximum tolerated dose to be 110 mg/m^2 for non-HCC tumors and 93 mg/m^2 for HCC after being administered to 47 stage IV cancer patients twice weekly for 3 weeks in 4-week cycles [62]. This clinical trial was

amended to include premedication with dexamethasone to reduce the toxicity and side effects triggered by MRX34. Despite this modification, dexamethasone was not sufficient to mitigate the inflammation and immune-mediated toxicity of MRX34. Unfortunately, due to five serious adverse events that resulted in the death of four participants, this clinical trial was terminated. Another phase I clinical trial (NCT02862145) was carefully designed for the treatment of melanoma patients with MRX34, but it was withdrawn due to the fallout from the other trial. Initial speculations suggested that the liposomal formulation may have contributed to these adverse events, but the use of the same formulation in other clinical trials without serious events makes this possibility less likely [63]. It appears that unanticipated on-target effects of MRX34 on immune cell activation could be driving the immune-mediated toxicity. Delivery of the drug to the tumors was confirmed, and target gene expression was found to have the expected dose-dependent response in white blood cells [63]. Of the 85 patients that received MRX34, 3 had a partial response and 16 showed stable disease. The recommended phase 2 dose was determined to be 70 mg/m^2 for HCC and 93 mg/m^2 for all other cancers. While a refined approach to target more selective cases with MRX34 may provide therapeutic benefits, the future clinical development of MRX34 is unclear [64].

3.4. Pancreatic Cancer

Ninety percent of pancreatic cancers are pancreatic ductal adenocarcinoma (PDAC), and 80%–85% of pancreatic cancer cases are stage III or IV when diagnosed. Therefore, the vast majority of patients are not candidates for curative surgery [65]. Chemotherapy, most commonly gemcitabine, with or without radiation, is the current gold standard of care [66][67]. Ribonucleotide reductase (RR) is a good target for tumor treatment as it controls a rate-limiting step essential for DNA replication and can therefore reduce PDAC growth potential [68]. While the RR M1 subunit expression is constant, the M2 subunit is mainly expressed during DNA replication. In vivo RRM2 siRNA therapies resulted in the presence of an mRNA fragment that indicates that mRNA cleavage occurred at the site consistent with the RNAi mechanism [69]. siRNA targeting RRM2 has also been shown to inhibit gemcitabine resistance in pancreatic adenocarcinomas [70]. The RRM2 siRNA is contained within a linear cyclodextrin-based polymer and polyethylene glycol nanoparticle with human transferrin (hTf) targeting ligands designed to circulate before accumulating in tumors [69]. It has been demonstrated that targeting through these nanoparticles inhibits tumor growth more significantly than the same nanoparticle without transferrin targeting [71]. When biopsies from treated melanoma patients were collected, the amount of intracellular nanoparticles correlated directly with the delivered dose. This was the clinical demonstration of this observation [69].

Ninety percent of PDACs have a *KRAS* mutation, most commonly the G12D mutation. Preclinical PDAC models have shown that KRAS inhibition leads to improved survival, slowed migration, and increased tumor necrosis in mouse models [72][23][67]. Natural or synthetic exosomes, similar to chemically-defined lipid nanoparticles, can be used as encapsulation and delivery systems for siRNAs and miRNA activity modulators in PDAC animal models [72][23][73][74][75][76][77][78][79][80][81][82]. Several of these exosome-loaded strategies directly target mutant KRAS mRNA [76][82]. Engineered exosome-loaded siRNAs against KRAS G12D offered a higher therapeutic benefit than the same siRNAs loaded in liposomes in several KRAS^{G12D}-driven orthotopic models [76]. The engineered exosomes can be produced at a large scale in bioreactors under the good manufacturing practice (GMP) standards that are required for clinical applications [82]. However, the first platform to reach the clinical stage relies on the local

delivery of the *KRAS*-targeting siRNA. The LODER™ (Local Drug EluteR) platform consists of a poly(lactic-co-glycolic acid) (PLGA) matrix that slowly releases siRNAs (**Figure 1**C). The siG12D-LODER™ is a very small biodegradable implant that releases siRNA targeting the G12D and G12V mutations on the KRAS gene and can be implanted directly into pancreatic tumors during endoscope ultrasound biopsy procedures [65]. The siRNAs are released at a rate of about 1 mm/day from the inner core of the tumor for 4 months, with full saturation of the tumor occurring around one week after commencing the treatment. The polymeric PLGA matrix is made entirely of materials generally recognized as safe and allows for prolonged release without the degradation of the drug over time [65][83]. Twelve patients with PDAC treated with siG12D-LODER™ combined with chemotherapy (gemcitabine and/or FOLFIRINOX) received CT scans after treatment that showed no tumor progression, and two of them showed partial response (https://www.silenseed.com/wp-content/uploads/2020/05/Silenseed-Presentation-21-Jan-2020-FC.pdf, accessed on 15 March 2022). The dose was set at a maximum of 3 mg, above which RNAi saturation occurred. Any adverse events that occurred were attributed to either the procedure or the chemotherapy rather than the siRNA nanodrug. Notably, two of the patients were still alive 27 and 30 months after treatment, respectively.

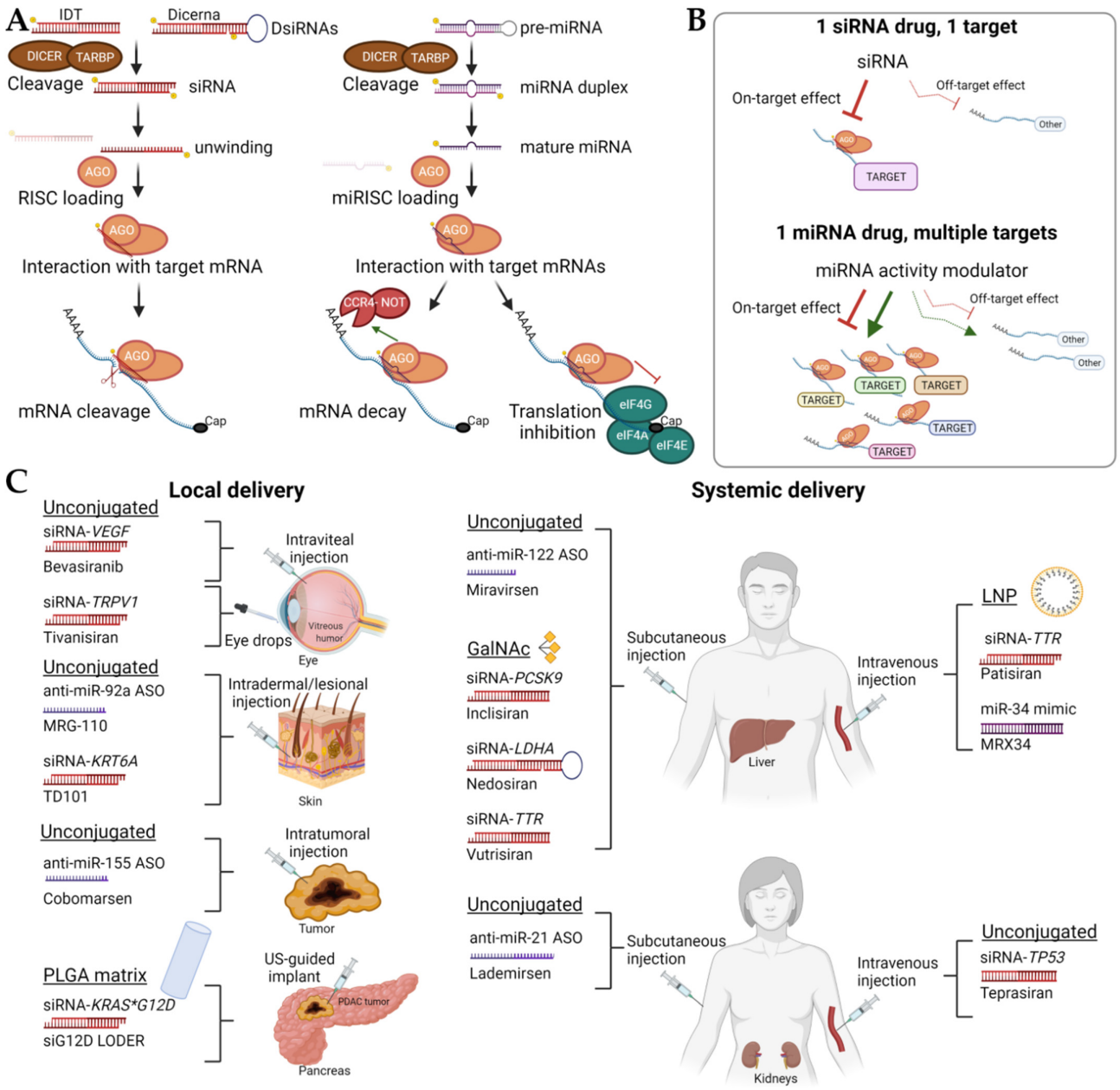

Figure 2. Processing, delivery strategies, and target engagement of RNA therapeutics. (**A**) miRNA precursor stem-loop hairpin and longer siRNA precursors (e.g., dicer substrate [DsiRNA] by IDT, or small hairpin RNAs such as the Dicerna nicked dsRNA stemloop) are processed by the DICER-containing complex. Transitory double-stranded product is similarly unwound and loaded into the ARGONAUTE-containing RNA-induced silencing complex (RISC). (**B**) While siRNAs are designed to specifically and perfectly match the complementary sequence of the cognate target mRNA, miRNAs bind to partially complementary sequences of multiple target mRNAs. (**C**) The researchers provide representative examples of local and systemic delivery strategies to enhance the accumulation of the RNA therapeutics in the intended site of treatment. For systemic delivery, chemical modifications (shown in **Figure 1**B), encapsulation, and/or targeting moieties can facilitate retention by a specific organ or cell type. Abbreviations: AGO = argonaute RISC component; CCR4-NOT = carbon catabolite repression-negative on TATA-less complex; DICER = ribonuclease III Dicer1; Dicerna = Dicerna Pharmaceuticals; eiF4 = eukaryotic translation initiation factor 4; GalNAc = *N*-acetylgalactosamine; IDT = Integrated DNA Technologies; LDHA = hepatic lactate dehydrogenase A;

LPN = lipid nanoparticle; *PCSK9* = proprotein convertase subtilisin/kexin type 9; PLGA = poly(lactic-co-glycolic acid); TARBP = TAR (HIV-1) RNA-binding protein 1; *TRPV1* = transient receptor potential cation channel subfamily V member 1; *TP53* = tumor protein p53; *TTR* = transthyretin; *VEGF* = vascular endothelial growth factor.

4. DOPC Nanoliposomes

Solid Tumors

EphA2 is a receptor tyrosine kinase associated with poor outcomes that are thought to play a significant role in the proliferation, survival, and migration of tumor cells [84]. It is mainly expressed in epithelial cells and overexpressed in many cancers. EPHARNA DOPC (18:1 PC *cis* 1,2-dioleoyl-sn-glycero-3-phosphatidylcholine) is a neutral nanoliposomal delivery for EphA2-targeting siRNA. It was found to be well tolerated overall, both in mice and non-human primates (macaques). Phase 1 safety trials of EPHARNA DOPC in solid tumors are underway and not yet completed [85].

5. Mini-Cells, Larger Encapsulation

Mesothelioma

Malignant pleural mesothelioma (MPM) is an incurable pleura cancer related to exposure to asbestos that is associated with a 2- to 5-fold downregulation of the miR-15/107 group [86][87]. Chemotherapy can reduce tumor burden and prolong life, but other therapies are needed. MesomiR1 is a drug for MPM using TargomiR technology, which delivers RNA mimics, miR-15/107 in this case, to targeted bacteria cells as an alternative to liposomal or nanoparticle delivery. These EnGeneIC Delivery Vehicle (EDV)™ minicells are nonviable bacterial cells of about 400 nm in diameter, modified with antibodies on the surface for tumor cell targeting. In this first-in-human trial, MesomiR-1 was administered intravenously to patients with MPM at doses of 5×10^9 TargomiRs over 20 min once weekly. The majority of patients had a short-term inflammatory response, lymphopenia, and neutrophilia after administration, and about half had short-term pain at the site of the cancer. After 8 weeks of the treatment described above, four patients had stable disease, one patient had improved, and one patient had progressive disease. The patient who improved significantly had, prior to treatment with MesomiR-1, undergone six rounds of chemotherapy with no response beyond the stabilization of disease. After treatment with the miRNA mimic, he showed evidence of partial response on computed tomography (CT) imaging and experienced a marked improvement in chest pain and respiratory function. This response occurred after 8 weeks of treatment, with the lowest dose administered in this trial.

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