

Progress on RNAi-based molecular medicines

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Abstract: RNA interference (RNAi) is a promising strategy to suppress the expression of disease-relevant genes and induce post-transcriptional gene silencing. Their simplicity and stability endow RNAi with great advantages in molecular medicine. Several RNAi-based drugs are in various stages of clinical investigation. This review summarizes the ongoing research endeavors on RNAi in molecular medicine, delivery systems for RNAi-based drugs, and a compendium of RNAi drugs in different stages of clinical development. Of special interest are RNAi-based drug target discovery and validation, delivery systems for RNAi-based drugs, such as nanoparticles, rabies virus protein-based vehicles, and bacteriophages for RNA packaging.

Keywords: RNA interference, delivery systems, liposome, nanoparticle, molecular medicines

Overview of RNA interference

RNA plays key roles in organisms beyond the traditional role of being a messenger bridging genetic information and biosynthesis of protein. Endogenous or exogenous double-stranded RNA can be cleaved into 21–23-nucleotide small interfering RNA (siRNA) by the endonuclease, Dicer. siRNA can unzip into a guide strand and a passenger strand. The latter will be subsequently degraded. The guide strand is then incorporated into the RNA-induced silencing complex and binds to the target mRNA, inducing mRNA degradation by Argonaute, a component of the RNA-induced silencing complex which affects protein synthesis (Figure 1). This phenomenon is known as RNA interference (RNAi)^{1–4} and is well established in diverse organisms.

There are three major types of small RNA used to silence gene function by RNAi technology, ie, microRNA (miRNA), siRNA, and short hairpin RNAs (shRNA).^{5,6} Although all small RNAs are 22–24 nucleotides in length,^{7–9} there are some important differences between them. First, siRNA, processed from exogenously introduced strictly base-paired siRNA duplexes, are completely complementary to their target mRNA and facilitate cleavage of bases 10–12 at the 5' end of the guide strand,^{10,11} resulting in transient silence of the target gene. Second, shRNA, inserting in a Pol III expression cassette, can be transfected or packaged into a recombinant virus and introduced into target cells.⁵ The advantage of shRNA is prolonged expression of the RNAi effect. Third, miRNA or chimeric miRNA/shRNA, derived from endogenously encoded shRNAs,⁵ can mediate gene silencing post-transcriptionally, and bind partially to complementary miRNA target sequences located in the 3' untranslated regions of target mRNAs,⁹ but the “seed” region located in bases 2–8 is highly complementary.¹² The seed region can

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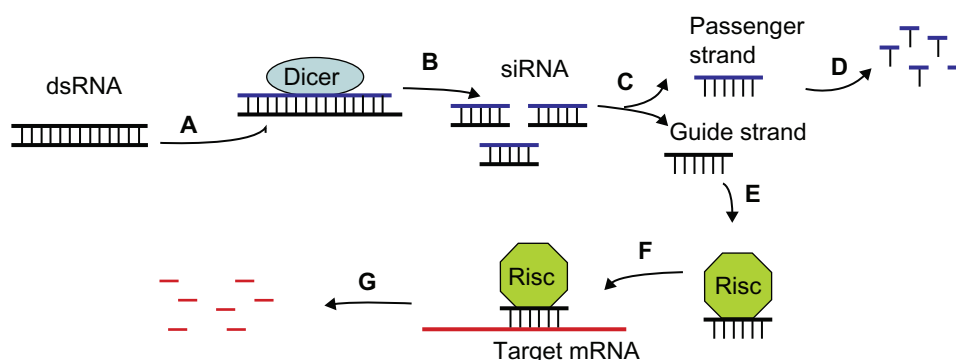


Figure 1 Schematic representation of RNAi. **(A and B)** Double-stranded RNA is cleaved into siRNA by Dicer. **(C and D)** siRNA is unzipped into a guide strand and passenger strand. The latter is subsequently degraded. **(E–G)** The guide strand is incorporated into a RISC and binds to target mRNA, inducing degradation of mRNA. **Abbreviation:** RISC, RNA-induced silencing complex.

result in deadenylation, translational repression, and decay of mRNAs.^{9,13,14} Interestingly, either strand of miRNA can silence gene expression post-transcriptionally⁶ and some translational repression mediated by miRNA is reversible.¹⁵ miRNA are the latest major breakthrough in the field of RNAi and represents an important tool for RNAi to overcome some of the limitations of shRNA.⁵

RNAi as a tool for discovery of drug targets

Most drugs function via blocking of their targets. The simplicity and stability of RNAi in gene silencing make it a powerful tool in drug research and development.¹⁶ RNAi-based drug target discovery includes the following steps.

Identification of target genes

The selection of target gene is crucial and, to great extent, determines whether subsequent RNAi silencing can be effective or not. The abundance of the target gene, the regulation of its expression, and the half-life of its products are several important parameters that should be factored into.¹⁷ For example, mutation of somatic cells and their unchecked proliferation are the main factors underlying tumors. Based on this phenomenon, the RNAi library and high throughput screening can be harnessed to find the genes or proteins involved in tumor development to pinpoint drug target candidates.¹⁸

Designing siRNA

Once the target is defined, siRNA can be used to evaluate potential candidates. siRNA-mediated target RNA cleavage is highly sequence-specific. Any mismatches in the siRNA duplex can abolish potential target RNA cleavage.¹⁷ Therefore, the success of RNAi-based therapeutics is strictly dependent

on siRNA which can silence target genes specifically and effectively. There are various online algorithms available for appropriate design of siRNAs.⁵ Some empirical characteristics of efficacious siRNA are summarized as follows:

- G/C content should be relatively low,^{17,19} ranging between 30% and 52%,¹⁹ because a high G content tends to form G quartet structures¹⁷
- Low internal stability of siRNA at the 5' antisense end is a prerequisite for effective silencing;¹⁹ internal repeats or palindromes in siRNA sequences may form internal fold structures;¹⁹ the silencing potential and effective concentration of siRNA can be reduced by these hairpin-like structures¹⁹
- Silencing efficiency is inversely proportional to high internal repeat stability¹⁹
- The sense strand has an A at positions 3 and 19^{5,19,20}
- The sense strand has a U at position 10^{5,19,20}
- The sense strand lacks a G or C at position 19¹⁹
- The sense strand lacks a G at position 13.^{5,19}

The efficiency of siRNA can be improved if the above-mentioned criteria are met.

Constructing an RNAi library

A library of artificial RNAi can induce RNAi to suppress expression of diverse genes. An RNAi library can be specific or random. A specific RNAi library is designed according to known genes, whereas a random RNAi library consists of random DNA fragments obtained by cleaving genomic DNA in specific reaction conditions.²¹ RNAi libraries have very much facilitated RNAi drug research.

SiRNA-transfected target cells

RNAi-based screening has singled out numerous candidate cancer targets, among which many have been or will be

validated by disease-relevant in vitro phenotypic assays. However, few have been validated in vivo, largely due to the lack of effective vehicles and high cost.¹⁶ siRNA has been shown to be effective in several localized disease models. However, weak stability and low transmission efficiency during in vivo delivery have severely limited its application.¹⁶

The “off-target” effect of RNAi is a severe drawback. To realize the benefit of RNAi fully, this effect must be eliminated or minimized.¹⁶ An ideal drug target would have high on-target efficacy and low toxicity.⁵ Generally, toxicity is caused by saturation of the endogenous RNAi machinery following transfection of siRNA or expression of shRNA. Reducing the intracellular concentration and/or cell-specific expression of shRNA can minimize side effects.^{5,22} This can be achieved by using a regulated RNAi expression cassette delivered either by a nonviral or a viral vector.⁵ The delivery vehicle is crucial to achieve the above goal and should be capable of circumventing the reticuloendothelial system, be effectively taken up by the target tissues, and be able to escape from the endosome after endocytosis.²³

Expression cassette for RNAi

Under the control of RNA polymerase III promoters, the expression of shRNA can introduce siRNA into cells.^{22,24,25} Heritable gene knockdown can be achieved by incorporating the shRNA cassette into viral vectors in cells and even in whole animals.^{22,26–28} Inducible systems which silence RNA expression by RNA polymerase III promoters containing operator sequences (tetO) of the *Escherichia coli* tetracycline resistance (*tet*) operon²² have been developed.²⁹ This tetracycline-inducible gene regulation system has become a powerful device for transient and dose-dependent regulation of target gene expression in vitro and in vivo.³⁰ A lentiviral based-system in which a tetO sequence is located between the H1 promoter and shRNA was developed. This system can cooperate with another cassette consisting of the codon-optimized tetR^{31,32} fused with enhanced green fluorescent protein by the T2A peptide under the control of the ubiquitin promoter,³³ which can be used to treat type 2 diabetes mellitus.²² In the absence of the doxorubicin inducer, tetR binding to tetO can block shRNA transcription. Addition of doxorubicin or tetracycline can release tetR, thereby facilitating initiation of shRNA expression.²²

Delivery systems

Nanoparticles have recently been recognized as a principal delivery vehicle for gene therapy.³⁴ Nanotechnology holds great promise for medicines for a number of

reasons, ie, multiple selectivity, desirable solubility, and permeability, a favorable pharmacokinetic profile, tunable tissue specificity, higher stability under physiological conditions, and ready scale-up during manufacture.^{35,36} Polymers are the basis of nanomedicine and their characteristics are constantly being improved to achieve better efficacy. Polymers can protect siRNA from serum nuclease degradation and escort the siRNA to the desired cells.³⁷ One drawback of synthetic nanoparticles is their relatively lower transfection efficiency.³⁸ This can be improved by modification. The primary, secondary, and tertiary amines within the cationic polymer polyethylenimine (PEI) enable it to complex with DNA or siRNA, creating a “proton sponge effect”,^{37,39} which can result in accumulation of PEI-siRNA complexes in the liver, lung, spleen, and kidney, and also in severe cytotoxicity, which limits its wider application.³⁷ Poly(ethylene glycol) (PEG)-modified PEI polymers can reduce this adverse effect, and achieve higher transfection efficiency, more solubility and stability, and a longer circulation time in vivo, as well as fewer non-specific interactions with serum protein.⁴⁰ An example of this is a PEG-PEI/siRNA nanoparticle targeting CD44v6, a risk factor for lymph node metastasis and highly expressed in patients with gastric cancer and liver metastasis.^{41,42} The morphology of the PEG-PEI/siRNA nanoparticle has been monitored by scanning electron microscopy, and the size and surface charge of the nanoparticle assessed by zeta potential measurement. The transfection efficiency, cytotoxicity, and interactions with the target were measured using SGC7901 human gastric carcinoma cells as a model. The results showed that the N/P charge ratio between the PEG-PEI amino groups and the siRNA phosphate groups largely determines the transfection efficiency. The peak transfection efficiency of the PEG-PEI/siRNA nanoparticle was $72.53\% \pm 2.38\%$ at an N/P of 15, and the cytotoxicity of PEG-PEI was lower than that of PEI using the MTT assay. Optimal silencing of CD44v6 expression was achieved at a PEG-PEI/siRNA N/P ratio of 15.⁴⁰ More recently, nanoparticle-based delivery systems have largely been viral or nonviral.⁵

Viral delivery systems

Viruses can integrate shRNA expression cassettes into the genome, thereby inducing prolonged gene silencing. Viruses can be customized to specific tissue tropism.³⁷ Delivery systems based on retrovirus, lentivirus, adenovirus, and adenovirus-related vectors have been validated in transgenic systems.³⁸ They have the advantages of delivering siRNAs to nondividing cells and of improving biosafety by diminishing

the risk of producing replication-competent viruses and evading dysregulation of endogenous genes by promoters and viral enhancers.^{43–45}

Lentiviral vectors, in contrast with adenovirus, can introduce short RNA into bone marrow cells and blood,⁴⁶ and are widely used in the biomedical field. Amyotrophic lateral sclerosis is a fatal and incurable degenerative disorder of motor neurons in the brainstem, spinal cord, and motor cortex, leading to generalized weakness and muscle atrophy.⁴⁷ Amyotrophic lateral sclerosis has a high incidence of approximately 6/100,000, and about 90% of cases are sporadic, with the remaining 10% being familial.⁴⁷ About 20% of familial cases arise from mutations in the Cu/Zn superoxide dismutase (*SOD1*) gene. *SOD1*^{G93A} and *SOD1*^{mis} mutants were designed as a control for *SOD1* silencing, and a specific shRNA (shSOD1) was applicable to most mutant *SOD1* proteins. In transgenic *SOD1*^{G93A} mice, intraspinal injection of a lentiviral vector (LV-shSOD1) led to a long-lasting and substantial decrease in mutant *SOD1* protein, thereby delaying the onset and progression of amyotrophic lateral sclerosis.⁴⁷

The transmissible spongiform encephalopathies or prion diseases are fatal neurodegenerative disorders prevalent among sheep, cattle, cervids, and humans. Transmissible spongiform encephalopathies arise from a cellular prion protein which misfolds and forms a protease-resistant isoform. The cellular prion protein is widespread among mammalian cells, and is highly expressed on glial and neuronal cells in the central nervous system.^{48,49} Prions migrate to the central nervous system, where a conformational change in the cellular prion protein causes a protease-resistant isoform.⁵⁰ Decreasing the cellular prion protein in mice infected with transmissible spongiform encephalopathy can reduce the incidence and severity of the disease, delay its development,^{51,52} and even reverse its pathological progression.⁵³ RNAi can effectively knock down the cellular prion protein in mice, but the effect on the outcome of prion disease remains to be determined.⁵⁴ Lentiviral vectors encoding prion protein-specific shRNAs can be injected intracranially to knock down cellular prion protein expression,⁵² but the clinical application of this delivery method is limited due to irreversibility of cellular prion protein suppression.⁵⁵

The chemokine receptor CCR5, a human immunodeficiency virus-type 1 (HIV-1) coreceptor, is essential for CCR5 tropic HIV-1 infection and serves as a desirable therapeutic target for inhibiting HIV-1.^{56–59} Hindering CCR5 expression protects against HIV-1 infection at the primary stage of the life cycle of the virus. $\Delta 32$ CCR5, mutants with a 32-bp

deletion in the *CCR5* gene, do not express CCR5 and are highly effective in preventing HIV-1 infection.^{60,61} Therefore, it is a promising strategy for reducing CCR5 expression in a stable manner when treating HIV-infected patients.⁶² A hu-BLT (bone marrow/liver/thymus humanized) mouse model showed that engraftment of lentiviral vector-mediated CCR5 shRNA led to stable and efficient *CCR5* knockdown in multiple lymphoid organs, and CCR5 expression was downregulated in systemic lymphoid organs without causing obvious adverse effects.⁶² In addition, the anti-HIV drug, BLT-HIV (rHIV7-shl-TAR-CCR5RZ) produced by Benitec Ltd, using lentivirus as a delivery tool has now entered into Phase Ib investigation.

The major drawbacks of viruses are their ready elimination by preexisting bloodstream antibodies, and their role in raising cytotoxicity. Furthermore, viruses can activate coagulation or complement factors, and can induce neutralizing antibody responses that prevent repeated administration.

Nonviral delivery systems

The advantages of nonviral delivery systems, compared with viral vectors, are their ease of synthesis, low toxicity, and limited immune response.⁶³ Nonviral vectors mainly contain liposomes and bacteriophages.³⁷

Liposome delivery systems

The delivery systems based on liposomes can protect the nuclease, penetrate the cell membrane, and deliver RNA to target cells.³⁸ This method can decrease immunogenicity and is much safer.⁶⁴ Well designed lipid delivery systems can bypass the endosome and release siRNA. The endosomal pathway is the main obstacle to drug delivery into the cytoplasm. siRNA can be released by neutralization.³⁸

More recently, a transvascular method which delivered siRNA across the blood-brain barrier by intracranial injection was reported.⁶⁵ The siRNA was fused to a short peptide of the rabies virus glycoprotein which can bind to acetylcholine receptors on neuronal cells,⁶⁶ and nine d-arginines were added to the C-terminal of the short peptide (RVG-9r), enabling it to interact electrostatically with siRNA. In this way, siRNA has been successfully delivered to neurons within the mouse brain and been shown to inhibit protein expression and protect against viral encephalitis. RVG-9r peptide within cationic liposomes can knock down cellular prion protein expression and dramatically decrease expression of the protease-resistant isoform in neurons infected with transmissible spongiform encephalopathy in vitro.⁵⁵ This combination integrates the advantages of resistance

of cationic liposomes to serum degradation and the target specificity of the RVG-9r peptide.

Stable nucleic acid-lipid particles (SNALPs) developed by Tekmira Pharmaceuticals, represent an efficacious siRNA delivery system. SNALPs are composed of a lipid bilayer containing a mixture of fusogenic and cationic lipids that enable cellular uptake and endosomal release of a nucleic acid payload. SNALPs can also be coated with a diffusible PEG-lipid conjugate providing a neutral or hydrophilic surface, and stabilizes the particle during formulation. The exterior coating also shields the cationic bilayer in vivo, blocking rapid systemic clearance.⁶⁷

In the study of hepatitis B virus (HBV), HBV263 is a siRNA molecule of HBV. HBV263M that placed one ribonucleotide on the 5' end of the antisense strand of HBV263 was incorporated into lipid nanoparticles to form SNALPs.⁶⁷ HBV263M-SNALP was intravenously injected into mice carrying replicating HBV to evaluate its biodistribution, half-life, immunostimulatory properties, and efficacy. The results showed that HBV263M-SNALP had improved efficacy and a longer half-life, and reduced serum HBV DNA to $>1.0 \log_{10}$ after three days of intravenous injections at a dose of 3 mg/kg/day. Furthermore, HBV263M-SNALP reduced toxicity, dosing frequency, and immunostimulatory side effects, and had more robust and persistent biological activity.⁶⁷ SNALP delivery systems for SNALP-apolipoprotein B and ALN-TTR01 have been used in several clinical trials due to their advantages.

Gold nanoparticle delivery systems

Excellent biocompatibility and easy surface chemistry make gold nanoparticles one of the most widely investigated nanomaterials, and they have received increasing attention in a wide range of biomedical fields,⁶⁸ including radiotherapy, photothermal cancer therapy, biomolecular sensing, drug delivery, imaging, and regulation of DNA.^{69–71} Gold nanoparticles have emerged, especially in drug delivery, as promising candidates because of their excellent biocompatibility, high surface-to-volume ratio, versatility in synthesis, optical properties, and easy surface functionalization.^{70,71}

Until recently, diverse types of gold nanoparticles, including antisense oligonucleotide, plasmid DNA, and siRNA, have been explored for their ability to deliver nucleic acid drugs into cells.^{71–78} Cationic lipid-coated gold nanoparticles have been developed for efficient intracellular delivery of therapeutic siRNA. In this system, gold nanoparticles served as a scaffolding material, inducing self-assembly of lipid elements, with an exterior cationic lipid shell surrounding a core of clustered gold nanoparticles. Lipid-coated gold

nanoparticles possessing a positively charged shell layer are believed to condense siRNA molecules effectively into stable nanosized polyelectrolyte complexes, enabling a gene silencing effect and efficient cellular internalization.⁷⁹

To validate further the efficacy of lipid-coated gold nanoparticles in the treatment of hepatitis B, siHBV targeting viral open reading frames encoding an X protein was complexed with the lipid-coated gold nanoparticles to induce an antiviral response. The gene silencing effect of lipid-coated gold nanoparticles-siHBV polyelectrolyte complexes was evaluated in HBV-expressing HepG2.2.15 cells.⁷⁹ After treatment with 200 nM of lipid-coated gold nanoparticles-siHBV complexes, release of HBV surface antigen decreased markedly to $37.0\% \pm 6.6\%$, showing that these complexes were able to inhibit HBV replication efficiently.⁷⁹ Furthermore, gold nanoparticles have been used in clinical diagnostics and therapeutics, such as cancer, tuberculosis, Alzheimer's disease, HIV, and sciatic nerve repair.^{80–84}

Bacteriophage delivery systems

Bacteriophages are promising delivery systems, as exemplified by the *Bacillus subtilis* phage phi29 encoding a 117-nucleotide packaging RNA molecule.⁸⁵ Packaging RNA can constitute a nanoparticle approximately 11 nm in size by folding into a unique and stable secondary or tertiary structure.⁸⁶ Further, it can encapsulate DNA into procapsids, thereby forming 10–30 nm polymers by base-pairing between two interlocking right-hand and left-hand loops.⁸⁷ A packaging RNA monomer has two functional domains, ie, a double-stranded helical DNA packaging domain and an intermolecular interacting domain.^{87–91} Although the intermolecular regions can hardly tolerate sequence variation, the double helix allows changes in lengths and sequences without compromising the structure of packaging RNA, as long as the double helix is maintained.^{90,92,93} siRNA is a double-stranded RNA helix which can replace the 3'/5' helical region of the packaging RNA without affecting folding of siRNA and packaging RNA, or the functions of the inserted moiety.⁸⁷ This method can deliver siRNA to cancer cells without degradation, with a longer half-life and significant efficacy.⁸⁷

Several features^{87,94,95} of RNA nanoparticles make nanodelivery platforms attractive:

- Two-dimensional, three-dimensional, and four-dimensional structures, which are diverse and stable
- Various biochemical/biological functions
- Metabolic stability accomplished by modification
- Ideal biodegradability, biocompatibility, and noninduction of antibodies, modularized design, and ready scale-up.^{34,96}

These delivery systems can be tailored to the characteristics of focal tissues, making artificial polymer-based and virus-based vehicles dispensable. For example, based on the fact that transferrin protein receptor expression was significantly upregulated in human tumors, a 70 nm nanoparticle containing transferrin protein was generated and could deliver siRNA to tumor cells by systemic injection. Target gene expression can be specifically silenced by RNAi.⁹⁷ Other novel immunoliposome methods can integrate nanoparticles and lymphocyte function-associated antigen-1 integrin, a molecule which is highly expressed on all leukocytes. A lymphocyte function-associated antigen-1 integrin-targeted and stabilized nanoparticle could lead to selective uptake of siRNA by T cells and macrophages, which are the principal target cells of HIV. Anti-CCR5 siRNA/lymphocyte function-associated antigen-1 integrin-targeted and stabilized nanoparticles could silence leukocyte-specific genes, thereby preventing HIV infection in BLT mice.⁹⁸

RNAi drugs and future opportunities

RNAi have been tested in various fields of medicine because of their specificity, simplicity, and stability in gene silencing.⁹⁹ RNAi drugs in different stages of clinical investigation are summarized in Table 1. Many obstacles remain to be overcome to bring RNAi-based therapeutics successfully to the bedside, eg, precise targeting to avoid potentially fatal off-target effects³⁸ and overcoming the potential cytotoxicity and immunoreactivity of delivery systems. Hopefully, more indepth exploration of the above questions will expedite realization of the full potential of RNAi-based therapeutics.

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Table 1 RNA interference drugs in clinical trials

Drug name	Diseases	Target	Phase	Delivery method	Company/Affiliation
Bevasiraib ¹⁰⁰	AMD and DME	VEGF	Phase III was stoped	Direct eye injection	Opko Health Inc
PF-4523655	AMD and DME	VEGF and RTP801	II	Direct eye injection	Quark
(REDD-14-NP or RTP-80li-14) ¹⁰¹					
TKM-PLK I ¹⁰²	Solid tumors	PLK I	I	LNP delivery technology	Tekmira
TKM-Ebola ¹⁰³	Ebola infection	Ebola virus	I	LNP delivery technology	Tekmira
ALN-RSV01 ¹⁰⁴	RSV infection	RSV nucleocapsids	II	Direct to lungs via inhaler	Alnylam
QPI-1007 ¹⁰⁵	Eye neuropathy	Capase 2	I	intravitreal administration	Quark
ALN-VSP02 ¹⁰⁶	Solid tumors	KSP and VEGF	I	Systemic (liver) liposomal conjugation	Alnylam
CALAA-01 ¹⁰⁷	Solid tumors	RRM2	I	Intravenous, nanoparticle delivery system	Calando Pharmaceuticals
TD101 ¹⁰⁸	PC	Keratin K6a	I	Intradermal injection (skin)	PC Project and TransDerm
Sirna-027 (AGN211745) ¹⁰⁹	AMD	VEGF-R1	II	Direct eye injection	Allergan/Merck
AKli-5 (15 NP or QPI-1002) ¹¹⁰	Acute kidney injury delayed graft function	P53	II	Intravenous injection (systemic)	Quark
SPC4955 ¹¹¹	Hypereholesterol	Apo B	I	Subcutaneous injections	Santaris Pharma A/S
ALN-PCS02 ¹¹²	Low density lipoprotein cholesterol (LDL-C)	PCSK9	I	Intravenous (IV) infusion	Alnylam
SPC5001 ¹¹³	LDL-C	PCSK9	I	Systemic (blood) liposomal conjugation	Santaris Pharma A/S
PRO-040201 (ApoB SNALP) ¹¹⁴	Hypercholesterol emia	Apo B	I	Lipid nanoparticles	Tekmira Pharmaceuticals
NUC B100 ¹¹⁵	HBV infection	HBV	I	Intravenous injection (systemic)	Nastech Pharmaceutical
BLT-HIV (rHIV7-shl-TAR-C CR5RZ) ¹¹⁶	HIV infection	HIV	I	Lentiviral (ex vivo)	Benitec Ltd
ALN-TTR01 ¹¹⁷	TTR-mediated amyloidosis (ATTR)	TTR	I	Lipid nanoparticles	Alnylam

Abbreviations: ApoB, apolipoprotein B; AMD, age-related macular degeneration; HBV, hepatitis B virus; DME, diabetic macular edema; HIV, human immunodeficiency virus; KSP, kinesin spindle protein; LNP, lipid nanoparticle; PC, pachyonychia congenita; RSV, respiratory syncytial virus; RMM2, M2 subunit of ribonucleotide reductase; VEGF, vascular endothelial growth factor; TTR, transthyretin; PLK I, polo-like kinase I; PCSK9, proprotein convertase subtilisin/kexin type 9.

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Disclosure

The authors report no conflicts of interest in this work.

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