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The current state and future directions of RNAi-based therapeutics

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Abstract | The RNA interference (RNAi) pathway regulates mRNA stability and translation in nearly all human cells. Small double-stranded RNA molecules can efficiently trigger RNAi silencing of specific genes, but their therapeutic use has faced numerous challenges involving safety and potency. However, August 2018 marked a new era for the field, with the US Food and Drug Administration approving patisiran, the first RNAi-based drug. In this Review, we discuss key advances in the design and development of RNAi drugs leading up to this landmark achievement, the state of the current clinical pipeline and prospects for future advances, including novel RNAi pathway agents utilizing mechanisms beyond post-translational RNAi silencing.

Small interfering RNAs
(siRNAs). Short (19–21 bp) RNA duplexes with two-base 3′ overhangs that trigger RNA interference without Dicer cleavage.

Hereditary transthyretin amyloidosis
(hATTR). A rare inherited condition caused by deposition of amyloid fibrils formed by misfolded transthyretin protein monomers.

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In 1998, Andrew Fire and Craig Mello published a seminal paper identifying double-stranded RNAs (dsRNAs) as the causative agents for post-transcriptional gene silencing (PTGS) in *Caenorhabditis elegans*[1], a phenomenon they termed RNA interference (RNAi). The discovery of RNAi (FIG. 1) explained puzzling observations of gene silencing in plants and fungi and kicked off a revolution in biology that eventually showed non-coding RNAs to be central regulators of gene expression in multicellular organisms. Three years later, Elbashir and colleagues[2] and Caplen and colleagues[3] reported that 21 and 22 nucleotide (nt) dsRNAs could induce RNAi silencing in mammalian cells without eliciting nonspecific interferon responses. These small interfering RNAs (siRNAs) soon became ubiquitous tools in biological research as they allowed facile inhibition of any gene by a base sequence alone.

For drug developers, the potency and versatility of siRNAs, the prospect of suppressing genes encoding proteins that are 'undruggable'[4–6] by classical small molecules[7] and the potential for 'programmable' drugs that can be re-targeted without changing in vivo pharmacokinetics proved too enticing to ignore. By 2003, multiple companies had formed to harness the therapeutic potential of RNAi.

Unfortunately, the first clinical trials using unmodified siRNAs resulted in immune-related toxicities and questionable RNAi effects[8,9]. A second wave of clinical trials using systemically administered siRNA nanoparticle formulations achieved important advances, such as the first confirmed RNAi effects in humans from systemically administered nanoparticles[10], but also showed significant, dose-limiting toxicities and insufficient therapeutic efficacy. As a result, major pharmaceutical companies exited from the RNAi space in the early 2010s[11], causing a funding crisis for the industry.

Despite these challenges, smaller RNAi companies and academic researchers absorbed the hard lessons[12] from prior clinical trial failures and persisted[13] in making steady improvements in trigger design, sequence selection, chemical formulation and delivery mechanisms. These substantive advances, combined with more judicious selection of disease indications, better validated intervention pathways, more mature clinical development processes (see Related links) and improved manufacturing capabilities[14], have created a new pipeline of safer and more efficacious therapeutic compounds.

On 10 August 2018, the US Food and Drug Administration (FDA) inducted RNAi drugs into medicine by approving patisiran (Onpattro; Alnylam Pharmaceuticals), an siRNA acting on the liver, for the treatment of hereditary transthyretin amyloidosis (hATTR) with polyneuropathy. Patisiran's approval brings new hope to patients with hATTR with dire unmet medical needs and heralds a new era in the RNAi therapy field. Today, multiple drug candidates for liver, renal and ocular indications are currently in phase I, II and III clinical trials, and Investigational New Drug (IND) applications targeting the central nervous system (CNS) and additional non-liver tissues are expected within the next 2 years. Over the next 5 years, newly discovered RNAi pathway functions, advanced RNAi payloads with enhanced specificity and potency and improved methods for systemic and local RNAi delivery may enable new breakthrough treatments.

In this Review, we introduce the mechanisms of RNAi and the early history of RNAi discovery; survey the current motifs, design rules and chemistries used in synthetic RNAi triggers; and discuss various routes of delivery. The current state of the clinical pipeline for RNAi drugs is assessed, and patisiran and follow-on drug candidates are examined. Finally, we discuss RNAi therapeutics beyond cytoplasmic mRNA silencing

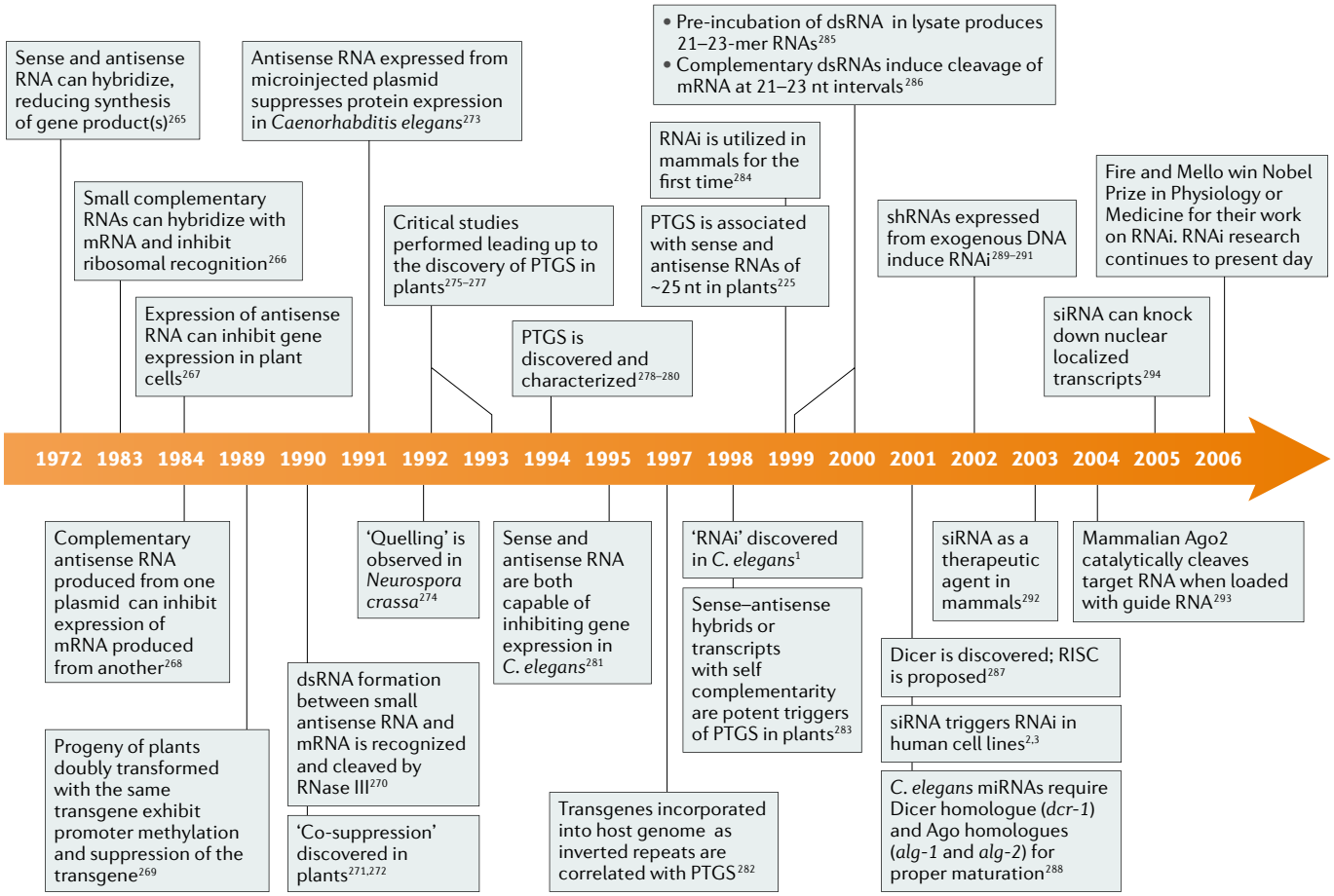

Fig. 1 | **Early events in the discovery and elucidation of the RNAi pathway.** A timeline of important developments leading up to the discovery of RNA interference (RNAi) in animals is shown[265–294]. Ago, Argonaute; dsRNA, double-stranded RNA; miRNA, microRNA; nt, nucleotide; PTGS, post-transcriptional gene silencing; RISC, RNA-induced silencing complex; shRNA, short hairpin RNA; siRNA, small interfering RNA.

Endosomal escape
The escape of RNA interference agents from endosomes into the cytosol.

RNA-induced silencing complex
(RISC). Protein RNA complexes that serve as the effectors of RNA interference. RISCs are composed of an Argonaute (Ago) protein with an inserted RNA guide strand and other proteins complexed with Ago.

Guide strand
An RNA strand that is inserted into an Argonaute protein to form a mature RNA-induced silencing complex.

Antisense strand
The strand in an RNA interference trigger that is complementary to the intended target.

Sense strand
The strand in an RNA interference trigger that is homologous to the intended target.

and the future opportunities and challenges for the expanded RNAi field.

Design and development of RNAi drugs

To utilize the mammalian RNAi pathway[15] (FIG. 2) for potent and specific inhibition of putative therapeutic targets, RNAi drug formulations must overcome pharmacodynamics-related challenges in targeting specificity, off-target RNAi activity, immune-sensor-mediated cytotoxicity (BOX 1) and pharmacokinetics-related challenges in systemic circulation, cellular uptake and endosomal escape. These challenges are addressed via structural motifs, sequence selection, chemical formulation of RNAi triggers and selection and engineering of delivery routes and excipients.

Structural motifs

Although RNAi pathway enzymes have restrictive structural requirements on the compatibility of dsRNA molecules, there is still a panoply of synthetic RNAi triggers with differing structural motifs (FIG. 3) and functional properties. Synthetic RNAi triggers are generally perfectly base-paired dsRNAs or short hairpin RNAs (shRNAs) ranging from 15 to 30 bp in overall length. dsRNAs smaller than 15 bp lose the ability to engage RNAi machinery, whereas those larger than 30 bp can induce nonspecific cytotoxicity via activation of the PKR pathway[16].

RNAi triggers with larger (>21 bp) RNA duplexes interact with the RNAi pathway enzyme Dicer for cleavage and handoff to the RNA-induced silencing complex (RISC) loading complex (RLC)[17]. Shorter (<21 bp) siRNAs and analogues can bypass Dicer cleavage and enter the RISC via interactions mediated by the TAR RNA-binding protein (TRBP)[18]. Although this second pathway may still involve Dicer, it can also function in Dicer's absence[19].

Important functional differences exist between different motifs in the efficiency of RNAi processing, the bias in guide strand selection between the antisense strand (correct) and the sense strand (incorrect) and the compatibility of the motifs with different patterns and types of chemical modifications. For example, 'asymmetric' siRNAs (a dsRNA with a blunt end on one side and a 2 nt 3′ overhang on the other) tend to bias guide strand selection to the strand with the 3′ overhang[20], and blunt-ended chemically modified RNAi triggers have been reported to have improved nuclease resistance compared with triggers with the canonical 3′ overhangs[21].

One of the most important functional differences may be between Dicer substrate siRNAs (DsiRNAs)

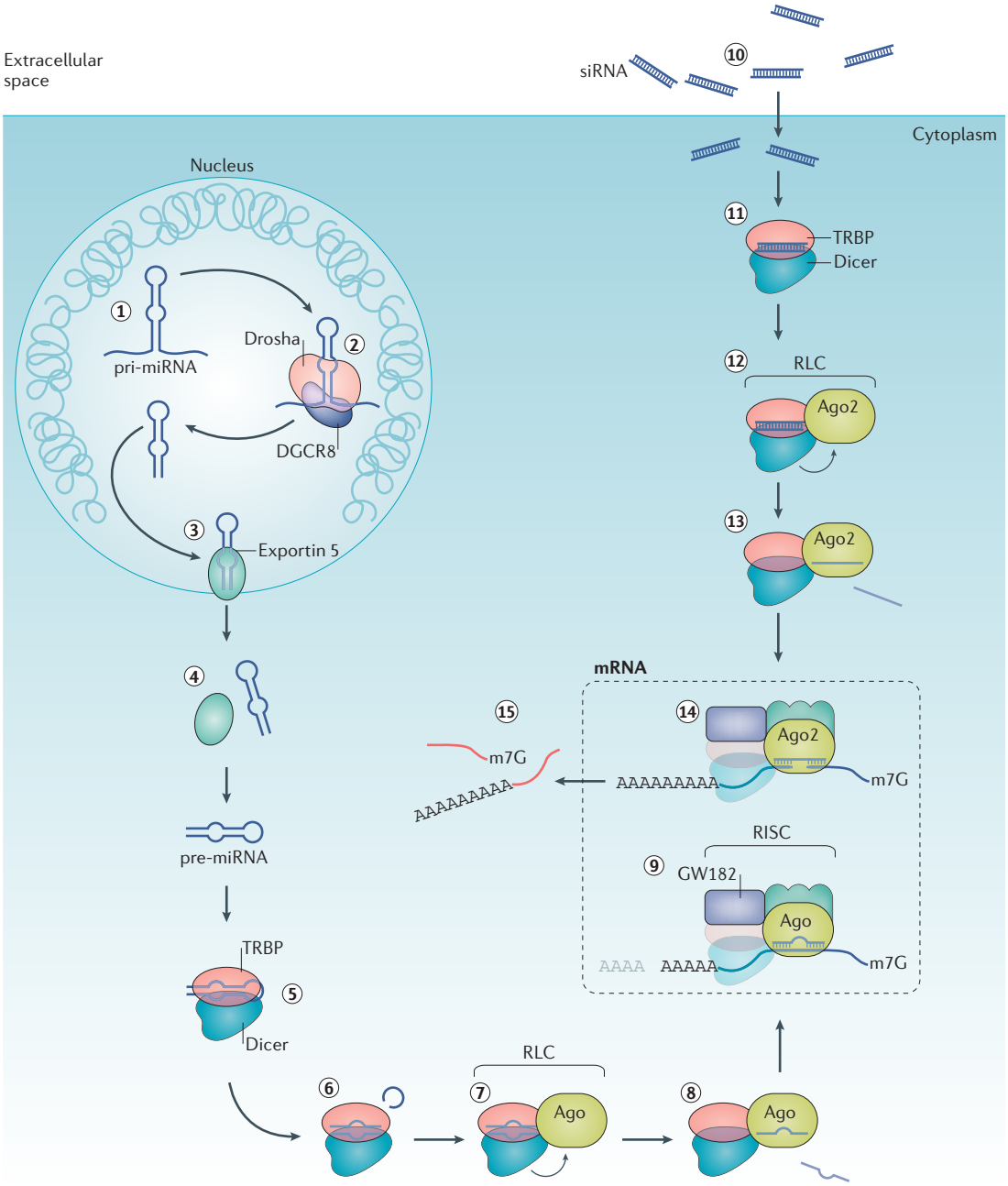

Fig. 2 | **Pathways for mammalian miRNA biogenesis, synthetic RNAi trigger processing and RNAi silencing.** (1) Mammalian primary microRNA (miRNA) transcripts (pri-miRNA) are transcribed in the nucleus (2) and cleaved by the Microprocessor complex (Drosha–DGCR8) to produce (~30 bp) short hairpin RNAs (shRNAs) called pre-miRNA. (3) Exportin 5 binds and transports the pre-miRNA to the cytoplasm (4) where it disengages from exportin 5 (5) and binds with Dicer and TAR RNA-binding protein (TRBP)[18,295]. (There are also non-Dicer-mediated pathways[296].) (6) Dicer cleaves the terminal loop of pre-miRNA (7) and induces formation of an RNA-induced silencing complex (RISC)-loading complex (RLC) with an Argonaute (Ago1–Ago4) protein. (8) A guide strand (antisense) is selected[295] and loaded into Ago1–Ago4 and the passenger (sense) strand is discarded. (9) The mature RISC can regulate gene expression by inhibiting mRNA translation, inducing mRNA sequestration in cytoplasmic P-bodies and/or GW-bodies[297–300], promoting mRNA degradation and directing transcriptional gene silencing[227] of the target gene loci. Argonaute, GW182 and the guide strand are essential for the mRNA-silencing activities of RISC. TRBP and DICER can dissociate from mature RISC after guide strand loading. mRNAs with as few as 7 bases of complementarity to the seed region (bases 2–8 from the 5′ end) of guide strands can be affected by RNAi[35]. (10) Synthetic small interfering RNAs (siRNAs) enter the cytosol via endocytosis followed by rare endosomal escape events. (11) siRNAs then interact directly with the cytosolic RNA interference (RNAi) enzymes (Dicer and TRBP) (12) to form the RLC via Dicer-mediated[295] or non-Dicer-mediated pathways[296] (13) and undergo strand selection to produce mature RISC. (14) siRNA guide strands usually have full complementarity to a single target mRNA to induce potent and narrowly targeted gene silencing. (15) Ago2 is particularly important for RNAi therapeutics as it has intrinsic slicer activity to efficiently cleave mRNA targets[293]. m7G, 7-methylguanosine. Adapted from REF.[255].

Box 1 | **Avoiding toxicity of RNAi-based drugs**

A critical challenge in the development of RNA interference (RNAi) therapeutics is avoidance of nonspecific toxicity. There are four main sources of toxicity that have considerably affected clinical RNAi drug development: immunogenic reactions to RNAi triggers by innate sensors of foreign double-stranded RNA (dsRNA)[8,256]; immunogenic and non-immunogenic toxic effects of excipient chemicals[12]; unintended RNAi activity due to off-target RNAi activity by the RNAi trigger (REF.[48]); and on-target RNAi activity by RNAi drugs that accumulate in non-target tissues.

Immunogenic reactions to dsRNA

Immunogenic reactions to dsRNA stem from sensing of dsRNAs by PKR, Toll-like receptor 3 (TLR3) and TLR7. Although a major problem for first-generation small interfering RNA (siRNA) drugs[8], recent RNAi triggers with extensive 2′-*O*-methyl base modifications have largely avoided this issue[45].

Toxicity of excipients

By contrast, the toxic effects of excipient chemicals have plagued nanoparticle drug formulations and are likely responsible for the dose-limiting toxicities seen in a number of nanoparticle-delivered drug candidates[12]. Clinical trials show that problems can arise directly from excipient components or from metabolic breakdown of the excipients. Complex multifunction nanoparticle formulations may also undergo degradation during storage that change their toxicity profile over time[12]. When toxicities do occur, the difficulty in determining the exact identity of the toxic component can be a major challenge. In addition, intravenous immunological infusion reactions[98,257] can be a considerable problem in nanoparticle formulations.

Despite these problems, patisiran, the first RNAi drug, uses a lipid nanoparticle (LNP) delivery formulation[93], and work continues on developing potent and non-toxic nanoparticles[124]. A key strategy for ongoing research may be to limit excipients to a small number of chemical components that are individually verified for low toxicity. Assembled nanoparticles need to be as uniform as possible as this correlates with an improved therapeutic index and reduced toxicity. Once manufactured, nanoparticle formulations may degrade over time and lead to increased toxicity. This may have played a role in the recent trial of Calando Pharmaceutical's CALAA-01 (REF.[12]). Thus, continuous quality monitoring of trial medication will likely benefit future trials.

Finally, pretreatment using corticosteroids and anti-allergy medications has helped to considerably attenuate infusion reactions with patisiran and may be necessary for other nanoparticle formulations delivered via intravenous infusion[104].

Unintended RNAi activity

Although the RNAi pathway can be specifically directed against target genes, off-target RNAi silencing can occur from unintended seed region matches between RNAi guide strands and non-targeted mRNAs[48]. This problem can be ameliorated by screening target sites against human genome sequences using tools such as BLAST and eliminating target sites with significant overlaps with genes of concern. However, the only way to ensure safety is via extensive testing. Here, it is critical to use primate models with large genomic sequence overlaps with humans as the genomes of other animals have too many significant differences to provide adequate screening (see Related links). Even with extensive testing, some off-target RNAi effects may be unavoidable. Recent evidence indicates that the dose-limiting liver toxicity observed in animal models at exaggerated RNAi doses can be attributed to off-target silencing[48]. Today, developers try to avoid these problems by minimizing the administered RNAi dose. Efforts are underway to further ameliorate these issues using novel base modifications (see Related links).

On-target RNAi activity in non-target tissues

A final problem with toxicity is that siRNAs delivered systemically to the body can accumulate in many tissues that are not the intended sites of drug activity. For example, RNAi silencing of endogenous transcription factors such as MYC[258] can have beneficial effects against tumours while causing unwanted side effects in healthy tissues. Today, RNAi drug developers mitigate these issues by choosing highly-disease-selective genes as the targets for RNAi silencing and by choosing delivery routes that reduce accumulation in non-target tissues. Future improvements in tissue-specific targeting of RNAi activities may ease these restrictions to allow development against additional indications. In addition, Alnylam has recently developed antisense oligonucleotides that can reverse the drug activity of siRNA drugs in vivo[181].

and non-Dicer-substrate siRNAs. Direct comparisons between 25/27 nt (25 bp) DsiRNAs and 21/21 nt (19 bp) siRNAs show that Dicer processing leads to more potent RNAi activity and more reliable selection of antisense strands as the RISC guide[17]. On the other hand, siRNAs that bypass Dicer cleavage can allow for the use of more extensive chemical modifications over the entire dsRNA and its 5′ terminal phosphates[22], thereby attaining better metabolic stability when administered without encapsulating nanoparticle excipients.

Seeking a compromise between efficiency of Dicer processing and the stability of siRNAs, Dicerna Pharmaceuticals has developed a motif that engages Dicer for handoff to the RLC without requiring cleavage of the putative guide strand (see Related links). This motif then allows more extensive chemical modifications over the entire duplex, including the use of a modified 5′ phosphate group on the putative guide strand. Whether this results in better clinical efficacy remains to be seen.

Other examples of RNAi triggers designed to accentuate particular functional properties include partially duplexed 'self-delivering' sd-rxRNAs (Phio Pharmaceuticals, formerly RXi Pharmaceuticals)[23] and small segmented siRNAs (sisiRNAs) that preclude sense strand loading by division into two segments.

One interesting exception to duplex RNAi triggers is Ionis Pharmaceuticals' single-stranded siRNAs (ss-siRNAs)[24]. These fully modified, partially phosphorothioated single-stranded oligonucleotides can enter cells gymnotically (nonspecific cellular uptake of single-stranded phosphorothioate (PS)-modified oligonucleotides through poorly understood mechanisms[25]) and insert into Argonaute (Ago) proteins to form mature RISCs. It is unknown how Ago loading occurs, but the presence of a 5′ phosphate was found to be crucial for RNAi activity[24], and the addition of a 5′-(*E*)-vinylphosphonate improved potency. In subsequent work, this modification improved the potency of more conventional siRNAs[22]. Although the ss-siRNA had significantly lower potency than conventional dsRNA triggers[24], the research elucidated necessary and sufficient elements for Ago association and RNAi activity and continues to influence the development of metabolically stabilized, fully modified RNAi triggers.

Sequence selection

The antisense strand in an RNAi trigger is the putative guide strand for RISC binding to the mRNA target. Thus, once the RNAi trigger is in the cell, the sequence of the antisense strand is the most crucial determinant

Dicer substrate siRNAs (DsiRNAs). RNA duplexes of 22–29 bp with a two-base 3′ overhang on the putative guide strand that trigger RNA interference via cleavage by Dicer.

Phosphorothioate (PS). A nucleic acid backbone modification in which one oxygen in the phosphodiester is replaced by a sulfur atom.

Argonaute (Ago). One of four different proteins, Ago1–Ago4, that bind to RNA interference guide strands to form RNA-induced silencing complexes.

of pharmacodynamics. Sequence selection has profound effects on strand selectivity, on-target potency and off-target spurious activities[26]. A recent review[27] provides an extensive listing of software packages and suggested usage protocols for siRNA design. Below, we discuss some of the pertinent issues.

During RNAi processing, both sense and antisense strands in dsRNAs can enter the RISC to direct RNAi

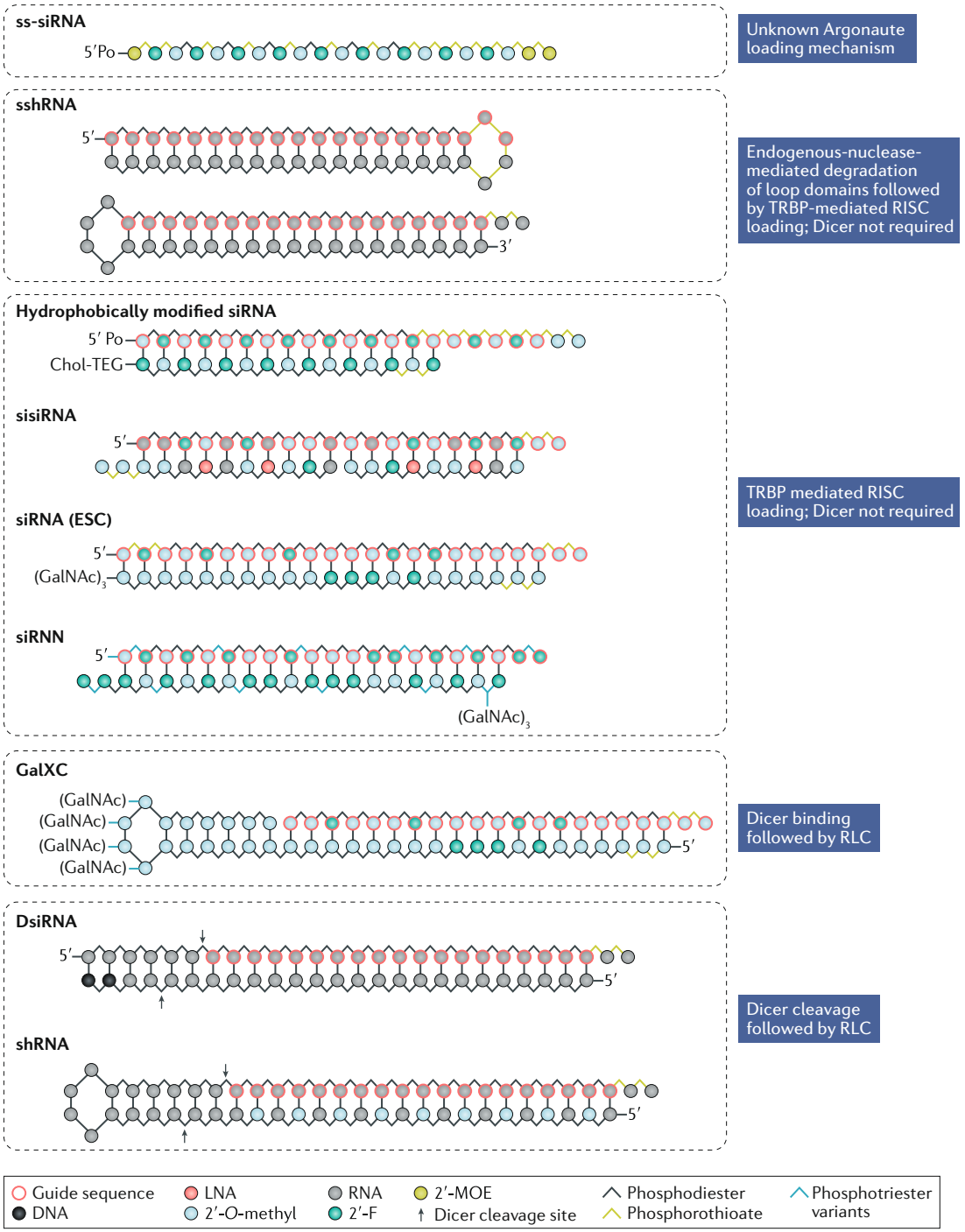

Fig. 3 | **Representative secondary structure motifs of different classes of synthetic RNAi triggers along with their primary mechanisms of entry into the RNAi pathway.** Coloured circles denote typical base and backbone modifications. In each motif, antisense (putative guide) strands are shown on top whereas sense strands are shown on the bottom. 5′-(*E*)-Vinylphosphonate (5′ Po), *N*-acetylgalactosamine (GalNAc) and tetra-ethylene glycol cholesterol (Chol-TEG) modifications are indicated by text labels. 2′-F, 2′-fluoro; 2′ MOE, 2′-*O*-(2-methoxyethyl); DsiRNA, Dicer substrate siRNA; ESC, enhanced stability chemistry; LNA, locked nucleic acid; RISC, RNA-induced silencing complex; RLC, RISC loading complex; RNAi, RNA interference; shRNA, short hairpin RNA; siRNA, small interfering RNA; siRNN, short interfering ribonucleic neutral; sisiRNA, small segmented siRNA; ss-siRNA, single-stranded siRNA; sshRNA, synthetic shRNA; TRBP, TAR RNA-binding protein. Adapted with permission from REF.[301], Elsevier.

activity[20,28]. For optimal safety and potency, RNAi drugs need to ensure exclusive selection of the antisense strand, which can be accomplished by tuning the dsRNA's thermodynamic stability. In general, the strand with weaker base pairing at its 5′ terminus will be preferentially selected for incorporation into the RISC[20,28]. Thus, ideal dsRNA triggers should be more AU rich at the 5′ end of the antisense strand than at the 5′ end of the sense strand (chemical modifications that can block sense strand loading will be discussed below).

Once loaded into the RISC, the guide strand needs to base pair with its binding site on the target mRNA to initiate RNAi activities. Ago proteins assist target binding by arranging guide strand bases to improve binding kinetics[29,30] and engaging in an efficient two-step dynamic search of mRNAs[31] for target sites. However, local secondary and tertiary structures[32], mRNA-bound proteins and transiting ribosomes can still substantially hinder access. Thus, modern RNA thermodynamic prediction software[33,34] cannot reliably predict the accessibility of cognate binding sites on mRNAs[27].

A further complication for sequence selection is the need to avoid off-target matches to other mRNAs. RISCs can potentially downregulate any mRNAs with perfect base-pairing complementarity to the guide strand seed region (bases 2–8 from 5′ end)[35]. Because this consists of only seven bases, the potential number of off-target matches is large for any guide sequence. Fortunately, efficient catalytic degradation of target mRNAs requires more extensive base pairing to engage the slicer activity of human Ago2 (REF.[36]). One tool useful for screening genomic sequence matches is NCBI BLAST[37]. In particular, the blastn algorithm can search genomic and transcriptomic sequence databases for matches to sequences as short as 12 bases and can be used to identify transcripts that have extensive complementarity to 21 nt putative guide strands and passenger strands. Some automated design tools, such as Horizon Discovery/Dharmacon's siDESIGN Center (see Related links), can incorporate BLAST search results against specific animal transcriptomes in the scoring mechanism used to rank candidate designs[27], whereas other websites such as Integrated DNA Technology's RNAi Design Tool (IDT DNA custom DsiRNA design center; see Related links) can display off-target BLAST matches to users[27]. For those requiring a more rigorous screen of shorter partial sequence matches, the siDirect web portal (see Related links) uses a custom sequence matching algorithm with relaxed 'mismatch tolerance' to find off-target matches[27].

Ultimately, owing to the intrinsic complexities of the biochemical processes underlying RNAi potency and targeting specificity, computational design tools[27] cannot be relied on to predict optimal target sites even when used according to optimized protocols[27,38]. Although new design algorithms based on convolutional neural networks[39] and deep reinforcement learning[40] may eventually improve design optimality, for the foreseeable future, developers of RNAi drugs will likely need to conduct extensive empirical target sequence screens around plausible target sites to identify the best drug candidates.

Chemical modifications

The history and evolution of chemical modifications for oligonucleotide therapeutics have been comprehensively reviewed in recent literature[41–44]. For RNAi drugs, chemical modifications (other than tissue-targeting ligands) serve two essential functions. First, they greatly improve safety by attenuating activation of endogenous immune sensors that detect dsRNA[45]. Second, they greatly improve potency by enhancing the ability of dsRNA triggers to resist degradation by endogenous endonucleases and exonucleases[46]. In addition to these functions, chemical modifications can also enhance antisense strand selectivity for RISC loading[47], improve sequence selectivity to reduce off-target RNAi activity[48] and change physical and chemical properties to enhance delivery[49].

The absolute necessity for chemical modifications was driven home by setbacks in the early days of RNAi clinical development. In the late 2000s, two unmodified 21 nt siRNAs (bevasiranib and AGN211745) entered clinical trials for the treatment of wet age-related macular degeneration via intra-ocular administration[8] and one unmodified siRNA (ALN-RSV01) was tested for the treatment of respiratory syncytial virus infection via nasal spray administration[9]. None of these candidates moved beyond phase II clinical trials. For bevasiranib and AGN211745, evidence suggested that the apparent therapeutic effect (inhibition of neovascularization) found in animal models could have resulted from the nonspecific activation of innate immunity via a previously unknown Toll-like receptor 3 (TLR3)-mediated pathway[8]. For ALN-RSV01, phase IIb clinical trials showed signs of therapeutic effect but did not meet the primary end point[9,50], and questions also persisted regarding whether the observed treatment effects were due to RNAi or nonspecific immune-related pathways.

At around the same time, CALAA-01, an unmodified siRNA delivered by a targeted polymer nanoparticle, showed clear evidence of in vivo RNAi silencing in human patients. However, a phase Ib clinical trial for treatment of advanced solid tumours was terminated owing to nonspecific toxicity and low rate of objective responses[12].

Fortunately, extensive research efforts since the 1980s[41,51] had already developed safe and versatile base, sugar and backbone modifications for antisense oligonucleotides (ASOs)[52] (BOX 2). Widely used modifications include 2′ sugar modifications (such as 2′-O-methyl, 2′-fluoro (2′-F) and 2′-O-(2-methoxyethyl) (2′-MOE)) that reduce immune activation, increase base pairing melting temperature (T_m) and improve nuclease resistance[41,53]; modified sugar groups that greatly increase (locked nucleic acid (LNA)[54]) or decrease (unlocked nucleic acid (UNA)[55]) binding affinity to RNA; and backbone changes that increase nonspecific protein binding (PS[56–58]), eliminate (morpholino and/or peptide nucleic acid (PNA)[59]) backbone charges, increase hydrophobicity, resist nuclease degradation and increase (PNA) or decrease (morpholino and/or PS-DNA and PS-RNA) binding T_m versus RNA.

For synthetic RNAi triggers, development efforts focused on identifying patterns of these existing modifications that can improve RNAi drug properties without compromising the compatibility of dsRNA triggers

Passenger strands
The complements to the guide strands that are discarded during strand selection.

Antisense oligonucleotides
(ASOs). Synthetic single-stranded oligonucleotides of varying chemistries for which the sequence specifically hybridizes with target RNAs.

2′-*O*-Methyl
(2′-O-me). A naturally occurring modification of RNA in which a methyl group is added to the 2′ hydroxyl of the ribose sugar.

2′-Fluoro
(2′-F). A synthetic analogue of RNA in which the 2′ hydroxyl on the sugar is replaced by a fluorine.

2′-O-(2-Methoxyethyl)
(2′-MOE). A synthetic analogue of RNA in which a 2-methoxyethyl group is attached to the 2′ hydroxyl.

Locked nucleic acid
(LNAs). A synthetic analogue of RNA in which a methylene bridge connects the 2′ oxygen and the 4′ carbon.

Unlocked nucleic acid
(UNA). A synthetic acyclic analogue of RNA missing the C2′–C3′ bond of the ribose ring.

Morpholino
A charge-neutral analogue of DNA in which backbone phosphodiesters are replaced with phosphorodiamidate linkages.

Peptide nucleic acid
(PNA). A synthetic analogue of DNA and RNA that has a peptide backbone.

Box 2 | **'Gapmer' antisense oligonucleotides**

Antisense oligonucleotides (ASOs) are synthetic single-stranded oligonucleotides of varying chemistries that contain complementary sequences to target RNA transcripts. ASOs have a range of applications, including blocking mRNA translation, competing for microRNA (miRNA) target binding, inhibiting target miRNA, exon skipping, exon inclusion and RNase H-mediated target degradation[259]. Of these applications, targeted RNase H-mediated degradation is the most utilized.

RNA strands in DNA–RNA duplexes are recognized and degraded by RNase H[260]. For recognition to occur, a short stretch of complementary single-stranded DNA (ssDNA) at least five nucleotides long (ideally eight or more) is required[52]. Because the phosphodiester backbone of ssDNA is highly susceptible to cleavage by endonucleases and exonucleases present in serum and in cells, the in vivo effectiveness of pure DNA ASOs is limited. Thus, phosphodiester backbones are usually replaced with phosphorothioate (PS) linkages, which are nuclease-resistant and compatible with RNase H activity[57]. However, PS linkages in ASOs are associated with nonspecific protein binding, which, in turn, leads to cytotoxicity[57]. PS linkages also introduce chiral centres into the backbone ASOs that lower melting temperature (T_m) by disrupting base pairing[57]. Typically, therapeutic ASOs use a central core of PS–DNA flanked by 5′ and 3′ nuclease-resistant modified oligonucleotides such as locked nucleic acid, 2′-*O*-(2-methoxyethyl), 2′-*O*-methyl or 2′-deoxy-2′-fluoro-β-D-arabinonucleic acid (FANA)[261]. These modified bases also stabilize base pairing, which lowers the number of nucleotides required to reach a sufficient T_m (REFS[52,262]). ASOs in these types of configurations are referred to as 'gapmers'.

Although ASO gapmers and small interfering RNAs (siRNAs) differ in their mechanisms of action, they both suppress gene expression by sequence-specific degradation of target mRNAs. Both are also limited by cell-specific targeting and inefficient endosomal escape[51]. However, major functional differences exist. ASO gapmers are most potent when knocking down transcripts in the nucleus, whereas siRNAs are most effective in the cytoplasm[263]. In addition, PS-modified ASOs can be delivered gymnotically (taken up by cells without the aid of a targeting ligand), although this method requires higher concentrations of ASOs and is nonspecific[25].

Owing to similarities in targets (mRNAs) and modes of action, ASO and siRNA drug candidates can directly compete against each other in some indications. For example, Ionis Pharmaceuticals' inotersen[264] is poised to compete with Alnylam's patisiran for the treatment of hereditary transthyretin amyloidosis. As the number of oligonucleotide therapeutic approvals increases in the coming years, it is likely that direct competition between ASO and siRNA drugs will only increase.

with RNAi pathway enzymes such as Dicer and Ago. In particular, Dicer and/or RLCs need to be able to cleave and/or unwind dsRNAs triggers for guide strand extraction and loading, and putative guide strands need their 5′ phosphates, seed region bases (22–8 from 5′ end) and 3′ tails to maintain favourable binding interactions with corresponding binding sites on Ago proteins[30].

Initial studies examined partial chemical modifications of dsRNA (for example, alternating patterns of RNA and 2′-*O*-methyl-modified nucleotides)[60] and found some patterns that could maintain acceptable RNAi activity while attenuating immune activation. However, partially modified dsRNAs were still vulnerable to degradation by serum nucleases[60]. With further development, fully modified dsRNAs with alternating 2′-*O*-methyl and 2′-F modifications (with 2′-F on one strand base pairing with 2′-*O*-methyl on the opposing strand)[46,53,60,61] were found to reduce immune activation and improve serum stability with acceptable penalties in RNAi potency[41]. These triggers also used DNA bases, 2′-*O*-methyl bases or PS backbone modifications on 3′ overhangs.

Follow-on research by RNAi drug companies and others[62,63] have identified increasingly potent variants of fully modified dsRNAs using irregular patterns of 2′-F and 2′-*O*-methyl modifications and PS backbone modifications. To some extent, optimized chemical modification motifs can be used with a variety of trigger sequences while maintaining basic pharmacokinetic and pharmacodynamic properties. This allows somewhat independent optimization of dianophores (modification patterns) and pharmacophores (sequences) during RNAi drug development[41].

For example, Alnylam has developed generations of 'enhanced' chemistries that improve the potency of siRNAs. A generation-one standard template chemistry (STC) positioned three consecutive 2′-*O*-methyls on the antisense strand (base paired to three 2′-Fs on the sense strand) at the eleventh, twelfth and thirteenth base from the 5′ end (near the endonucleolytic cleavage site of Ago2). This was followed by generations of enhanced stability chemistry (ESC) motifs that extended the antisense strand to 23 nt and added PS backbone modifications at the two 5′ terminal bases on the sense and antisense strands and further increased the use of consecutive 2′-*O*-methyl base modifications throughout the dsRNA. In in vivo and clinical testing, the changes have allowed a drop of two orders of magnitude in the administered doses of *N*-acetylgalactosamine (GalNAc)-conjugated siRNAs while improving RNAi activity and maintaining low toxicity[48,64–66].

In addition to advances in the patterning of well-studied modifications, novel chemistries have also been used to improve siRNA potency and safety. In one example, the 5′ terminal phosphate on an RNAi guide strand is critical for binding of the guide strand to the PAZ domain of Ago proteins[30]. Recent experiments show that the incorporation of a 5′-(*E*)-vinyl-phosphonate modification[24] on the antisense strand of metabolically stabilized siRNAs significantly improves the potency and duration of RNAi activity in vivo[22]. In a second example, new research shows that the hepatotoxicity of fully modified, GalNAc-conjugated siRNAs mainly stems from seed-domain-mediated off-target RNAi activities[48]. The incorporation of a single base-pair-destabilizing (*S*)-glycol nucleic acid[67] base into the seed region of antisense strands can attenuate hepatotoxicity while improving potency[48]. In a third example, the incorporation of a single 5′ terminal UNA[47] (or two 5′ terminal 2′-*O*-methyls[68]) has been shown to block undesired selection of sense strands during RISC loading.

Finally, chemical modifications can assist delivery by changing the physical and chemical properties of RNAi triggers to improve cellular uptake and endosomal escape. For example, sd-rxRNA (Phio Pharmaceuticals)[23] is a cholesterol-conjugated, shortened (~15 bp) dsRNA with extensive PS backbone modifications in the extended 3′ overhang of the antisense strand. These modifications improve systemic circulation via albumin binding and help induce cellular uptake by gymnosis[25]. Phosphotriester backbone chemistries have also been developed to improve delivery[49,69]. In this approach, a number of backbone phosphodiesters on an siRNA are replaced by charge-neutral phosphotriesters to create short interfering ribonucleic neutrals (siRNNs). The reduced backbone charge of the siRNNs and the pendant ligands attached to the phosphotriesters can aid in delivery. Once the siRNN enters the cytosol, cytoplasmic thioesterases

2′-Deoxy-2′-fluoro-β-D-arabinonucleic acid
(FANA). A synthetic nucleotide in which the 2′ sugar position is a stereoisomer of DNA with an additional fluorine group.

Dianophores
Molecular features that determine pharmacokinetics.

Pharmacophores
Molecular features that determine pharmacodynamics.

N-Acetylgalactosamine
(GalNAc). A sugar derivative of galactose that binds to the asialoglycoprotein receptor on hepatocytes.

Gymnosis
The nonspecific cellular uptake of single-stranded oligonucleotides, especially those with phosphorothioate backbones.

cleave the phosphotriester, reverting it back to a canonical phosphodiester. siRNNs are currently undergoing discovery-stage development by Solstice Biologics.

Delivery excipients

Regardless of chemical modifications, the size, hydrophilicity and charge of dsRNA triggers present major challenges for systemic circulation[70], extravasation, tissue penetration, cellular uptake[71,72] and endosomal escape[73]. Many chemical excipients have been developed to overcome these barriers. These include nanoparticles[12,74], lipid nanoparticles (LNPs)[75–77], polymers[78,79], dendrimers[80], nucleic acid nanostructures[81], exosomes[82,83] and GalNAc-conjugated melittin-like peptides (NAG-MLPs)[84,85]. Common targeting ligands for siRNA include aptamers[86,87], antibodies[88,89], peptides[90] and small molecules (for example, GalNAc). These approaches will not be discussed in depth here as they have been extensively reviewed elsewhere[12,51,87,91] (TABLE 1).

Site of administration

In addition to excipients, the method and site of administration can have a profound impact on both the bioavailability and biodistribution of RNAi drugs[92]. RNAi drugs in clinical development have been delivered systemically via intravenous (IV) infusion[93] and subcutaneous (SC) injection[94] and locally via inhalation (to the lungs)[9], site-specific injection (for example, in the eye or the cerebral spinal fluid)[8] or topical administration[23].

IV administration has been widely used with nanoparticle and LNP-formulated siRNAs. This route avoids first-pass metabolism in the liver and affords quick access to target tissue through the systemic circulation. However, siRNA-sized oligonucleotides can exit the body within 30 minutes through renal clearance[95], and nanoparticle formulations can be cleared in hours or days by the mononuclear phagocyte system (MPS)[96]. Rapid clearance may give insufficient time for drugs to extravasate, permeate target tissues and enter targeted cells. Cell surface receptors can also become saturated with drugs during administration, blocking further cellular uptake[97]. For patients and doctors, IV administration can be cumbersome and often requires lengthy hospital visits. Also problematic is the propensity for occurrence of infusion reactions[98], stemming from immune activation upon rapid mixing of pharmacological moieties with blood. The prevalence of these reactions may require prophylactic treatment of patients with steroids before injections. Despite these challenges, the bioavailability and biodistribution benefits of IV administration are critical to the functioning of some formulations.

Owing to the complications of IV administration, SC injection into the adipose tissue below the epidermis and dermis has been gaining favour in conjunction with the growing use of GalNAc-conjugated 'naked' RNAi triggers. Drugs administered subcutaneously have a slower release rate into the systemic circulation and can also enter the lymphatic system[66,99], giving more time for recycling of cellular receptors that mediate uptake. SC injections are also much faster and easier to administer, reducing treatment burden. Although SC administration is typically associated with ligand-conjugated dsRNAs, there has been at least one report of an SC-administered LNP formulation with efficient target silencing in mice[100].

Despite advances in ligands and excipients, the intrinsic pharmacokinetic properties of RNAi drugs make it very difficult to reach every diseased tissue via systemic delivery. Many diseases may best be tackled via local administration, and this is a very active area of investigation. For example, polymer matrix formulations for extended-release topical administration of siRNAs to body surface wound sites have recently been developed[101,102], and intracardiac administration of siRNAs has been tested in vivo[103]. Alnylam recently announced intentions to enter clinical development of ligand-conjugated siRNA drugs for CNS indications using injection into cerebral spinal fluid. These localized delivery strategies could broaden the reach of RNAi therapeutics into tissues where the potency of drug effects from systemic administration is currently insufficient.

RNAi in the clinic: patisiran and beyond

The approval of patisiran heralds a vibrant new era for RNAi therapeutics in which safe and potent RNAi payloads and delivery strategies are moving through more mature clinical research and development (see Related links) and current Good Manufacturing Practice (cGMP)[14] pipelines, and promising drugs against numerous indications in both liver and non-liver tissues are being produced. Multiple drug candidates for liver and non-liver indications are currently in phase I, II and III clinical trials (TABLE 2), and INDs targeting the CNS (Alnylam presentations; see Related links) and additional non-liver tissues are imminent (see Related links). Beyond these, novel technologies for RNAi payloads and delivery excipients will likely lead to enabling advances for additional tissues and indications within 5 years. Together, these developments suggest that RNAi therapy has numerous paths for impactful innovations over the next decade.

Patisiran

Patisiran (also known as ALN-TTR02) is an siRNA LNP for the treatment of transthyretin (TTR) amyloidosis (FIG. 4). hATTR is a rare, inherited, life-threatening neurodegenerative disease driven by deposition of TTR amyloids in the peripheral nervous system, heart, gastrointestinal tract and other organs[104]. Patients suffer progressive neuropathy, cardiomyopathy, impaired ambulation and a variety of other debilitating symptoms, with median survival between 5 and 15 years from diagnosis[104].

The majority of TTR proteins are produced in the liver. There are >120 mutations in *TTR* that can cause hATTR[104]. Before the development of patisiran and its ASO competitor, inotersen[105], drug treatment options were limited to small molecules that stabilize TTR tetramers in their native conformation[93,105]. Although these approaches can slow disease progression, there has been great need for more efficacious treatment options.

The patisiran siRNA (ALN-18328) works by silencing both wild-type and mutant *TTR* mRNAs in hepatocytes to reduce serum levels of the TTR protein.

Extravasation
The exit of pharmaceutical agents from the systemic circulation into the extracellular space.

To achieve broad silencing activity against *TTR* mRNA variants, the antisense (putative guide) strand targets the 3′ untranslated region of the mRNA[93,106,107], which presumably has less variability in the patient population (FIG. 4). Unlike most siRNAs in clinical development, ALN-18328 is not a fully modified, metabolically stabilized siRNA and does not have a targeting ligand for enhanced uptake by hepatocytes. Delivery is instead achieved by encapsulation of ALN-18328 in an LNP formulated for hepatocyte uptake[93].

Two generations of LNP formulations were tested in the clinic: ALN-TTR01 and ALN-TTR02 (REF.[108]). ALN-TTR02 (now known as patisiran) is a 'second-generation' pegylated LNP containing cholesterol, a polar lipid (DSPC), a pegylated lipid (PEG2000-C-DMG) and an ionizable amino lipid (DLin-MC3-DMA)[75] that is neutral at pH 7 but becomes cationic under acidic pH (at an optimized pK_a of 6.44). siRNA-LNPs[77] are assembled by electrostatic interactions between the siRNA and the lipid excipients at acidic pH (when DLin-MC3-DMA is cationic). Once assembled, PEG2000 lipids on the surface of the LNP maintain particle stability during storage. In systemic circulation, the PEG2000-C-DMG is lost and replaced by serum proteins[109], notably apolipoprotein E (ApoE)[110], which interacts with cholesterol molecules incorporated in the LNP lipid matrix. In the liver, hepatocytes take up ApoE-covered LNPs and send them to the endosome, where the acidic pH induces re-ionization of the amino lipid component, causing particle disassembly. Electrostatic and hydrophobic interactions between disassembled lipid globules (helped by the splayed lipid tail configuration of DLin-MC3-DMA) and the endosomal membrane then help the siRNA escape into the cytosol. Compared with ALN-TTR01, which used an earlier ionizable lipid (DLin-DMA), patisiran achieved greater than ten times improvement in potency in vivo[108].

In phase II clinical trials, patisiran administered intravenously once every 3 weeks achieved >80% mean sustained reduction in circulating TTR protein in serum[108]. A subsequent phase III, double-blind, placebo-controlled clinical trial demonstrated impressive safety and efficacy in controlling progression of hATTR (BOX 3).

The approval of patisiran for the treatment of hATTR with polyneuropathy (and the approval of patisiran's ASO competitor, inotersen) brings a new era of hope. For the first time, patients with hATTR have treatment options with considerable probabilities of halting disease progression[111]. Comparing the two drugs, patisiran appears to have better results for safety and efficacy[93,105], but significant proportions of patients on both patisiran (~44%)[93] and inotersen (74%)[105] show continuing declines in index scores for polyneuropathy (~44% and ~74%) and quality of life (~49% and ~50%). Furthermore, the safety and efficacy of both drugs over durations significantly longer than 18 months remain uncertain, and it is also unknown whether there are distinctive populations of patients who are better suited to either drug. Thus, additional trials and longer-term clinical experience may be needed to stratify patient populations and determine the best treatment strategies using one or both drugs.

Metabolically stabilized, GalNAc-conjugated siRNAs

Approximately one-third of RNAi drugs currently in clinical trials (TABLE 2) are single-molecule, chemically modified RNAi triggers conjugated to multivalent GalNAc ligands[112] targeting the asialoglycoprotein receptors (ASGPRs)[65,66,94]. GalNAc is a sugar derivative of galactose found on damaged glycoproteins that have lost terminal sialic acid residues from their pendant oligosaccharides. The liver functions to clear these proteins from the systemic circulation by expressing trimeric ASGPRs at very high levels (order of 10^5–10^6 per cell) on the surface of hepatocytes (see Related links)[113]. ASGPRs bind specifically to GalNAcs at neutral pH for endocytosis of circulating macromolecules from the blood and release GalNAc at acidic pH (~5–6) for cargo drop-off in the early endosome. Freed ASGPRs are then recycled back to the cell surface for reuse[113]. The suitable physiology of the liver, the unique properties of ASGPRs, the non-toxic nature of the GalNAc ligand and the simplicity of GalNAc–siRNA conjugates make this a near-ideal approach for systemic RNAi delivery to hepatocytes. A further advantage compared with patisiran and similar nanoparticle-based delivery formulations is that GalNAc-conjugated oligonucleotides can achieve efficient delivery from SC injection rather than the more burdensome IV infusion with pre-medication. Research into GalNAc-mediated delivery of nucleic acids has been underway for >25 years[114]. A key problem for RNAi delivery has been development of non-toxic RNAi triggers with sufficient metabolic stability and potency.

Revusiran. Revusiran (Alnylam) was the first metabolically stabilized GalNAc–siRNA conjugate to enter clinical trials. Similar to patisiran, revusiran targeted *TTR* mRNA for the treatment of hATTR[94]. Every base position was chemically modified. Duplex regions had an asymmetric, irregular pattern of 2′-*O*-methyl and 2′-F modifications in the duplex and DNA bases used in the overhangs. This motif considerably reduces degradation by serum nucleases and attenuates immunogenic toxicity[41,45].

In phase II clinical trials, SC injection of revusiran achieved 55–90% mean knockdown of serum TTR levels[94,115]. Side effects were mild, with injection-site reactions being the most common adverse event and there was no evidence of systemic immune activation.

Following these results, a phase III, double-blind, placebo-controlled clinical trial of revusiran (ENDEAVOUR, NCT02319005) was initiated, with patients treated for 18 months. In October 2016, following reports of peripheral neuropathy and elevated blood lactate levels in the revusiran phase II open-label extension study, a review of data for the ongoing phase III ENDEAVOUR trial revealed an imbalance of mortality in the revusiran arm versus the placebo arm. Although there was no imbalance in peripheral neuropathy or lactic acidosis, development of revusiran was halted[116].

However, in a post-trial investigation of the mortality imbalance (see Related links), no clinical evidence of revusiran-related mortality was found. Evidence suggested that the imbalance in mortality could be explained by an unusually low rate of deaths in the

Table 1 | **Delivery methods and excipient chemicals for RNAi drugs**

Delivery method	Mechanisms	Tissues	Examples	Status
Naked siRNA	Nonspecific uptake, unknown escape routes	Ocular[8,302], respiratory[9,50]	Bevasiranib[302], ALN-RSV01 (REF.[50])	• Withdrawn from clinical testing owing to safety and potency problems • Replaced by fully modified siRNA
Small-molecule ligand conjugated to fully modified RNAi agent	Receptor-mediated endocytosis, systemic circulation via subcutaneous injection	Liver[91], CNS (see Related links), solid tumours[303], other tissues	Folate[303], GalNAc[48,94]	• Preferred route for hepatocyte delivery • One active application for FDA approval • Clinical development by Alnylam, Dicerna, Arrowhead and Silence Therapeutics
Lipid nanoparticles	EPR, increased circulation half-life, receptor-mediated endocytosis, pH-induced lipid ionization and membrane disruption, peptide-induced membrane fusion	Liver[93], haematopoietic[123], solid tumours, other tissues[123,304]	Patisiran[305], TKM-Ebola[306], review[123,304]	• Patisiran received FDA approval • Liver-targeted lipid nanoparticles favour GalNAc–siRNA conjugates[91] • New development of biodegradable lipids decreasing toxicity[156]
Antibody and antibody fragments conjugated to siRNA or nanoparticles	Receptor-mediated endocytosis, long circulation half-life mediated by Fc domain, release of payload by catabolism of antibody	Muscle[307], haematopoietic[88,308], solid tumours[309], other tissues[89]	Early examples[88,309], THIOMAB-siRNA[89], Avidity patent[143], review[310]	• Preclinical development of variants with fully modified siRNA and excipients by Avidity Biosciences • In vivo studies by a number of groups
Polymer nanoparticles, PAMAM dendrimers	EPR, increased circulation half-life, receptor-mediated endocytosis or nonspecific uptake, enhanced endosomal escape, encapsulated siRNA	Solid tumours[12], other tissues[311]	Poly (lactic-co-glycolic acid)[312], polyethylene imine[313], nanohydrogel[314], CALAA-01 (REF.[10]), clinical review[12], dendrimer review[80]	• One compound under clinical development for skin application • Several compounds withdrawn from clinical testing owing to safety or efficacy problems • Newer-generation particles being actively developed and undergoing in vivo and in vitro testing
Aptamer–siRNA conjugates	Receptor-mediated endocytosis	Haematopoietic[87,315,316], solid tumours[317]	Transferrin receptor[318], CCR5 aptamer–DsiRNA[315], gp120 aptamer–DsiRNA[319], review[87]	• In vivo testing in rodent models by academic researchers • Rapid clearance challenge for systemic applications • May benefit from using fully modified siRNA
Phosphorothioate-modified single-stranded oligonucleotides and CpG oligonucleotides	TLR9 binding to CpG (immune-stimulatory), nonspecific uptake via gymnosis	Haematopoietic versus acute myeloid leukaemia[320,321]	CpG(A)–STAT3 siRNA[321]	• In vivo testing in rodents by academic researchers • Early stage of development • May benefit from fully modified siRNA
Cell-penetrating peptides, endosomolytic peptides, dynamic polyconjugates	Targeted or non-targeted uptake, enhanced endosomal escape	Liver[78,85], nonspecific uptake into other tissues unless targeted[322]	Penetratin–siRNA[323,324], polyarginine–siRNA[90,325], dynamic polyconjugates[78], NAG-MLP[84], review[322]	• NAG-MLP withdrawn from clinical testing owing to toxicity concerns • Others under active in vivo and in vitro development
Inorganic nanoparticles and other nanomaterials	EPR, increased circulation half-life, receptor-mediated or nonspecific uptake, siRNA attached to surface or encapsulated	Solid tumours[307,308], skin[326], CNS glioblastoma[327]	Gold nanoparticles[328], SNA[326,327,329], carbon nanotube–siRNA[330], mesoporous silicon nanoparticles[331,332]	• In vivo and in vitro testing on rodents in numerous indications by academic researchers • SNA siRNAs were dropped from clinical development by Exicure Inc.
Hydrophobically modified siRNAs	Binding to serum proteins, nonspecific interactions with cell surface proteins, gymnosis-mediated uptake	Skin, eye, placenta[150], other tissues[150]	siRNN[49], sd-rxRNA[23], improved hydrophobic siRNAs[63]	• Phio Pharmaceuticals has two compounds in clinical development • siRNNs under development by Solstice Biologics • Significant in vivo potency improvements seen for fully modified siRNAs[63]
Polymer matrices, hydrogels	Retention and extended-duration release in a localized area	Solid tumour, topical wound[101,102], bone[333], other tissues	siG12D-LODER, layer-by-layer wound dressing[101,102], hydrogel siRNA for bone fracture[333]	• siG12D-LODER in phase II clinical trial for solid tumours • Other applications tested in vivo on murine models • Active development by academic researchers

Table 1 (cont.) | **Delivery methods and excipient chemicals for RNAi drugs**

Delivery method	Mechanisms	Tissues	Examples	Status
Exosomes	Increased circulation half-life via CD47 expression, receptor-mediated uptake	Solid tumours[82], other tissues	Exosome delivery of KRAS siRNA[82], exosome delivery to CNS[159], review[83]	• Promising in vivo activity in rodent models • Manufacturing-scale and homogeneity challenges[151,152]
Nucleic acid nanostructures	Molecularly homogeneous multifunctional nanostructures, receptor-mediated endocytosis	Solid tumours[171], CNS[334], other tissues[81]	RNA nanorings and nanoparticles[81], DNA nanostructures[335]	• In vivo experiments in rodent models • Early stage of development, rapid clearance, in vivo stability and immunogenicity challenges for some structures[145] • May benefit from fully modified siRNAs

CNS, central nervous system; DsiRNA, Dicer substrate siRNA; EPR, enhanced permeability and retention; FDA, US Food and Drug Administration; GalNAc, *N*-acetylgalactosamine; NAG-MLP, GalNAc-conjugated melittin-like peptide; RNAi, RNA interference; siRNA, small interfering RNA; siRNN, short interfering ribonucleic neutral; SNA, spherical nucleic acid; TLR9, Toll-like receptor 9.

placebo arm rather than excess deaths in the revusiran arm. However, the investigation could not definitively exclude the possibility of drug-related effects.

Enhanced stability chemistries. Despite the setback with revusiran, Alnylam has continued clinical development of GalNAc–siRNA conjugates. A key reason for continued optimism is the dramatic gains in the RNAi efficiency of follow-on siRNA formulations. Since revusiran, Alnylam has announced development of two generations of siRNA ESCs[66]. ESC involves the addition of PS backbone modifications to the two 5′ terminal nucleotides on each strand of the siRNA, whereas ESC$^+$ centres on more extensive use of 2′-*O*-methyl modifications on both strands of the siRNA and the use of novel base-pair-destabilizing modifications to reduce toxicity[66] (see Related links). In preclinical and clinical studies, ESCs resulted in impressive increases in the potency of siRNA-GalNAc conjugates, enabling a corresponding reduction in administered doses by up to ten times. The dosage reduction enabled by ESCs further ameliorates concerns for toxicity.

Several Alnylam clinical candidates have reported exciting results using ESC, including a new PCSK9 inhibitor, inclisiran, and the successor to revusiran, ALN-TTRsc02 (see Related links). In late 2018, Alnylam announced positive top-line results from an interim analysis of the phase III ENVISION trial of givosiran in patients with acute hepatic porphyria and initiated a rolling New Drug Application with the FDA on the basis of the 6-month results (see Related links).

The impressive potency and safety of ligand-conjugated, metabolically stabilized siRNAs with optimized patterns of well-tolerated chemical modifications (2′-*O*-methyl, 2′-F and PS) point to an attractively simple and low-risk strategy for developing RNAi drugs.

Other fully modified RNAi triggers. Fully chemically modified, metabolically stabilized RNAi triggers with differing secondary structures and chemical modification patterns have been adopted by other leading companies in the RNAi space, including Arrowhead Pharmaceuticals, Dicerna and Silence Therapeutics.

For example, clinical trials of ARO-HBV for hepatitis B infection and ARO-AAT for liver disease associated with α1-antitrypsin (AAT) deficiency (Arrowhead) have shown that these GalNAc-conjugated, subcutaneously administered siRNAs are well tolerated and produce striking reductions in HBsAg (hepatitis B surface antigen) and serum AAT levels, respectively (see Related links).

Similarly, preliminary phase I clinical trial data of DCR-PHXC (Dicerna) — a subcutaneously administered GalNAc-conjugated RNAi trigger (based on a proprietary GalXC Dicer substrate) designed to treat primary hyperoxalurias by silencing lactate dehydrogenase, an enzyme essential to oxalate production — revealed a reduction in 24-hour urine oxalate to levels close to those seen in healthy individuals. Clinical trials for a separate agent for the treatment of hepatitis B are also planned for Q1 2019 (see Related links).

In addition, SLN124 (Silence Therapeutics) — a GalNAc–siRNA targeting TMPRSS6 for the treatment of iron regulation disorders — is undergoing preclinical development. Dicerna has also developed 'generation 4' constructs with reduced use of 2′-F-modified nucleotides and optimized utilization (number and placement) of GalNAc conjugates (see Related links).

Moving beyond the liver

Although the majority of current drug candidates under clinical development target the liver, RNAi therapy is moving into other tissues using metabolically stabilized siRNAs. A reviving trend in clinical RNAi development is the use of localized delivery to circumvent limitations in systemic drug distribution.

For example, Quark Pharmaceuticals currently has multiple candidates for kidney injury[117] and eye diseases[118] in phase II and phase III clinical trials (see Related links). QPI-1002 is an siRNA targeting the *TP53* gene that is administered via IV injection to prevent acute kidney injury following cardiac surgery and delayed graft function following renal transplantation. Phase I and II clinical trials have demonstrated QPI-1002 to be well tolerated at doses up to 10 mg per kg during cardiac surgery[117]. QPI-1002 reduced the incidence, grade and duration of acute kidney injury in high-risk individuals, leading to initiation of a phase III clinical trial. Similarly, a phase I trial of QPI-1007 — a chemically modified siRNA targeting caspase 2 for the treatment of non-arteritic anterior ischaemic optic neuropathy — showed it to be well tolerated following single intravitreal injections up to 6 mg, with study participants displaying plausible signs of vision protection[119]. A phase III clinical trial has been initiated.

Table 2 | **Selected RNAi-based therapies currently in clinical trials**

Sponsor: RNAi therapy	Indication: target	Administration route	Clinical trial phase; NCT number	Comments
AIDS Malignancy Consortium/ National Cancer Institute: lentivirus vector shRNA-transduced autologous CD34$^+$ HSCs	HIV infection with lymphoma: CCR5	Ex vivo transduction, cell injection	Phase I/II; NCT02797470	Estimated primary completion date September 2019
Alnylam Pharmaceuticals/ Genzyme (Sanofi): fitusiran/ALN-AT3SC (GalNAc ESC siRNA)	Haemophilia A/ haemophilia B: antithrombin	Subcutaneous	Phase III; NCT03417245, NCT03417102, NCT03549871, NCT03754790	One patient death in phase II open-label extension; FDA clinical hold lifted in November 2017; phase III trials recruiting
Alnylam Pharmaceuticals/the Medicines Company: inclisiran/ ALN-PCSSC (GalNAc ESC siRNA)	Hypercholesterolaemia, atherosclerotic cardiovascular disease, renal impairment: PCSK9	Subcutaneous	Phase I, II, III; NCT03060577, NCT03159416, NCT02963311, NCT03705234	Phase II ORION-1 trial achieved reduction in low-density lipoprotein cholesterol up to 52.6% at 180 days
Alnylam Pharmaceuticals/ Vir Biotechnologies: VIR-2218 (GalNAc ESC siRNA)	Chronic hepatitis B: all HBV mRNAs	Subcutaneous	Phase I/II; NCT03672188	Estimated primary completion date August 2020
Alnylam Pharmaceuticals: givosiran/ALN-AS1 (GalNac ESC siRNA)	Acute intermittent porphyria: ALAS1	Subcutaneous	Phase I, I/II, III; NCT02949830, NCT03338816, NCT02240784, NCT03547297	Positive interim results of phase III ENVISION trial, New Drug Application initiated
Alnylam Pharmaceuticals: cemdisiran/ALN-CC5 (GalNAc ESC siRNA)	Atypical haemolytic uraemic syndrome: complement component 5	Subcutaneous	Phase II; NCT03303313	Terminated owing to lack of enrolment
Alnylam Pharmaceuticals: lumasiran/A16ALN-GO1 (GalNAc ESC siRNA)	Primary hyperoxaluria: hepatic glycolate oxidase	Subcutaneous	Phase I/II, II, III; NCT02706886, NCT03350451, NCT03681184	Appears well tolerated in phase I and II trials, 50% of patients achieved plasma oxalate levels within normal range
Alnylam Pharmaceuticals: vutrisiran/ALN-TTRsc02 (GalNac ESC siRNA)	hATTR: transthyretin mRNA 3' untranslated region	Subcutaneous	Phase III; NCT03759379	Phase I well tolerated, 83% mean knockdown of TTR
Arbutus Biopharma Corp.: ARB-1467 (LNP siRNA combo)	Chronic hepatitis B: all HBV RNA transcripts	Intravenous	Phase II completed; NCT02631096	Phase IIb trial showed average HBsAg reduction of 1.4 $\log_{10}$
Arrowhead Pharmaceuticals: ARO-AAT (GalNAc ligand siRNA)	α1-Antitrypsin deficiency liver disease: α1-antitrypsin	Subcutaneous	Phase I; NCT03362242	93% maximum AAT reduction at 6 weeks following single dose, no severe AEs up to 300 mg single dose
Arrowhead Pharmaceuticals: ARO-HBV (GalNAc ligand siRNA)	Hepatitis B: HBV mRNAs	Subcutaneous	Phase I, II; NCT03365947	100% of patients achieved >1 $\log_{10}$ reduction in HBsAg; well tolerated up to 400 mg q4w
Boston Children's Hospital: lentivirus vector shRNA-transduced autologous CD34$^+$ HSCs	Sickle cell disease: BCL-11a	Ex vivo transduction, cell injection	Phase I; NCT03282656	Estimated primary completion date February 2020
City of Hope Medical Center/ National Cancer Institute: lentivirus vector shl-transduced haematopoietic progenitor cells	HIV infection with lymphoma: CCR5	Ex vivo transduction, cell injection	Phase I; NCT00569985	Estimated primary completion date July 2019
Dicerna Pharmaceuticals, Inc.: DCR-PHXC (GalNAc GalXC)	Primary hyperoxaluria: hepatic glycolate oxidase	Subcutaneous	Phase I; NCT03392896	Appears well tolerated, most patients reach normal circulating oxalate
Gradalis, Inc. and collaborators: DNA vector shRNA-transduced autologous tumour cells	Ewing sarcoma, advanced gynaecological cancer, stage III/IV ovarian cancer: furin	Ex vivo transfection, cell injection	Phase I, III; NCT03495921, NCT03073525, NCT02725489, NCT02511132, NCT02346747	Phase II showed improved progression-free median survival (8.2 months) versus historical median (<3 months)
Gradalis, Inc.: pbi-shRNA EWS/FLI1 lipoplex DNA vector	Ewing sarcoma: EWS–FL1 fusion mRNA	Intravenous	Phase I; NCT02736565	Estimated primary completion date January 2019
M. D. Anderson Cancer Center: mesenchymal-stromal-cell-derived exosomes with KRAS G12D siRNA	Pancreatic cancer: KRAS G12D	Intravenous	Phase I; NCT03608631	Registered 1 August 2018, not yet recruiting

Table 2 (cont.) | **Selected RNAi-based therapies currently in clinical trials**

Sponsor: RNAi therapy	Indication: target	Administration route	Clinical trial phase; NCT number	Comments
M. D. Anderson Cancer Center: EPHA2-targeting DOPC-encapsulated siRNA	Advanced solid tumours: EPHA2	Intravenous	Phase I; NCT01591356	Estimated primary completion date July 2020
MiNA Therapeutics: LNP MTL-CEBPA saRNA	Hepatocellular carcinoma/liver cancer: activation of CEBPA	Intravenous	Phase I; NCT02716012	Preliminary data show it to be well-tolerated, mediate RNA activation in white blood cells and evidence of antitumour activity
miRagen Therapeutics, Inc: cobomarsen/MRG-106 (LNA anti-mir)	Lymphoma and leukaemias: miR-155	Intravenous/ subcutaneous	Phase I; NCT02580552	Generally well tolerated, 52% of patients achieved partial response in tumour burden
miRagen Therapeutics, Inc.: MRG-110 (LNA anti-mir)	Heart failure: miR-92	Local administration	Phase I; NCT03603431	Estimated primary completion date February 2019
miRagen Therapeutics, Inc.: MRG-201 (miRNA mimic)	Keloids: miR-92	Intradermal injection	Phase II; NCT03603431	Estimated primary completion date August 2019
Olix Pharmaceuticals, Inc.: OLX10010 (asymmetric siRNA)	Hypertrophic cicatrix	Subcutaneous/ intradermal	Phase I; NCT03569267	Estimated primary completion date May 2019
Peking University/Marino Biotechnology Co., Ltd.: shRNA-modified CAR-T cells	Relapsed or refractory B cell lymphoma: PD-1	Ex vivo transduction, cell injection	Phase I; NCT03208556	Estimated primary completion date June 2019
Phio Pharmaceuticals, Corp.: RXI-109 (A45sd-RxNA)	Hypertrophic scar: CTGF	Intradermal	Phase II; NCT02246465	Well tolerated, reduced recurrence of hypertrophic scar formation in some cohorts
Phio Pharmaceuticals, Corp.: RXI-109 (sd-RxNA)	Age-related macular degeneration, retinal scarring: CTGF	Intravitreal	Phase I, II; NCT02599064	Estimated primary completion date April 2018
Quark Pharmaceuticals: QPI-1002 (siRNA)	Cardiac surgery: kidney expression of p53	Intravenous	Phase III; NCT03510897	Phase II demonstrated protection against acute kidney injury
Quark Pharmaceuticals: QPI-1007 (siRNA)	Non-arteritic anterior ischaemic optic neuropathy: caspase 2	Intravitreal	Phase II, III; NCT02341560	Single intravitreal injections well tolerated up to 6 mg, showed plausible vision protection
Regulus Therapeutics Inc./ Genzyme (Sanofi): RG-012 (anti-mir)	Alport syndrome: miR-21	Subcutaneous	Phase I, II; NCT02855268, NCT03373786	Phase II trial suspended owing to business restructuring, will continue under Sanofi
Shanghai Public Health Clinical Center/Kanglin Biotech (Hangzhou) Co. Ltd.: lentiviral vector shRNA-transduced $CD34^+$ cells	HIV infections/AIDS: CCR5 and HIV mRNAs	Ex vivo transduction, cell injection	Phase I; NCT03517631	Estimated primary completion date July 2018
Silenseed Ltd: siG12D-LODER polymer matrix siRNA	Pancreatic ductal adenocarcinoma/ pancreatic cancer: mutant *KRAS* (NCT03517631)	Intratumoral	Phase II; NCT01676259	Data in phase I suggest median overall survival may be improved versus gemcitabine
Sirnaomics: STP705 peptide nanoparticle siRNA	Hypertrophic scar: TGFβ1 and COX2	Intradermal	Phase I, II; NCT02956317	Investigational New Drug application approved and granted orphan designation for advanced cholangiocarcinoma
Sylentis, S.A.: SYL1001 siRNA solution	Dry eye disease: TRPV1	Topical eye drop	Phase III; NCT03108664	Phase I, II demonstrated reduced pain and conjunctival hyperaemia, agent well tolerated
Wake Forest University Health Sciences/National Cancer Institute: APN401 (siRNA-transfected peripheral blood mononuclear cells)	Various solid tumours: CBLB silencing in peripheral blood mononuclear cells to increase antitumour activity	Ex vivo transfection, cell injection	Phase I; NCT03087591	Estimated primary completion date March 2019

AAT, α1 antitrypsin; AEs, adverse effects; CAR, chimeric antigen receptor; CEBPA, CCAAT/enhancer-binding protein-α; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphatidylcholine; ESC, enhanced stability chemistry; FDA, US Food and Drug Administration; GalNAc, *N*-acetylgalactosamine; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; HSC, haematopoietic stem cell; hATTR, hereditary transthyretin amyloidosis; LNP, lipid nanoparticle; PD-1, programmed cell death 1; q4w, dosing once every 4 weeks; RNAi, RNA interference; saRNA, small activating RNA; shRNA, short hairpin RNA; siRNA, small interfering RNA; TTR, transthyretin.

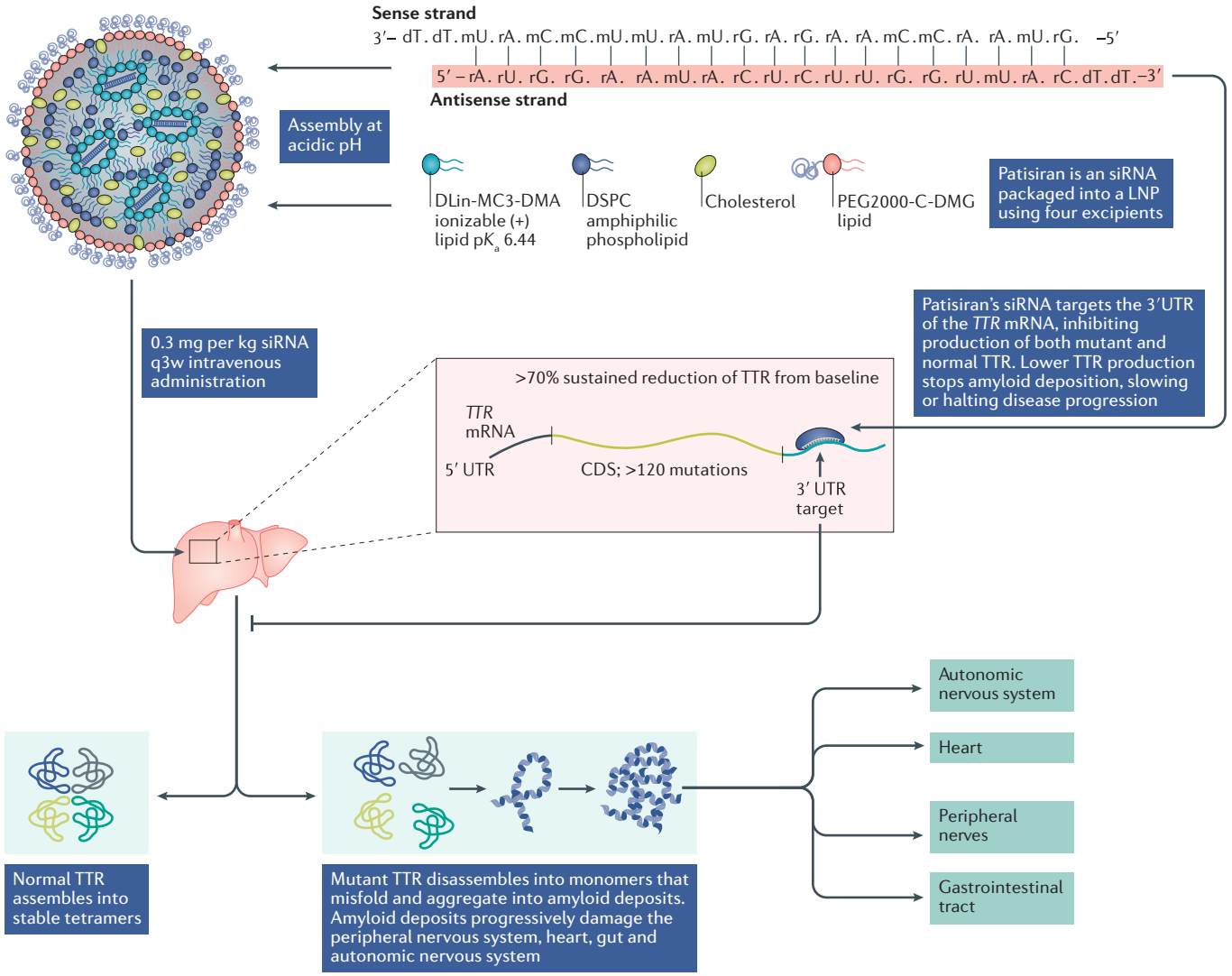

Fig. 4 | **The therapeutic mechanism of patisiran.** Patisiran consists of the small interfering RNA (siRNA) shown in complex with lipid excipients. The components are assembled under acidic pH into lipid nanoparticles (LNPs) and injected intravenously once every 3 weeks (q3w) at dosages of 0.3 mg per kg. The siRNA targets the 3′ untranslated region (UTR) of the *TTR* gene, which encodes transthyretin, to silence all possible mRNAs with coding region mutations. RNA interference (RNAi) silencing results in sustained >70% reductions of circulating TTR proteins, effectively stopping deposition of TTR amyloids. For the siRNA, 'm' = 2′-*O*-methyl-modified bases, 'r' = RNA and 'd' = DNA. Adapted from REF.[336] and with permission from Alnylam Pharmaceuticals.

In addition, Alnylam recently reported positive preclinical findings in non-human primates for CNS-targeted RNAi drug candidates and expects IND filings by 2020. Although specific programme details have not been released, the Alnylam 2018 Research and Development Day presentation mentions ALN-APP as a new agent targeting the amyloid precursor protein (APP) for the treatment of hereditary cerebral amyloid angiopathy (see Related links and TABLE 2). Injection into the cerebrospinal fluid via lumbar puncture is expected as the means of delivery for these CNS-targeted drugs (see Related links).

Lessons learned in drug development

The advances and setbacks in the preclinical and clinical development of RNAi drugs have led to increasing maturity and sophistication in RNAi drug development processes. Key lessons learned include the benefits of empirical testing and continuous evolution of dianophores, the importance of using the correct animal models to predict safety and drug activity and the need to minimize the complexity and toxicity of excipients in order to simplify manufacturing, storage and clinical testing and decrease the risk of significant adverse events.

Although there are a deceptively low number of commonly used base and backbone chemistries for RNAi triggers, the presence of ≥20 modifiable nucleotides in each strand of the RNAi trigger leads to an astronomical number of possible modification patterns. The development of generations of ESCs by Alnylam and the corresponding gains in clinical potency and safety show the power and potential of evolutionary development through empirical screening. With additional RNAi-compatible base and backbone chemistries emerging, the development of dianophores will continue unabated.

Endosomolytic
Disrupts the integrity of the endosomal membrane, leading to membrane rupture.

When carrying out empirical testing, the importance of using the correct in vitro and in vivo models for accurate prediction of toxicity and potency cannot be overstated. Different model organisms can have differing reactions to oligonucleotides and excipients. A particular problem with RNAi drugs is that their toxicity and activity depend largely on sequences present or absent in the transcriptome of the animal model. An siRNA that elicits no off-target effects in mice could well have intolerable off-target RNAi activity in humans. Conversely, an siRNA that works well in silencing a mouse gene can have little activity against the human gene, which may also have differing functions. Adding to these complications, for some indications there may be no available rodent disease models with sufficient target homology with humans. This then requires careful selection of animals used in the preclinical development process. For example, Alnylam has used non-primate models such as rodents for platform-wide testing of chemistry-related toxicity but has found the cynomolgus monkey more viable for testing pharmacological-related toxicity and potency owing to better genomic homology with humans (see Related links).

Other important considerations are the complexity, uniformity, stability and toxicity of excipients such as nanoparticles, polymers, peptides and proteins. Lipid and polymer nanoparticles have been widely used to improve pharmacokinetic properties, but they can be challenging to manufacture, and the resulting products can often have some degree of heterogeneity in particle composition, particle properties and drug loading, making it more difficult to establish therapeutic windows during clinical development[12]. Furthermore, particles can become unstable during storage[12] or after administration[120] and release breakdown products that can then cause toxicities that are difficult to trace[12]. Similarly, although endosomolytic excipients such as melittin[84,121] can substantially improve the endosomal escape of RNAi agents, they can also be potentially very toxic. In 2016, Arrowhead reported that their EX1 excipient, a GalNAc-conjugated, melittin-like, polyethylene glycol masked endosomolytic peptide (a version of Arrowhead's dynamic polyconjugate (DPC) platform[78] known as NAG-MLP) caused the death of several non-human primates when administered at high doses in a safety study (see Related links). Although the exact cause of the trial animal deaths was not disclosed, clinical development of three drugs using EX1 — ARC-520, ARC-521 and ARC-AAT (see Related links) — was discontinued despite promising initial clinical trial results[122].

Addressing ongoing challenges

Despite the advances in clinical RNAi drug development, there is still much room for further improvements in pharmacokinetics, pharmacodynamics and strategies to limit toxicity. Towards these ends, there has been continuing progress in well-established technologies such as polymer nanoparticles or LNPs[123,124], aptamers[87], molecular ligands[91] and oligonucleotide base and backbone modifications[41]. In addition, there are novel approaches that could change the existing paradigms in systemic and local RNAi delivery and RNAi payload specificity and safety. Below, we highlight some of these emerging technologies in the context of their application areas.

Improving endosomal escape

Endosomal escape is a major barrier to the expansion of RNAi-based therapeutics beyond the liver. Recent reviews of the topic[51,125,126] have noted previous work and analysis suggesting a passive siRNA escape rate of <0.01% and a requirement for approximately 2,000 (REF.[73]) to 5,000 (REF.[51]) cytoplasmic siRNAs for maximum target knockdown, and have highlighted the futility of reaching a therapeutic threshold of siRNAs in the cytoplasm given that most surface receptors are expressed in the range of 10,000–100,000, or less, with receptor recycling durations of ~90 minutes[97]. However, the ASGPR is an obvious exception, with hepatocyte expression levels of ~500,000, or higher, and a recycling rate of <20 minutes[127]. Given this, it is reasonable to anticipate that enough GalNAc–siRNA conjugates will accumulate in the cytoplasm of a hepatocyte to reach therapeutic levels during treatment. Although this bodes well for future RNAi-based therapeutics targeting the liver, it still leaves the escape problem unsolved for other cell types. Attempts at using endolytic agents such as chloroquine[128], similar agents[129] or pore-forming peptides such as melittin[121] and DPC and/or NAG-MLP[78,84] have thus far failed to adequately uncouple cytotoxicity from enhanced endosomal escape. The setbacks with the DPC platform were particularly disappointing, as prior reports pointed to reduced toxicity from the innovative use of acid-labile linkers to

Box 3 | Phase III clinical trial for patisiran

The phase III, double-blind, placebo-controlled clinical trial (APOLLO) for patisiran began recruiting in December 2013 (REFS[93,104]). 225 patients with hereditary transthyretin amyloidosis (hATTR) with polyneuropathy were enrolled at 44 sites in 19 countries, with a 1:2 randomization placing 77 patients in the placebo arm and 148 patients in the patisiran arm.

Study patients received an intravenous injection every 3 weeks of 0.3 mg per kg (small interfering RNA) patisiran or placebo over 18 months. Both groups received pre-medication before each injection to attenuate infusion reactions.

Over the course of the trial, the patisiran group achieved >70% sustained reduction in transthyretin (TTR) from baseline and substantial improvements versus placebo occurred for both primary and secondary end points[93].

On the primary end point of modified neuropathy index score +7 (mNIS +7; range 0 to 304, higher score indicates more impairment), the least-squared mean change from baseline at 18 months was –6.0 ± 1.7 in the patisiran group versus 14.4 ± 2.7 in the placebo group ($P < 0.001$). 56% of patients in the patisiran group had improvements in mNIS + 7 versus just 4% in the placebo group[93].

On the secondary end point of Norfold Quality of Life–Diabetic Neuropathy questionnaire (Norfold QOL-DN; range –4 to 136, higher scores indicate worse quality of life), the patisiran arm achieved a least-squared mean change from baseline at 18 months of –6.7 ± 1.8 (patisiran) versus 14.4 ± 2.7 (placebo) ($P < 0.001$). 51% of patients in the patisiran arm reported an improvement in the quality of life versus just 10% in the placebo arm[93].

Study patients with cardiomyopathy (36 in placebo and 90 on patisiran) showed statistically significant ($P < 0.02$) improvements in cardiac structure and function.

Patisiran also showed good safety, with patients in the patisiran arm reporting similar incidences of severe (28%) or serious (36%) adverse events as the placebo arm (36% and 40%, respectively). Patisiran treatment was not associated with a higher risk of death. The most common adverse effects that occurred more frequently in the patisiran arm were peripheral oedema and infusion-related reactions, which were mild or moderate in severity[93].

reversibly mask endosomolytic motifs[78] and clinical trials were well underway[122]. This suggests that agents that can cause mild or localized membrane destabilization with reduced risk of endosomolysis should be investigated. However, thus far, these technologies have shown only modest increases in endosomal escape[130].

An alternative to disrupting the endosome might be the exploitation of cellular retrograde transport[131–133], perhaps by taking inspiration from the AB_5 toxin family members, which enter a cell through endocytosis and ultimately localize to the cytoplasm[131,134]. Several reports utilizing a retrograde transport strategy claim to have achieved target gene knockdown using siRNAs or ASOs conjugated to retrieval peptide motifs in cultured cells[135–138]. Tracking of these constructs by fluorescent microscopy showed that a significant proportion localized to the targeted compartment; for example, constructs bearing the Golgi-to-endoplasmic reticulum (ER) retrieval signal 'KDEL' were found to colocalize with ER markers[135,137,138]. Interestingly, the luminal side of the ER might harbour a small yet functional pool of Dicer[139]. Therefore, this approach might benefit from swapping the conjugated 21-mer siRNA for a DsiRNA, which can then be freed from the retrieval signal. Although these ideas are currently at an early stage of development, new non-toxic approaches to endosomal escape would clearly help broaden the applications of RNAi therapy.

Antibody–siRNA conjugates

In addition to enhancing endosomal escape, the potency and safety of RNAi therapeutics could also benefit from improved systemic circulation and targeted delivery. Immunoglobulin G (IgG) antibodies are highly privileged macromolecules that have extended systemic circulation (weeks) and pervasive tissue distribution, courtesy of Fc–FcRn interactions that promote recycling and transcytosis[140]. Conjugation of drug moieties with antibodies can dramatically improve pharmacokinetics and achieve tissue-specific targeting[141]. IgG–siRNA conjugates have been under investigation for more than a decade[142], with coupling of siRNAs to antibodies achieved by non-covalent interactions with modified IgGs[141] or via covalent linkage to lysine or cysteine residues[89]. In the mid-2010s, Genentech achieved quantitative, site-specific, scalable conjugation of siRNA to IgGs via their THIOMAB platform[89]. However, potency was disappointing, with conjugation often leading to decreased or abolished RNAi activity, depending on the target antigen. Testing indicated that entrapment of IgG–siRNA within endosomal compartments was an important limiting factor.

Recently, Avidity Biosciences (see Related links) announced an antibody–siRNA conjugate that purportedly achieved >90% knockdown of myostatin mRNA in unspecified muscle tissues in vivo after a single IV dose. Although there is not yet a peer-reviewed publication reporting the details of this research, a 2017 Avidity patent filing[143] disclosed moieties comprising antibodies conjugated to different combinations of metabolically stabilized siRNAs and endosomal-escape-enhancing peptides[144,145], notably INF7 (REF.[146]), a fusogenic peptide, or melittin[121,147], a cell-membrane-disrupting peptide. If the announced results are confirmed and the patent disclosure is indeed relevant, then the combination of siRNAs, metabolically stabilized siRNAs[91] and endosomal-escape-enhancing excipients may prove a viable strategy for antibody-based delivery of siRNAs to non-liver tissues. Such moieties could also take advantage of recent advances in cleavable linker chemistries[141], such as a newer generation of pH-sensitive linkers with greater stability near-neutral (pH 7.4) and faster dissociation at endosomal (pH 5.5) pH[148,149]. Avidity's IgG–siRNA conjugates are currently in discovery-stage development for Duchenne muscular dystrophy and myotonic dystrophy type 1.

Improving potency with hydrophobic siRNAs

The increasing potency of metabolically stabilized siRNAs is also contributing to renewed interest in siRNAs with hydrophobic modifications (for example Phio's sd-rxRNAs[23]). Recent reports have elucidated specific motifs for improving the potency of cholesterol-conjugated PS-modified self-delivering siRNAs[62] and have demonstrated the superior potency of fully modified siRNAs in this application[63]. Fully modified anti-soluble FMS-like tyrosine kinase 1 (FLT1; also known as VEGFR1) siRNAs with hydrophobic modifications (a conjugated cholesterol at one end and a short PS-modified single-stranded RNA overhang at the other) exhibited widespread tissue distribution, significant placental accumulation and efficient target silencing when administered systemically to pregnant mice[150]. Furthermore, in a baboon model of preeclampsia, a single siRNA injection modulated sFLT1 serum levels and reduced hypertension and proteinuria. Although the injected dose of siRNAs was still fairly high, the wide tissue distribution and potent RNAi silencing from an untargeted, systemically administered siRNA is exciting, and recent reports suggest that versions with non-cholesterol lipid conjugates could have applications in the CNS[151,152] and other non-liver tissues[153]. Because testing has already shown favourable activity in non-human primates, hydrophobic siRNAs may soon enter preclinical development.

Stereoselective phosphorothioate synthesis

A possible drawback of the increasing use of PS modifications in metabolically stabilized siRNAs and hydrophobically modified siRNAs is that each PS modification introduces a stereo centre with two possible chiral orientations. Thus, an oligonucleotide with n number of PS modifications is a mixture of $2n$ racemers. The two orientations have meaningfully different pharmacokinetic-related and pharmacodynamic-related properties: whereas *Sp*-oriented PS linkages provide better resistance to nuclease cleavage, they also tend to reduce the base-pairing T_m of the flanking base compared with *Rp*-oriented linkages[58]. Because molecular heterogeneity is often detrimental to clinical development[12], future RNAi agents may well benefit from recently developed technologies for stereoselective synthesis of PS-modified oligonucleotides[58].

Reducing the toxicity of LNPs

Despite the prevalence of metabolically stabilized RNAi agents, the success of patisiran suggests that there could still be applications for LNPs. However, the toxicity

Fusogenic
Induces the fusion of lipid vesicles. These are typically less disruptive of endosomal membranes than endosomolytic agents.

of ionizable lipid excipients currently limits maximum tolerable dosages and causes concern in scenarios that require long-term use. To circumvent these issues, researchers are attempting to facilitate the metabolic breakdown of ionizable lipids by incorporating ester groups into their hydrophobic tails. An ionizable lipid (L-319; Alnylam) with symmetric enzyme-cleavable ester-linked alkyl tails was reported to accelerate clearance from tissues, reduce toxicity and maintain in vivo potency in murine models[154,155]. However, L-319 was later found to exhibit lower particle stability in storage and reduced potency in non-human primates compared with DLin-MC3-DMA, and variants with branched tails that show improved stability and potency in non-human primates (L-369) are now being investigated (see Related links). An alternative approach involving an asymmetric ionizable lipid with a cleavable ester in the longer tail was reported to be well tolerated, and injection of an anti-PCSK9 siRNA using this formulation led to 90% protein silencing in cynomolgus monkeys that was maintained at ~50% at the 60-day time point[156]. Although current RNAi drug pipelines are dominated by fully modified unencapsulated RNAi agents, the FDA approval of patisiran and the potential gains in safety and potency with newer-generation lipids will likely spur further interest. This may be especially true for non-liver, systemically administered applications, where higher tolerable doses could translate into better efficacy. Thus, biodegradable ionizable lipids may enter into preclinical development in the next 2–5 years.

Using exosomes for systemic siRNA delivery

Although rapid tissue clearance is a desirable feature of excipient chemicals, increasing the circulation half-life and widening the tissue distribution of intact nanoparticles could substantially improve RNAi potency. Recently, exosomes have shown considerable promise as vehicles for systemic RNAi delivery. Exosomes are natural nanoparticles produced by endogenous cells for transport of cargo such as microRNA (miRNA) to distal tissues[157,158]. Synthetic siRNAs can be transfected into exosomes for delivery to target tissues[82,151,159]. The key advantage is that surface expression of CD47 (REF.[82]) and other endogenous signalling ligands on exosomes can increase systemic circulation half-life by inhibiting MPS clearance and improving cellular uptake[83]. Exosomes produced using cGMP methods have successfully delivered anti-KRAS siRNA to metastasized pancreatic cancer tumours in mice, significantly increasing overall survival[82].

These biological nanoparticles may thus offer a less toxic and more potent alternative to synthetic nanoparticles in systemic delivery applications. However, exosomes face considerable challenges in manufacturing scale-up and particle heterogeneity[152]. Although some researchers are perfecting cGMP manufacturing methods[151], others are investigating the viability of taking exosomal components and reforming them into well-controlled synthetic nanoparticles[160].

Currently, Codiak Biosciences is undertaking preclinical development of exosome-delivered siRNAs for immune-oncology and autoimmune targets in macrophages (see Related links), and the M. D. Anderson Cancer Center has registered a phase I clinical trial testing an exosome-delivered anti-KRAS siRNA against pancreatic cancers (TABLE 2).

Enhancing local delivery

With the intrinsic difficulties of systemic delivery, localized delivery of RNAi payloads may be a better option for many applications. One key challenge is retention of RNAi agents within a local area for extended release. Recent research with layer-by-layer electrostatic assemblies[101,102] and injectable, biodegradable hydrogels[154,155,161] shows promise in the extended local release of siRNAs for therapeutic effects. These approaches may be especially applicable to wound healing and tissue regeneration[101,102,162], although current efforts are still at the discovery stage.

For topical applications to mucosal membranes, a major problem is that mucus has a dense polymer gel structure that can entrap nanoparticles and macromolecules, preventing homogeneous drug distribution and effective interactions with mucosal membrane cells. Recent developments show that nanoparticles with appropriate charge-neutral hydrophilic surface coatings can penetrate mucus and effectively reach underlying tissues[163–165]. This work is currently an area of active academic research that may substantially improve RNAi delivery to lung[165], gastrointestinal tract[166] and other tissues[164].

Nucleic acid nanostructures

A major barrier to the clinical success of multifunctional nanoparticles in systemic and localized applications has been the intrinsic chemical and physical heterogeneity of such nanoparticles[12,152]. Recent developments in DNA[144,145,167,168] and RNA[81,169–171] nanotechnology offer the ability to assemble siRNA-carrying nanostructures with exact molecular composition, well-defined shape and size and precise control over the number, type, pattern and even orientation[169] of surface ligands. In addition, nucleic acid nanostructures can be designed to react dynamically to local biochemical signals in order to deliver drugs to specific targets[172,173]. Both RNA[81] and DNA[174] nanostructures have been used to deliver siRNA in vivo. However, major hurdles remain. Nucleic acids have highly negative backbone charges that could accelerate clearance from systemic circulation[175]. Unmodified DNA and RNA are vulnerable to serum nuclease degradation and can induce immunogenic toxicity[145]. Large DNA nanostructures, such as DNA origami[176], need to use hundreds of component DNA strands that vastly increase chemical complexity, and some structures may not be sufficiently thermodynamically stable in the salt and temperature conditions found in serum[177]. However, efforts are underway to understand and resolve many of these problems by improving the thermodynamic stability of nucleic acid nanostructures via crosslinking[178], modifying nucleic acids to reduce toxicity and increase nuclease resistance[41,145,179] and adding protecting adjuvants such as lipid-based[180] and peptide-based[177] coatings. These efforts may ultimately result in molecularly well-defined, multifunctional nanostructures for RNAi delivery. For now, this technology is still at the academic research stage.

Reversal of RNAi activity

Because many recently developed RNAi drugs have effects lasting weeks after a single administration, there is some need for agents to rapidly reverse RNAi activity in case of adverse events. New research shows that 9 nt LNA-modified ASOs fully complementary to the seed region of guide strands[181] can reverse siRNA activity in murine models after SC administration by engaging guide strands within mature RISCs. Although such antagonists against specific RNAi drugs would likely each require FDA approval, this costly process may be viable for RNAi drugs used in large patient populations, such as PCSK9 inhibitors[64,181], and may enter preclinical development in the next 2–5 years.

Conditional RNAi activity

Although anti-guide-strand ASOs offer improved ability to control the duration of RNAi activity, emerging developments in the field of dynamic nucleic acid nanotechnology[144,182,183] may offer new ways to restrict the activity of RNAi agents to specific populations of cells. Since the early 2000s, researchers have sought to use nucleic acid logic switches to sense RNA transcripts (such as mRNAs and miRNAs) and output oligonucleotide therapeutics in mammalian cells exhibiting specific gene expression signatures[184–186]. Recent work shows that chemically modified nucleic acid switches can sense mRNAs in mammalian cells[187], and we and others have long proposed schemes for turning such switches into conditionally activated RNAi triggers[188–193]. Such riboswitches could open up new paradigms for RNAi therapy by making it easier to restrict RNAi silencing to very specific populations of disease-related cells. In turn, the activated RNAi triggers could conceivably target critical endogenous genes to manipulate cell function or cell fate. To date, there is not yet a published system that can reliably couple cellular RNA inputs to RNAi outputs in mammalian cells. However, recent papers have demonstrated the detection of mRNAs by strand displacement switches in live mammalian cells[187] and signal transduction from mRNA input into siRNA output in human cell lysate[194]. These and other developments[192] suggest that enabling advances may be imminent.

Alternative preclinical models

A key complication in discovery-stage and preclinical testing of RNAi therapeutics is that non-primate model organisms tend to have insufficient genomic sequence overlap with humans to predict pharmacodynamic effects (see Alnylam presentations in Related links). This insufficient overlap necessitates the expanded use of non-human primate models, increasing development cost, risk and ethical considerations.

This problem might be ameliorated if human tissues could be grown and cultured in in vitro environments that better recapitulate the tissue organization, biochemical signals and mechanical stimuli present in real human organs. Recent advances in the generation of organoids[195,196] and organ-on-a-chip technologies[197,198] are providing opportunities in this direction. Organoids are self-organized collections of cells that recapitulate features of human organs in miniature. They are usually formed from pluripotent stem cells or isolated organ progenitors using 3D cell culture techniques and culture media with appropriate biochemical signals[195]. Their microscopic structures often closely mimic those of real human organs, suggesting that they may better predict responses to drugs. To date, organoids have been generated for a number of tissues and diseases[199], and personalized organoids can be created for individual patients[196]. In some tissues, proper cellular differentiation and functioning may require additional spatial patterning cues and dynamic stimuli (mechanical, biochemical or electrical). Specialized microfluidic chips have been developed to supply these stimuli and mimic human organs. For example, a variety of microcontact printing, soft lithography, electrical stimulation and micromechanical device techniques have been used to re-create injured heart tissues on microfluidic chips[200]. Practitioners envision connecting a number of different organ-on-a-chip platforms to simulate the physiological response of human patients[201].

A current challenge with organoids and organs on chips is that the technologies are still fairly early in development; hence, there is still insufficient knowledge regarding how well drug responses in these models will really predict in vivo responses. However, as these technologies develop, they may take on growing importance for RNAi drug developers looking to increase screening throughput and fidelity and decrease development cost, risk and complexity.

Beyond siRNA

Insights into the mechanism of miRNA-induced RNAi have given rise to synthetic miRNA therapeutics as well as constructs, collectively termed anti-mirs and block-mirs, which inhibit the activity of a specific miRNA or prevent silencing of a specific miRNA target, respectively (FIG. 5). Additional functions of short dsRNAs have also been discovered. For instance, guide RNAs generated from small dsRNAs can induce gene silencing or gene activation at the transcriptional levels in an Ago-dependent manner (FIG. 4). Maturation of the following technologies based on these discoveries is expected to greatly expand the number of disease indications treatable by future therapeutics.

miRNA mimics

miRNA mimics are synthetic versions of endogenous miRNAs[202]. They are often perfectly base-paired, chemically modified siRNAs that have the same guide strand sequence as an endogenous miRNA. When the guide strand is loaded into Ago1–Ago4, the resulting RISC begins mimicking the corresponding miRNA by modulating its many target genes, most of which are only partially complementary to the guide strand via target sites in the 3′ untranslated region[203,204]. These large numbers of targets can include transcription factors and genes coding for other miRNAs[205], leading to effects over entire gene expression networks. Thus, similar to normal miRNAs, miRNA mimics can regulate critical developmental programmes and pathways that maintain cellular identity[206,207]. Because the disruption of specific tumour suppressor miRNAs or the miRNA maturation

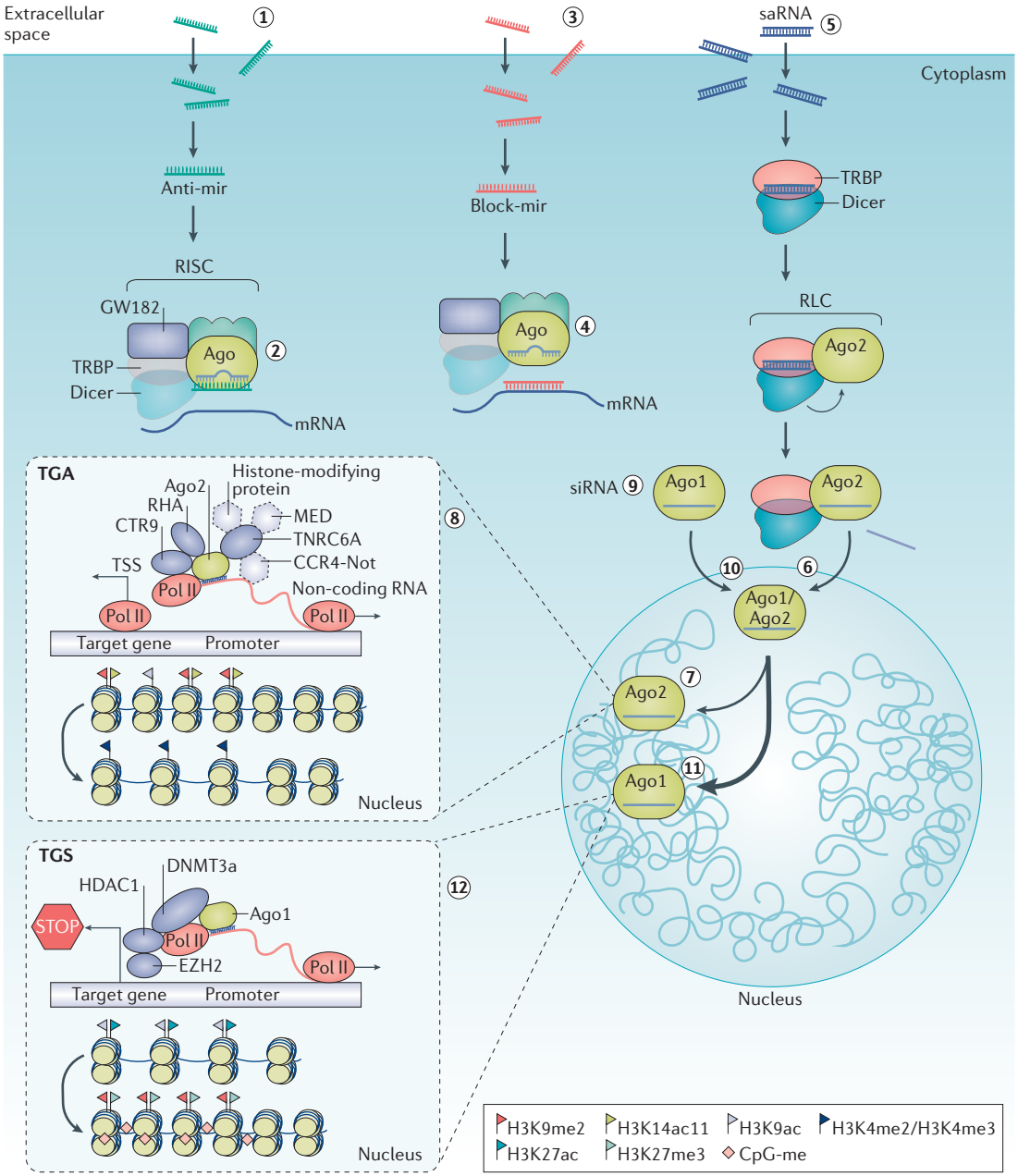

Fig. 5 | **RNAi-based therapeutics beyond siRNA.** (1) Anti-mirs enter a cell, depending on construct chemistry, by endocytosis of lipid nanoparticles, receptor binding or gymnosis. (2) The anti-mir binds to the guide strand of a microRNA (miRNA)-loaded RNA-induced silencing complex (RISC), preventing miRNA suppression of all targets of that particular miRNA. (3) Block-mirs enter a cell in a similar manner to anti-mirs. (4) The block-mir binds to a complementary sequence in a target RNA and blocks the recognition and binding of an miRNA-loaded RISC. (5) Small activating RNA (saRNA) enters a cell and is loaded into Argonaute 2 (Ago2) in a similar fashion to small interfering RNA (siRNA) (FIG. 2). (6) saRNA–Ago2 is passively entrapped in the nucleus after cell division or actively shuttled into the nucleus by import factors. (7) saRNA–Ago2 binds to a complementary chromatin-bound RNA such as promoter-associated transcripts (PROMPTs) or antisense RNA. (8) saRNA–Ago2 forms a complex of proteins (CTR9, RNA polymerase II (Pol II), RHA, TNRC6A and TNRC6A-associated CCR4-Not complex proteins, histone-modifying proteins and Mediator complex proteins) that are associated with gene transcription. Increased gene expression is correlated with increased Pol II occupancy (possibly by complex-facilitated loading of Pol II onto chromatin) and H3K4 dimethylation (H3K4me2) and/or H3K4me3 as well as decreased H3K9me2, H3K9 acetylation (H3K9ac) and H3K14ac11 at the target loci. (9) siRNA enters the cell and is loaded into Ago1 (FIG. 2). (10) siRNA–Ago1 is passively entrapped inside of the nucleus after cell division or actively shuttled into the nucleus by import factors. (11) siRNA–Ago1 binds to complementary chromatin-bound RNA such as PROMPTs or antisense RNA. (12) siRNA–Ago1 recruits a complex of proteins that are associated with transcriptional repression, resulting in lower target gene expression and correlating with increased H3K9me2, H3K27me3 and CpG methylation (CpG-me) and decreased H3K27ac and H3K9ac. DNMT3a, DNA methyltransferase 3a; EZH2, histone methyltransferase EZH2; GW182, a scaffold protein that interacts with Ago in RISC to mediate mRNA silencing; HDAC1, histone deacetylase 1; RLC, RISC loading complex; RNAi, RNA interference; TGA, transcriptional gene activation; TGS, transcriptional gene silencing; TRBP, TAR RNA-binding protein; TSS, transcriptional start site. Adapted from REF.[255].

machinery (FIG. 2) can contribute to tumour development and progression[208], miRNA mimics can potentially contribute to cancer therapy by restoring proper regulation of gene expression. This strategy can also be extended to diseases that exhibit aberrant miRNA expression profiles, including hepatitis and cardiovascular diseases[209,210]. The field of miRNA-based therapeutics, including ongoing clinical trials, has been recently reviewed[202].

miRNA inhibitors (anti-mirs)

Anti-mirs, also known as antagomirs, are chemically modified ASOs made complementary to the active strand of a target miRNA. In effect, they act as a 'decoy' miRNA target that prevents the bound miRNA from suppressing the expression of endogenous targets (FIG. 5). Anti-mirs were first described in 2005 in a study by Krützfeldt et al.[211] in which the activity of miR-16, miR-122, miR-192 and miR-194 was inhibited in mice using chemically modified, cholesterol-conjugated single-stranded RNA analogues complementary to the active strand of the target miRNA.

The hepatitis C virus (HCV) was one of the first proposed indications for anti-mir therapy[212]. In a twist on typical miRNA function, miR-122 in hepatocytes actually protects HCV from nuclease attack and recognition by the innate immune system by binding to and stabilizing the 5′ non-coding region of the viral genome[213,214]. Inhibition of miR-122 by 2′-*O*-methyl or LNA ASOs significantly lowers HCV copy number in infected cells[212] in vitro and in mice[215], non-human primates[216] and humans[203,204,217]. Examples of ASO drug candidates include miraversin[203] (Roche), a 15-mer PS and LNA modified ASO delivered gymnotically, and RG-101 (Regulus), a PS and 2′-MOE modified ASO conjugated to GalNAc[218]. Clinical development of RG-101 was halted in 2017, whereas phase II clinical trials for miraversin are ongoing. Market prospects have been considerably diminished by the breakthrough success of Gilead Sciences' ledipasvir plus sofosbuvir in curing HCV genotype 1 infection. Thus, oligonucleotide therapeutics are not entirely immune to competition from traditional small-molecule drugs[219]. Nevertheless, miraversin will serve as an important proof of concept for anti-mir therapy.

miRNA competitor (block-mir) agonist for mRNA

Block-mirs are ASOs that act by 'masking' the miRNA binding site on a specific target RNA (FIG. 5). An early example of the block-mir approach was reported in 2007 using phosphorodiamidate morpholino oligomers complementary to miR-430 binding sites of *ift2* and *sqt* transcripts in *Danio rerio*[220]. Protection of the Lefty or Squint mRNAs from miR-430 binding resulted in increased expression of the respective proteins. Currently, Mirrx, in collaboration with Biolink, is working to develop a preclinical 15-mer oligonucleotide block-mir therapeutic, CD5-2. CD5-2 is claimed to selectively increase vascular endothelial cadherin (VE-cadherin) by binding to VE-cadherin mRNA and blocking miR-27a-mediated downregulation[221]. Increased VE-cadherin expression is claimed to improve vascular perfusion and pericyte coverage and reduce permeability, hypoxia and programmed cell death ligand 1 (PD-L1) expression[221]. CD5-2 is thus intended to be used in combination with immunotherapies such as checkpoint inhibitors, adoptive T cell transfer therapies and cancer vaccines.

Block-mirs and anti-mirs are complementary technologies. The block-mir strategy is ideal when the intention is to prevent miRNA binding to only one or a few targets. Such a situation may arise when regulation of other targets by a given miRNA (for example, miR-122 in hepatocytes[222]) is otherwise beneficial or even required for homeostasis. Alternatively, when a particular miRNA is aberrantly overexpressed, it might be more appropriate to reduce the target miRNA activity via an anti-mir.

Small dsRNA-mediated transcriptional gene silencing

RNA-mediated de novo methylation was first observed in plants[223]. Six years later, it was found that dsRNA was a trigger for de novo DNA methylation, a process termed transcriptional gene silencing (TGS)[224]. The trigger was a ~23 nt dsRNA complementary to a promoter sequence[224]. Similar sized siRNA products (~25 nt) had previously been observed to play a role in PTGS in plants that were infected by potato virus X[225]. Repressed gene expression was found to be Ago-dependent and correlated with H3K9 histone methylation and DNA methylation at the promoter[226]. In *Schizosaccharomyces pombe*, small dsRNAs produced by Dicer processing and loaded into Ago1 can direct TGS by forming the RNA-induced initiation of TGS (RITS) complexes with the proteins CHP1 and TAS3 (REFS[200,201]). siRNA[227] and miRNA[228] induction of TGS also occurs in mammals, where it appears to require Ago1 (REF.[229]) and possibly Ago2 (REF.[230]). In this context, Ago1 and Ago2 serve dual functions by binding to chromatin-associated promoter transcripts and associating directly or indirectly with DNA methyltransferase 3A (DNMT3A), histone methyltransferase EZH2 and histone deacetylase HDAC1 (REFS[231–233]) (FIG. 5). Thus, Ago1 and Ago2 recruit repressive machinery to the target promoter, resulting in target-specific TGS. Mammalian TGS has recently been reviewed[234].

siRNA-induced TGS (FIG. 5) offers a unique advantage over PTGS as it shuts down transcription for prolonged periods of time through repressive epigenetic modification of a target promoter[235,236]. Genome-integrated viral genes were quickly identified as ideal targets for long-term TGS[235,237–239]. Indeed, initial in vitro results showed that siRNAs targeting the HIV 5′ long terminal repeats induced DNA methylation and subsequent long-term suppression of HIV transcription and infectivity[235]. In some cases, inhibition of infection lasted >30 days in chronically infected Magic-5 cells[235]. However, initial studies of siRNA-induced TGS in primary human $CD4^+$ T cells, although positive for inhibition, proved to not be as robustly protective against increasing viral burden as was shown in non-primary cells[237]. To improve suppression, TGS-inducing shRNAs can be constitutively expressed when transduced into cells by lentiviral vectors[238]. Although a good proof of concept, the safety of lentiviral-based gene therapy is still under investigation. As an alternative approach, an HIV gp120-specific

Transcriptional gene silencing
(TGS). Direct epigenetic silencing of a target gene's promoter induced by either small interfering RNAs or microRNAs.

Small activating RNAs (saRNAs). Short double-stranded RNAs that induce transcription of a target gene in an Argonaute 2-mediated process called RNA activation.

RNA aptamer conjugated to a TGS-inducing anti-HIV-1 DsiRNA has recently been developed. This siRNA–aptamer conjugate was shown to protect $CD4^+$ T cells from HIV infection in HIV-1-infected humanized NOD/SCID/IL2Rγ^{null} (hu-NSG) mice[86]. Ultimately, it will be interesting to see whether siRNA-induced TGS is superior to PTGS as an antiviral therapeutic strategy. Currently, this area is still being developed in academic research.

Small dsRNA-mediated transcriptional gene activation

Small activating RNAs (saRNAs) (FIG. 5) were first described by Long-Chen Li and colleagues in 2006, when they observed that dsRNAs targeting a promoter sequence activated, rather than suppressed, transcription of E-cadherin, $p21^{WAF1/CIP1}$ and VEGFA[240]. The new phenomenon was coined RNA activation (RNAa) and the short dsRNAs responsible were called 'saRNAs' to differentiate them from siRNAs. Although RNA-mediated transcriptional gene activation holds promise, activation is limited to target genes that have not undergone a loss-of-function mutation.

saRNAs and siRNAs are structurally identical to one another but differ in function. However, RNAa is exclusively dependent on Ago2 (REFS[240,241]). Unlike siRNAs, saRNAs act only in the nucleus and are designed to contain sequences homologous to regions near or within gene promoters[240,241]. In the nucleus, Ago2–saRNA binds to complementary sequences within chromatin-bound RNA transcripts and possibly to complementary DNA as well[242–248]. Surprisingly, Ago2 'slicer activity' is not required for RNAa[248,249]. Following saRNA treatment, target loci show increased levels of H3K4 dimethylation (H3K4me2) and/or H3K4me3 (REFS[240,241]) and RNA polymerase II occupancy[247,250,251], reduced levels of H3K9me2 (REF.[240]) and reduced acetylation of H3K9 and H3K14[241]. How saRNA–Ago2, in complex with binding partners, activates gene transcription is still under investigation. Current evidence suggests that Ago2 forms a structure called the RNA-induced transcriptional activation (RITA) complex with RHA and CTR9, the latter being part of the PAF1–RNA polymerase II complex[247]. TNRC6A binds tightly to nuclear Ago2 and has recently been indicated as a critical RNAa factor[252]. Nuclear TNRC6 proteins have also been shown to bind to proteins involved in histone modification, the Mediator complex and the CCR4–Not complex[252]. Knockdown of TNRC6 proteins abolishes the effect of saRNA[252]. Therefore, TNRC6 proteins might bridge Ago2–saRNA with the tentative chromatin remodelling factors involved in RNAa.

The potential for RNAa is becoming increasingly recognized. To date, >14 genes have been activated by saRNAs[253]. The first saRNA therapeutic, MTL-CEBPA[245,248,254] (see recent review[255]), entered clinical trials in 2016 for the treatment of inoperable hepatocellular carcinoma (HCC). MTL-CEBPA is a liposomal formulation of an saRNA that increases expression of the transcription factor CCAAT/enhancer-binding protein-α (CEBPA). Treatment with MTL-CEBPA in mice has been shown to lower HCC tumour burden and improve clinically relevant parameters of liver function[254]. If successful, saRNAs will provide gene-expression-activating therapeutics that complement siRNA-mediated gene-expression-suppressing therapeutics.

Conclusions

With the approval of patisiran, systemic delivery of RNAi therapeutics to the liver is now a clinical reality. Although follow-on drugs based on GalNAc-conjugated, metabolically stabilized siRNAs[91] are still in clinical development, the consistent potency and safety of these agents across diverse liver indications warrant considerable optimism, as does the progression of other RNAi candidates for ocular[118], renal[117] and CNS indications (see Related links). Many current clinical drug candidates target rare diseases, but Alnylam's PCSK9 inhibitor inclisiran and Quark's QP-1002 for kidney injury and PF-655 for wet age-related macular degeneration could affect much larger patient populations if approved.

Despite current successes, it is also clear that RNAi therapeutics could have substantially expanded impact if systemic delivery to non-liver, non-kidney tissues becomes viable in clinical settings. Key challenges include simultaneously avoiding both renal and reticuloendothelial clearance, enhancing extravasation and tissue perfusion, increasing uptake in cell types that do not have highly expressed cargo internalization receptors and improving endosomal escape[51]. Some of these problems may be amenable to approaches that work more synergistically with complex biological pathways such as those responsible for intracellular cargo sorting and trafficking following endocytosis. This would require closer collaboration between chemists and biologists than in the past to develop solutions that make use of subtle aspects of biological pathways. It may also require utilization of multifunctional excipients with the type of chemical complexity that has thus far vexed the clinical development of RNAi-carrying nanoparticles[12]. Alternatively, further development of metabolically stabilized RNAi triggers and conjugated ligands could allow effective delivery to an increasing number of extra-hepatic tissues without the need for more complex formulations. These are challenging problems, but the 20-year development history of the RNAi therapeutics field is a testament to the power of persistence. With many innovative technologies under development for both excipients and payloads, there will undoubtedly be many more breakthroughs to come.

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Competing interests
J.J.R. is a co-founder of Dicerna Pharmaceuticals and MiNA Therapeutics. S.-p.H. and J.J.R. are inventors on US patents and patent applications for conditional RNA interference-related technologies.

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