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RNAi-based drug design: considerations and future directions

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Abstract

More than 25 years after its discovery, the post-transcriptional gene regulation mechanism termed RNAi is now transforming pharmaceutical development, proved by the recent FDA approval of multiple small interfering RNA (siRNA) drugs that target the liver. Synthetic siRNAs that trigger RNAi have the potential to specifically silence virtually any therapeutic target with unprecedented potency and durability. Bringing this innovative class of medicines to patients, however, has been riddled with substantial challenges, with delivery issues at the forefront. Several classes of siRNA drug are under clinical evaluation, but their utility in treating extrahepatic diseases remains limited, demanding continued innovation. In this Review, we discuss principal considerations and future directions in the design of therapeutic siRNAs, with a particular emphasis on chemistry, the application of informatics, delivery strategies and the importance of careful target selection, which together influence therapeutic success.

Introduction

RNAi is an intrinsic post-transcriptional gene regulation mechanism that has been harnessed for therapeutic development since its discovery in *Caenorhabditis elegans* (Box 1). Double-stranded small interfering RNAs (siRNAs) can trigger the RNAi process to induce sequence-specific gene silencing. Upon cellular uptake, the guide strand of the siRNA is assembled into an RNA-induced silencing complex (RISC) capable of searching and degrading complementary mRNAs, thus aborting target protein translation. The current clinical success of *N*-acetylgalactosamine (GalNAc)-conjugated siRNAs targeting the liver, with multiple approved drugs and many clinical trials in process, required a long path of innovation. The technology progress from bench to clinic can be defined by three major milestones: animal proof of concept (POC) (statistically significant silencing of the target at an acceptable

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Competing interests

A.K. is a founder of Atalanta Therapeutics and Comanche Biopharma; serves on the Scientific Advisory Board of Aldena Therapeutics, Prime Medicine, Alltrna, Advirna and Evox Therapeutics. A.K. and Q.T. are Listed as inventors of RNAi technology patents and patent applications.

dosing and safety range in vivo); clinical POC (demonstration of robust safety and disease target modulation at a tolerable dose in humans); and regulatory approval (Table 1).

In liver, lipid nanoparticles (LNPs) were first used to deliver patisiran, currently the only siRNA drug on the market that uses this technology platform. The animal POC studies of LNP-mediated siRNA delivery were explored in the early 2000s by multiple groups^{1–7}. It took several technology generations to reach an acceptable clinical POC (2012–2013)⁸ and a few more years to attain regulatory approval (2018)⁹. The dominant platform for liver delivery today is GalNAc conjugation. The first animal POC was reported in 2014 (ref. 10), and clinical success required two reiterations of the technology. The first iteration, using Alnylam's 'standard template chemistry' (STC) platform, achieved clinical POC with revusiran¹¹, but had to be discontinued owing to unexpected toxicity in the phase III trial¹². The clinical efficacy and safety of GalNAc technology was later demonstrated in the next-generation products using the 'enhanced stabilization chemistry' (ESC) platform, in which the necessity for additional siRNA stabilization was realized. Givosiran was the first compound in the category to receive regulatory approval (2019). The recent development of an advanced ESC strategy (ESC+), which incorporates a glycol nucleic acid (GNA) modification¹³, further improved the safety profile of GalNAc–siRNAs (discussed later in 'Medicinal chemistry of siRNA design'). Aspects of the chemistry, structure and function of the currently approved siRNA drugs were recently reviewed¹⁴. More recently, the siRNA drug *nedosiran* (see Related links) – developed using a chemical stabilization concept similar to ESC but with a different GalNAc conjugation strategy (discussed in a later section) – received FDA approval for the treatment of primary hyperoxaluria type 1 by targeting lactate dehydrogenase A in hepatocytes.

So far, there have been no approved siRNA drugs to target extrahepatic tissues, but animal POC studies have demonstrated promising efficacy in multiple tissues. Several delivery technologies and strategies – for example, lipophilic conjugates, protein-antibody conjugates and manipulation of the size and structure of the siRNA molecule itself – are in development and show robust efficacy in various animal tissues with some platforms moving towards clinical translation (Table 1). For central nervous system (CNS) and muscle, efficacy has also been achieved in humans. In the near future, some technologies that have shown robust animal data might be translated into the development of drug classes in these tissues. The RNAi drug development field is very active and rapidly advancing. Every aspect of the diverse preclinical and clinical evaluations cannot be covered here, and much of the mechanistic details of RNAi biology and RNAi therapeutics have been reviewed elsewhere¹⁵. Instead, this Review will assess fundamental aspects of siRNA drug development – siRNA chemistry, the application of informatics, delivery strategies and target and indication selection – that may encourage future innovation and clinical translation of RNAi-based drugs.

Medicinal chemistry of siRNA design

Advances in nucleic acid chemistry have greatly accelerated the clinical development of oligonucleotide-based drugs. These approved products encompass a range of therapeutic modalities, including antisense oligonucleotides (ASOs), splice-switching oligonucleotides

(SSOs), aptamers and siRNAs¹⁴. Although these modalities use various mechanisms of target recognition and gene modulation, their molecular configurations highlight the importance of chemical modifications in improving drug-like properties and long-term durability of oligonucleotides.

For siRNAs, initial biodistribution is usually completed within a few hours after injection. The first obstacle for productive delivery is the metabolic degradation and clearance during absorption and circulation. With sufficient accumulation in target tissues, cellular uptake of siRNA occurs primarily through membrane endocytosis. Internalized compounds become entrapped within endosomal and lysosomal compartments, creating an intracellular drug depot that gradually releases siRNA into the cytoplasm for RISC loading, offering prolonged durability on target silencing (Fig. 1). Endosomal escape is a rate-limiting step in the onset of silencing¹⁶, but slow release is the basis for the multi-month durability of GalNAc-conjugated siRNA drugs in the clinic. Strategies have been explored to improve the endosomal escape rate¹⁷; successful strategies might be advantageous for siRNA therapies designed to treat viral infections or cancers that require a rapid onset of silencing. For therapies in which only short-term RNAi activity is desired, designing partially modified compounds with degradable chemical composition is straightforward.

Necessity for chemical modifications

Unmodified siRNAs exhibit poor metabolic stability and are susceptible to immediate degradation (>50% within a minute) *in vivo*¹⁸. Double-stranded siRNAs can also trigger innate immune responses¹⁹ and cause off-target effects²⁰. These issues were largely resolved by introducing certain 2'-ribose and terminal backbone modifications, including 2'-*O*-methyl (2'-OMe), 2'-fluoro (2'-F) and phosphorothioate (PS)²¹ (Fig. 2a). These three modifications – now used in nearly all advanced siRNA designs – were originally applied in the early 1990s to enhance drug-like properties of ASOs (for example, fomivirsen, approved in 1998)^{22,23} and aptamers (for example, pegaptanib, approved in 2004)²⁴. Nevertheless, nucleotide modifications were not a primary focus in the initial development of siRNA therapeutics. In hindsight, it is perhaps unsurprising that early trials using unmodified or slightly modified siRNA compounds yielded limited efficacy and dose-limiting toxicity.

For an extended period, the prevailing approach – LNP-mediated delivery – involved only partial modification of siRNAs to improve stability²¹. Partially modified compounds (for example, patisiran) are fully functional in the context of LNP formulation, driven by the mechanism of fast internalization and endosomal release of lipid-encapsulated drug molecules. Lipid formulations protect siRNA from nuclease degradation and enable faster onset of silencing, but when being used to deliver partially modified compounds they have limited durability *in vivo*. For conjugate-mediated delivery to achieve multi-month durability without LNP formulation, compounds must survive prolonged exposure to the highly aggressive, nuclease-rich endosomal and lysosomal environments²⁵. Extensive modification of the siRNA scaffold is therefore essential for long-term stability. Indeed, full stabilization of the scaffold by incorporating modifications at every nucleotide position and several terminal backbone modifications profoundly improves siRNA accumulation and potency in tissues²⁶.

Basic scaffold stabilization

To prevent metabolic degradation during tissue distribution and intracellular entrapment, scaffold optimization is required to modulate siRNA interactions with cellular processes, mostly 2'-hydroxyl (2'-OH)-mediated hydrolysis, endonucleases, as well as 5' and 3' exonucleases. Various chemical strategies were developed early for oligonucleotide-based therapeutics, including modifications of nucleobases, ribose sugars and backbones¹⁴. Studies revealed that a selectively small set of RISC-compatible modifications (2'-OMe, 2'-F and PS) are sufficient to confer nuclease resistance on the siRNA duplex^{18,27-29} and can also reduce immunogenicity and off-targeting effects. 2'-OMe and 2'-F modifications reduce 2'-OH vulnerability, while maintaining the pre-organized A-form helix of the guide strand, which is necessary for successful RISC recognition and loading³⁰. 2'-OMe and 2'-F also enhance the affinity of target interactions, likely owing to more favourable pre-organization of the backbone in a 3'-endo conformation³¹.

The bulky 2'-OMe is generally well tolerated, but its effect on activity depends in part on sequence and position. For example, 2'-OMe at positions 2 and 14 of the guide strand consistently reduces activity³². The smaller 2'-F is tolerated in most positions of an siRNA; moreover, 2'-F enhances hydrophobicity and improves siRNA cellular uptake and potency^{28,33}. Simultaneously, because 2'-F is not a natural modification, there was some concern that it might be incorporated into mitochondrial DNA by polymerases and cause toxicity. The exact cause of clinical toxicity of [revusiran](#) (see Related links) – the first generation of STC GalNAc-conjugated siRNA – during the phase III trial remains unknown. Detailed toxicology studies provide proof that long-term administration of 2'-F modified siRNAs is safe³⁴. Although the levels of detectable 2'-F metabolites were significantly higher in revusiran than in its next-generation ESC compound, the only differential structural finding was enlarged mitochondria in hepatocytes and certain myocytes. Although the levels of detectable 2'-F degradation products were marginal, the hypothesis that the administration of an extremely high dose (~25 g per year) of oligonucleotides and highly inefficient, but detectable incorporation of 2'-F metabolites into mitochondrial DNA might be involved, cannot be completely ruled out.

The most commonly used fully 2'-modified siRNA configuration was initially introduced as an alternating 2'-OMe and 2'-F scaffold pattern²⁷. 2'-OMe provides a substantially higher level of stabilization, and indeed more methyl-rich configurations result in better efficacy and durability in vivo³². The 5' nucleotide of the guide strand does not interact with the target and is predominantly modified with 2'-OMe uridine, which provides an optimal fit in the Argonaute 2 (Ago2) MID domain³⁵. Increasing the 2'-OMe content through the remaining positions of the guide strand enhances durability in vivo, but also introduces challenges. The extreme example of the methyl-rich scaffold uses compounds in which all but two positions (positions 2 and 14 of the guide strand) are 2'-OMe modified³⁶. This scaffold provides high stability in vivo, but it is compatible with only a limited number of targeting sequences.

When modified siRNAs are delivered in vivo, long-term efficacy relies on the continuous release of the drug from endosomal and lysosomal compartments. Upon release into the cytoplasm, they compete with naturally occurring microRNAs (miRNAs) to be loaded into

de novo synthesized Ago2 (ref. 37). Thus, even a minor reduction in RISC-entering ability might negatively affect long-term biological efficacy. Indeed, identification of optimal siRNA configurations requires iterative screening of sequence and modification patterns. Interestingly, while the pharmacokinetic and pharmacodynamic (PK–PD) profile of the siRNA scaffold is believed to be mainly defined by the general chemical configuration²¹, the exact clinical durability varies significantly between approved drugs (1- to 6-month dosing intervals), at least in part owing to differences in the 2'-OMe and 2'-F patterns used. Although the exact modification patterns may vary, recently approved drugs and clinical programmes predominantly adopted fully stabilized scaffolds.

Exonuclease stabilization

Replacing 2'-OH groups with 2'-OMe and 2'-F modifications is a proven strategy to protect siRNA scaffolds against endonuclease-mediated cleavage. However, this approach is insufficient to provide stability against 5' and 3' exonucleases, which are generally effective in degrading both RNA and DNA and do not rely on 2'-OH recognition. Incorporating PS modifications, usually at the ends of both the guide and passenger strands, is currently the dominant strategy for terminal stabilization. Indeed, the simple addition of two PS-modified backbone linkages at the 5' ends provides orders of magnitude improvement in exonuclease stability and is the primary difference between Alnylam's STC and ESC platforms^{32,38}. The first-generation GalNAc-conjugated siRNAs, such as revusiran, lacked these simple additional stabilizations, and the weekly 500 mg doses of revusiran in clinical trials resulted in cumulative toxicity³⁹.

The 3' end of the passenger strand of siRNA is commonly conjugated to targeting entities such as the tri-antennary GalNAc or small-molecule lipids, providing some protection from 3' exonuclease-mediated degradation. In currently approved siRNA drugs, no PS modifications are incorporated at the 3' end of the passenger strand. This design may be acceptable for certain 3'-conjugated siRNAs, but for internal or 5'-conjugated siRNAs, stabilizing the 3' end is essential for protection against 3' exonucleases. The PS-free design at the 3' end might help to release the siRNA from membranes (for example, where the conjugate is tightly bound) for RISC loading, but it might also reduce the durability of the siRNA. Introducing a cleavable linker⁴⁰ (for example, the labile phosphodiester bond) after PS stabilization of the 3' end might be a strategy to improve both conjugate release and 3' stability (Fig. 2a). Notably, nedosiran uses a longer variant of the passenger strand, in which the 3' sequence forms a loop from which four nucleotides are linked via their 2' positions to GalNAc moieties for hepatocyte delivery. In general, the modifications and structure motifs at the 3' end of the passenger strand can be tailored to the purpose.

The 3' end of the guide strand is more susceptible to 3' exonuclease degradation, particularly in scaffolds with a longer overhang design than the typical dinucleotide overhang. Increasing the number of PS modifications in the single-stranded overhang region can enhance siRNA cellular uptake and potency⁴¹. As PS modifications do not completely eliminate the vulnerability of siRNA to exonucleases, developing strategies to further stabilize the backbone might improve siRNA in vivo durability.

5'-Terminal modification of guide strand

The 5'-terminal phosphate of the guide strand is crucial for efficient RISC loading and interaction with the MID domain of the Ago2 protein^{42,43}. The currently approved siRNA drugs lack chemical incorporation of the 5'-terminal phosphate or its analogues, with the exception of nedosiran, which uses a methyl-protected 4'-phosphate analogue. These drugs likely rely on intracellular phosphorylation of the 5'-hydroxyl for RISC loading. Interestingly, in liver, the effects of the terminal phosphate are observed for some compounds but not for others⁴⁴. The differences might be explained in part by the sequence effect on efficiency of intracellular phosphorylation. For extrahepatic delivery, in which overall accumulation is lower, chemical introduction of phosphatase-resistant 5'-phosphate analogues significantly improves siRNA potency⁴⁵.

The most commonly used metabolically stable analogue of 5'-terminal phosphate is 5'-(*E*)-vinylphosphonate (5'-VP), which substitutes the bridging oxygen with carbon in a fixed stereo configuration optimal for RISC loading (Fig. 2a). This modification was originally described in the context of single-stranded siRNA variants^{46–48} and is currently a key component in most conjugated siRNA designs, enabling prolonged durability for extrahepatic silencing^{49–52}. The 5'-VP-mediated enhancement of *in vivo* efficacy is likely due to two main factors. First is the metabolic stabilization conferred by the phosphonate bond, which is not cleaved by phosphatase. Second is the enhanced fit of the 5'-VP into the Ago2 MID domain⁵³, resulting in an energetically favourable configuration. This can partially provide an added advantage to artificial siRNAs in competition with naturally generated miRNAs for Ago2 loading. However, the 5'-VP does not add significantly to the stabilization of phosphorothioates against 5' exonucleases. The development of 5'-terminal phosphate modifications to enhance resistance to phosphatase and 5' exonuclease, as well as to facilitate Ago2 loading, is an important direction⁵⁴.

Guide strand design for RISC selection

Tuning the global scaffold of siRNA has significant implications for its functionality. The optimal configuration of the scaffold, achieved through 2'-OMe, 2'-F, PS and 5'-VP modifications, enables the efficient formation of RISC, guide strand loading and target recognition. Advanced designs to improve guide strand selection by RISC complex have mostly adopted asymmetrical scaffolds, meaning that the modification pattern of the guide strand differs in length (with 2- to 5-nucleotide overhangs at the 3' end) compared with the passenger strand (Fig. 2a). In some scaffolds, the guide strand is significantly longer than the passenger strand, generating an extended 3' end overhang that may also enhance tissue distribution in a mechanism similar to that of ASOs⁵⁵. Asymmetry⁵⁶ can be chemically introduced to ensure the selective recognition of the guide strand and prevent the passenger strand from entering the RISC⁵⁷, thus reducing passenger strand-mediated off-target effects. Another variable is the length of siRNA; longer (~27 nucleotides) asymmetrical variants^{58,59} require processing of the scaffold into the optimal length by an endogenous RNAi pathway enzyme called Dicer before RISC loading^{60,61}. Many strategies have been explored to improve guide strand selection, including shortening the asymmetrical duplex⁶², introducing paired 2'-OMe⁶³, including a 5'-morpholino analogue⁶⁴ on the 5' end

of the passenger strand, 5'-O-methylation of the passenger strand⁶⁵, terminal bridging of the siRNA duplex^{66,67} and other chemical approaches⁶⁸.

Fine-tuning siRNA chemistries

Advanced chemical configurations typically include a fully modified duplex with a combination of 2'-OMe, 2'-F, PS and 5'-VP modifications, applicable for most sequences. In addition to the four core chemistries, other strategies can selectively enhance particular siRNA properties.

Off-target effects from the passenger strand can be blocked by strand asymmetry and specific structural modifications, but guide strand-mediated off-target silencing through seed complementarity can induce undesired effects⁶⁹. Off-target effects are typically derived from seed complement frequency⁷⁰ and high affinity to the targets⁷¹. Although the exact off-target profile is hard to predict and is often cell-type and species specific, the overall 'specificity' of a compound can be partially predicted bioinformatically and validated experimentally⁷⁰. In many cases, in vivo off-target signatures are barely detectable^{49,72}, with only a handful of targets showing marginal effects. Nevertheless, off-target effects can drive in vivo toxicity⁷³. In these cases, the introduction of modifications in the seed region to reduce affinity to off-target sites can significantly improve specificity and reduce toxicity while maintaining the primary target activity.

Various strategies can be used to minimize off-targeting effects. Early studies explored the introduction of a 2'-OMe at position 2 of the guide strand, which reduced off-targeting effects through two potential mechanisms⁶³. First, 2'-OMe modifications at positions 1 and 2 make the 5' terminus a poor substrate for intracellular phosphorylation. This significantly reduces the efficiency of RISC loading of the modified strand, thus limiting passenger strand off-targeting. Second, the modification of the 2'-hydroxyl of ribose with a methyl group in the context of a chemically phosphorylated guide strand enables efficient RISC loading, but restricts miRNA-mediated target recognition. This limitation provides better discrimination between full-length complementarity-based target cleavage and seed interaction-driven off-targeting. Position 2 of the guide strand is the primary hydroxyl contact maintained in the RISC crystal structure, and disruption of this interaction is likely to have a strong negative impact on the seed-mediated off-targeting. More advanced chemical modifications, such as GNA, in the seed region (for example, at position 6 or 7 of the guide strand; Fig. 2a) substantially reduces seed-mediated off-targeting with minimal impact on primary target activity⁷⁴⁻⁷⁶. Indeed, the GNA modification has been applied to several of the latest clinical candidates, including Alnylam's VIR-2218 for the treatment of chronic hepatitis B virus infection.

Many clinically relevant scaffold configurations use only PS modifications for backbone stabilization. There is additional space to further advance chemistries to increase backbone stability. Extension of the phosphate backbone by inserting an extra carbon, named extended nucleic acid (exNA) (Fig. 2a), was recently demonstrated to profoundly increase stability against 3' exonucleases. Indeed, replacing only two PS modifications with exNAs at the 3' end of the guide strand significantly improves extrahepatic tissue accumulation and

efficacy⁷⁷. Interestingly, delayed clearance of exNA-modified siRNAs indicates that 3' exonuclease degradation during the distribution phase significantly limits efficacy.

Currently, the most advanced siRNA scaffolds can achieve high potency and multi-month durability in on-target silencing, which are the most attractive features of this class of medicine. However, the potent and prolonged pharmacological effects may raise safety concerns, for example, by causing unexpected on-target side effects. Such siRNA-induced side effects can be mitigated by introducing a single-stranded oligonucleotide with high affinity for the seed region of the guide strand. These oligonucleotides, termed antagomirs (or anti-miRs)⁷⁸, bind to the loaded guide strand in RISC, thus blocking its target recognition and silencing activity (Fig. 2b). Indeed, REVERSIR (GalNAc-modified antagomir) can effectively reverse siRNA activity in GalNAc–siRNA-treated animals⁷⁹. This concept is not clinically realized and, so far, has not been requested by regulatory agencies. Another variant of this idea can be used to improve tissue selectivity. GalNAc enables selective delivery of siRNA to hepatocytes, but other classes of conjugate are significantly less selective. As liver and kidney are the main tissues responsible for oligonucleotide clearance, some extent of liver and/or kidney exposure is always observed, regardless of the conjugate and route of administration. For example, REVERSIR could be used to improve CNS-selective targeting by reducing liver clearance-related siRNA activity⁸⁰.

Most of the siRNAs in the clinic target both alleles of a disease-causing gene. In some genetically defined disorders, selective targeting of a mutant allele to unleash the wild-type allele is desired. Therefore, it is beneficial to design compounds^{81–84} that can efficiently discriminate between the mutant and wild-type alleles, which often differ in only a single nucleotide. Typically, a series of consecutive complementary guide strands around the SNP site of the mutant allele transcript are first screened to identify whether any of the designed siRNAs complementary to the SNP position are active. A single mismatch to the wild-type allele often fails to provide sufficient selectivity for the mutant allele; therefore, incorporation of an additional mismatch is necessary (Fig. 2c). A single mismatch between the mutant allele and the guide strand (in a tolerated position) and two mismatches between the wild-type allele and the guide can provide 50- to 80-fold discrimination⁸³. Selectivity can be further enhanced by chemically constraining the backbone with an internucleotide (*E*)-vinylphosphonate (iE-VP) modification next to the allele-discriminating mismatch⁸⁵. In the iE-VP modification, a bridging oxygen of a phosphodiester bond is substituted with a double bond in a locked stereo conformation, which supports proper guide strand target recognition, but prevents guide strand flexibility to accommodate the mismatch. Although the design of allele-specific siRNAs is feasible for many sites, it can often come at the cost of lower potency compared with some non-selective compounds, thus potentially requiring more frequent dosing.

Another interesting concept is the development of a unimolecular scaffold for multi-gene targeting. In this case, a 'passenger' strand carries two or more different sequences and is annealed to the corresponding guide strands. The resulting configuration can also be conjugated and can efficiently silence multiple genes simultaneously *in vivo*⁸⁶. Chemically, there are two strategies to generate this type of molecule: linear synthesis, whereby passenger strands are separated by a linker; and the use of orthogonal protection groups,

whereby the strands are synthesized in a step-wise manner (Fig. 2d). The utility of this approach remains to be established clinically. In general, as PK–PD properties of the siRNAs are driven mainly by the molecular architecture, incorporating multiple siRNAs into a defined scaffold can provide a viable alternative for multi-gene modulation. Indeed, although this approach requires synthesis of two or more compounds and a slightly more complex preclinical development plan, the robust scientific rationale for co-administration justifies the complexity. Notably, the FDA has previously permitted the administration of a pool of more than one siRNA as a single product in a few clinical trials: [NCT00882180](#), [NCT04669808](#) and [NCT05881993](#) ([clinicaltrials.gov](#)). This pooling approach provides more flexibility, including the ability to differentially tune the ratio of the compounds and thus, the relative levels of modulation. In addition, the chemistry, manufacturing and controls (CMC) plan for manufacturing of the smaller, less-complex entities is easier.

In silico design and biological screening

The design of siRNA drugs requires thorough analysis of genetic information, including mRNA expression profile, presence of SNPs, sequence specificity and homology. Informatics can help to predict siRNA functionality, specificity and cross-species targetability before experimental validation, eventually increasing the success rate for identification of siRNA leads. However, this approach faces several challenges (Box 2). Multiple factors can affect therapeutic siRNA efficacy; some are easy to predict with high confidence and others require extensive experimental screening. A combined approach is usually necessary to identify the optimal siRNA sequence. Below, we discuss key factors that influence the overall performance of bioinformatics in predicting hyper-functional siRNAs.

Unmodified versus modified siRNA

Extensive chemical stabilization is essential to maximize the durability of siRNA in vivo, but modification patterns affect siRNA functionality. A significant fraction of active non-modified siRNAs do not tolerate extensive modification. Currently, a cellular screen is usually performed in the context of a fully modified scaffold of one configuration. Identified leads may be further optimized by fine-tuning the modification pattern to the sequence. Although the correlation between the efficacy of unmodified and modified siRNA is relatively low, the data generated with one chemical modification pattern can be, at least partially, translated into a different chemical or structural scaffold.

Most publicly available siRNA prediction algorithms are based on unmodified siRNA datasets, which limits their ability to predict modified siRNAs. For example, one of the earliest robust algorithms was developed by machine learning trained on a large set of unmodified siRNAs⁸⁷. When the algorithm developed with the Huesken dataset was applied to the chemically modified siRNAs, it had limited predictive power⁸⁸. Conversely, algorithms developed on the basis of modified siRNAs do not show significant predictive power for non-modified compounds. The difference is likely due to the different factors driving efficacy. For example, thermodynamic bias^{56,89} – also called strand asymmetry – is a principal component of efficacy for unmodified siRNAs, but in modified siRNAs

asymmetry is usually chemically defined and thus contributes less. Primary screening must be conducted using modification patterns that resemble clinically applicable scaffolds. Most laboratories and companies now use a preferred modification pattern as a starting point and then optimize the chemistry for maximal potency. The final choice of the pattern is often defined by the lead compound sequence and the target tissue.

siRNA potency in vitro versus in vivo

Identification of an siRNA that silences a preferred target in vitro is often an easy task, and many commercial resources provide off-the-shelf solutions. Unfortunately, optimal clinical efficacy of a compound is highly dependent on potency, which can vary significantly depending on chemical modifications and delivery entities. Generally, compounds with better half-maximal inhibitory concentration (IC_{50}) values in vitro tend to show enhancement of in vivo activity, but the correlation between in vitro and in vivo efficacy is not always straightforward. Many unpublished cases exist in which highly potent siRNAs in vitro exhibit minimal in vivo efficacy, as the complexity of the in vivo environment can profoundly impact siRNA behaviour. The precise relationship between in vitro, in vivo and clinical potency can be difficult to resolve, as available data are often not well matched, especially in compounds for which the sequence, modification pattern and dosing regimen are changed. Hyper-functional sequences are well documented, but a fundamental understanding of the factors that drive hyper-functionality and informatics of their prediction is not well established. For example, mRNA local structure, the presence of miRNA binding sites or RNA-binding proteins might be contributing factors.

Another crucial factor that could influence the efficacy of siRNA is prevalent adenosine methylation, particularly N^6 -methyladenosine (m^6A), in target mRNA transcripts, which have a significant role in various cellular processes⁹⁰. Recent reports suggest that the presence of m^6A at miRNA binding sites in target mRNAs enhances miRNA-mediated silencing^{91,92}. Most of the current clinical leads target 3' untranslated regions (UTRs), which are enriched for miRNA binding sites, offering higher chances to enhance local context for gene silencing. Although many approaches have been explored to improve prediction accuracy, the correlation between computational outputs and actual activity remains limited mainly owing to biological complexities, demanding continued innovations. The use of machine learning methods^{93,94} to help identify patterns or correlations between sequences, modifications and efficacy would advance the development of functional siRNA informatics. This will require large-scale, high-quality datasets derived from biological validation under consistent experimental conditions.

Specificity

Target specificity is another key factor to consider. Compounds should be selected with at least one – preferably more – mismatch to potential off-target sites. In addition, factors such as seed complement frequency and the presence of natural miRNA seed⁹⁵, or sequence factors that affect CMC, all contribute to design. Seed-based off-targeting is a major challenge in the application of genome-wide RNAi-based screening. Indeed, in vitro, each siRNA will cleave the target mRNA but may also silence other mRNAs through the seed-based complementarity in their 3' UTRs^{69,70,96}. The exact identity of off-targets will depend

on cell type and species⁶⁹, but whether an siRNA has a few or many off-target sites can be partially predictable⁷⁰ by informatics and is consistent between species.

Interestingly, off-target miRNA-like regulation by siRNAs seems to be less of a problem in vivo than in vitro. Although siRNAs have been shown to cause toxicity in vivo by seed-based off-targeting⁷³, most do not^{49,72}. Apparent differences in the magnitude of off-targeting effects observed in vitro versus in vivo might be due to reported differences in the molecular weight and intracellular localization of RISC⁹⁷. Indeed, only a high-molecular-weight RISC is capable of miRNA-like function and is enriched in actively dividing cells in vitro; its presence in vivo is highly variable between tissues and cell types.

Cross-species targeting

siRNAs with cross-species targetability can streamline and expedite the translation of drug candidates from preclinical development to the clinic. Identification of such siRNAs requires analysis of the homology of target sequences between humans and species of interest⁹⁸. Databases such as NCBI BLAST, Ensembl and The UCSC Genome Browser are commonly used for the initial alignment of mRNA transcripts of different species. Regions of sequence identity are typically preferred targets for cross-species siRNA design and will serve as the basis for subsequent analyses. When there is limited identity, 'cross-species' siRNAs can be developed by carrying out additional screenings in human cells to identify hits that are partially complementary (with a few mismatches) to the mRNA of the species of interest as a first step. These human-active compounds indicate sites in the other species that, although not perfectly conserved by sequence, are nevertheless accessible to the RNAi machinery. Human-active siRNA sequences can then be reprogrammed to fully match the target sites in the species of interest for testing. Informatics and biological validation will still be needed to confirm efficacy and identify potential off-target effects. This approach may help in the development of closely related 'tool' compounds for human siRNA preclinical development. In other cases, tool compounds with different sequences but an identical scaffold configuration to the active human compound may be developed.

Oftentimes, there is a degree of variation in transcriptomics between humans and experimental species. Making the prediction to minimize off-target effects in both species is challenging. This is, in part, due to the limited availability of well-annotated sequencing information, necessitating open access to more validated datasets. Sometimes cross-species siRNAs effectively silence human targets but show little-to-no activity in other species or vice versa^{99,100}. In such cases, target silencing is typically validated in vitro using human cell- or tissue-based models, whereas molecular toxicity can be assessed in animal species, even in the absence of target engagement. A separate 'tool' compound with a similar or different sequence but an identical scaffold to the human lead can be used to assess the safety of on-target silencing in vivo. The biological mechanisms that mediate cross-species differences in activity remain poorly understood and could be multifactorial. Computational tools to fully address these challenges are not yet available, and their development represents a highly important direction.

Delivery principles

The design principles of optimized chemical scaffolds are similar for all delivery strategies. Without delivery conjugates, fully chemically stabilized siRNAs would essentially be ineffective. More than 90% of the systemically injected siRNA (which is smaller than the renal clearance threshold of 40–60 kDa¹⁰¹) would be rapidly eliminated by the kidney, resulting in low bioavailability^{102,103}. Moreover, in the absence of serum protein binding and targeting entities, compounds would not be efficiently distributed through target tissues or taken up by cells.

Functional delivery of siRNA to target tissues or cell types is affected by various factors, including route of administration, biological barriers, extravasation, tissue or cellular uptake and endosomal escape. LNPs and conjugates are clinically approved approaches for siRNA delivery. The development of LNPs has a long history with many chemistries available. LNP-mediated delivery represents one of the earliest methods to demonstrate effective gene silencing in humans^{8,104} and is the basis of the first approved siRNA drug, patisiran^{9,105}. Significant advances have recently been achieved in delivering nucleic acid-based therapeutics (for example, mRNA, CRISPR–Cas gene-editing systems and siRNA) to extrahepatic tissues, including lung, spleen and solid tumours, with improved selectivity to certain cell types^{106–110}; this will potentially open new avenues for the functional delivery of siRNAs to these tissues using the established LNP platforms. Currently, conjugate-mediated delivery is the dominant platform for siRNA delivery in the clinic.

Current tissues amenable to delivery are expanding following the success of LNP- and GalNAc-mediated liver delivery. For non-liver tissues, the central nervous system (CNS), eye, lung, muscle and skin show promising progress; additional tissues, including kidney, heart, fat, placenta and pancreas, are under active exploration. This expansion is mainly attributed to recent innovations in delivery platforms and modes of administration. Whereas selective delivery of siRNA to the liver is straightforward with a GalNAc conjugate, selective delivery to CNS, eye, lung and skin is achieved via local administration. Systemic delivery is used to reach muscle, kidney, heart, fat, placenta and pancreas; although this approach is not selective, siRNAs preferentially accumulate in some cell types in these tissues at acceptable dose levels.

Systemic delivery

Conjugate-mediated delivery.—Conjugation of targeting entities to siRNA enables targeted or preferential tissue delivery without complex formulation. GalNAc-conjugated siRNA binds to the surface asialoglycoprotein receptor (ASGPR) to prompt hepatocyte uptake through endocytosis¹⁶. Currently, five products that use GalNAc for delivery (that is, givosiran, lumasiran, inclisiran, vutrisiran and nedosiran) are on the market. Among them, vutrisiran targets transthyretin in the liver for the treatment of hereditary transthyretin-mediated amyloidosis and is given every 3 months through subcutaneous injection. Patisiran targets the same gene but is formulated in LNP for intravenous (i.v.) infusion every 3 weeks. Although the two drugs show comparable clinical efficacy¹¹¹, the convenient and less-frequent dosing regimen of vutrisiran will likely increase its popularity.

GalNAc-mediated delivery to hepatocytes represents a unique case, whereby together the natural filtering function of the liver, high blood flow, fenestrated endothelium and fast recycling of ASGPRs¹⁶ result in robust efficacy and durability. Notably, GalNAc conjugation also enables the delivery of various other types of oligonucleotide to the liver, making this approach applicable to a wide range of conditions¹¹². Currently, several targeting conjugates, including antibodies, engineered proteins, peptides, aptamers and lipids, are under development for siRNA extrahepatic delivery (Fig. 2a).

Extrahepatic delivery.—Systemic delivery of siRNA to non-liver tissues is conceptually complex and requires a better understanding of the principles that drive clearance and biodistribution (Fig. 3a). The biodistribution of siRNA can be influenced by routes of administration, nuclease stability and physiological features of clearance organs. Liver and kidney are the main clearance organs for systemically injected siRNAs. The high rate of blood flow, discontinuous endothelium of the liver and the natural filtration function of the kidney eliminate most of the injected compounds before they reach sufficient systemic accumulation (Fig. 3b).

In general, the overall hydrophobicity-to-hydrophilicity ratio of the scaffold defines the siRNA clearance mode. Highly hydrophobic compounds are cleared more in the liver, whereas highly hydrophilic compounds are cleared more by the kidney. Interestingly, unconjugated siRNAs can efficiently accumulate in kidney proximal tubule epithelial cells^{103,113,114}. Achieving accumulation in kidney is relatively easy, but most of the compound is tissue-entrapped and nonproductive. Indeed, target silencing in the kidney requires almost two orders of magnitude higher accumulation ($>100 \mu\text{g g}^{-1}$) of siRNA than tissues such as muscle and the CNS, where typically $1\text{--}2 \mu\text{g g}^{-1}$ is sufficient^{49,103}. Thus, for the kidney, additional chemical advances, such as enhanced stability and endosomal escape, are needed to improve efficacy. The kidney is an organ of interest for many renal disorders; successful delivery of siRNAs to additional cell types, such as glomerular endothelial cells and podocytes, would unlock unprecedented opportunities^{115,116}.

The liver-to-kidney ratio related to siRNA clearance mode appears to be more complicated when it involves other factors, such as conjugate variants and molecular size, which can all impact the in vivo behaviours of the siRNA. Below, based on the available literature and unpublished data, we propose several models of plasma clearance kinetics typically observed in siRNA therapeutic designs.

Unconjugated siRNAs are quickly cleared from the bloodstream after i.v. injection. With conjugation, their systemic distribution can be enhanced with an improved area under the clearance curve (Fig. 3c). By comparison with subcutaneous injection, i.v. injection results in a higher maximal plasma concentration (C_{max}), but it is associated with faster clearance^{38,117}. Designs that allow the incorporation of PK modifiers can increase the overall size of an siRNA; approaching a molecular weight of 40–60 kDa can lead to a significant reduction in renal clearance¹⁰² (Fig. 3d). Likewise, multivalent scaffold designs (Box 3) can significantly influence siRNA clearance kinetics owing to the multiplication of the molecular weight of monomeric compounds, thereby may also reduce kidney filtration (Fig. 3e). A primary mechanism by which hydrophobic conjugates improve the plasma half-

life of siRNAs is binding to serum lipoproteins¹¹⁸, such as high-density lipoprotein (HDL) and low-density lipoprotein (LDL). This trafficking mechanism enhances the delivery of siRNAs to tissues enriched with lipoprotein receptors. Serum albumin – the most abundant protein in the blood – can also serve as an excellent natural carrier of siRNA for systemic distribution¹¹⁹. Therefore, conjugates or molecular characteristics that promote siRNA association with lipoproteins and albumin can greatly benefit extrahepatic delivery (Fig. 3f).

Indeed, lipid conjugates, such as docosanoic acid (DCA) and phosphocholine (PC)–DCA, profoundly improve siRNA systemic distribution, demonstrating efficacy in several extrahepatic tissues, including muscle, heart and fat¹⁰³. The level of extrahepatic accumulation is superior to that of cholesterol conjugation. How the structure of the lipid conjugate affects distribution is not fully elucidated, but the higher level of DCA-driven extrahepatic uptake is likely due to a combination of lipoprotein association, preferential endothelial interaction and trans-vascularization properties^{103,118}. In addition to demonstrating better extrahepatic uptake, this class of compounds affords improved safety profiles; whereas cholesterol-modified compounds are toxic at doses approximating 100 mg kg⁻¹, saturated lipid-conjugated compounds are safe at this dosing level⁵⁰. Further chemical engineering of lipid conjugates can enhance efficacy or safety. Several strategies are being used, including increasing valency, modification of the conjugate head group¹²⁰ and building complex artificial architectures. Several PC–DCA-conjugated siRNAs are currently being evaluated for the modulation of sFLT1 in a phase I clinical trial to treat pre-eclampsia^{36,121}. A class of hydrophobic siRNAs conjugated to the 2'-*O*-hexadecyl (C16) lipid is widely used both preclinically and clinically. Although this conjugate shows limited systemic efficacy, it demonstrated robust clinical results upon local administration into the CNS, eye and lung (discussed in the following section)⁵¹.

Other types of conjugate that demonstrate robust systemic extrahepatic efficacy include antibodies or antibody fragments^{122–124} and engineered proteins¹²⁵. These conjugates also significantly increase the overall size of the siRNA construct, thus promoting systemic distribution by reducing renal clearance, similar to PK-modifier and multivalent designs.

The most advanced clinical programme of the antibody-siRNA conjugate platform uses a monoclonal antibody to the transferrin receptor 1 (TfR1) for skeletal, cardiac and smooth muscle delivery. In a preclinical study, i.v. administration of a single 6 mg kg⁻¹ dose of the anti-TfR1–siRNA targeting the *SSB* mRNA (encodes small RNA-binding exonuclease protection factor La) resulted in up to 75% target downregulation¹²³. A phase I/II study in human patients using anti-TfR1–siRNA to target the myotonin-protein kinase (*DMPK*) for the treatment of myotonic dystrophy type 1 demonstrated robust improvement in disease outcome (see Related links).

Centyrins are a class of small, engineered human protein derivatives that are being harnessed for siRNA extrahepatic delivery¹²⁵. The stability and favourable in vivo properties of this type of targeting entity can be achieved by varying certain amino acids within select structural regions. The centyrin–siRNA conjugate platform has shown promising systemic delivery properties. An advanced clinical programme that recently moved to phase I trial

uses centyrin-conjugated siRNA (**ABX1100**) for muscle delivery to silence the glycogen synthase 1 (*GYS1*) gene for the treatment of Pompe disease (see Related links). Led by the strong promise of this strategy, multiple other pipelines are currently under development to further advance the technology.

Peptide conjugates are expected to expand as an siRNA delivery platform. Peptides can be chemically synthesized, which may support less-complex CMC development. Glucagon-like peptide 1 (GLP1), a ligand of the GLP1 receptor, has been successfully used to deliver ASOs into pancreatic islet cells¹²⁶. This early work resulted in significant effort in exploring peptide chemical space for extrahepatic delivery of siRNA. Although there is limited information available publicly, many academic laboratories and pharmaceutical companies are working on focused and unbiased selection strategies to further explore the potential of peptide conjugation as a promising strategy for siRNA delivery.

Aptamers were among the first conjugates to be explored for delivery of siRNAs and other oligonucleotide-based therapeutics^{24,127–134}, but they have not gained significant traction in the translational space, possibly owing to limited durability observed in early attempts. More recently, however, aptamers have yielded notable advancements in animal models of disease, especially for targeting certain biomarkers involved in oncology^{135,136} and thus hold potential for future clinical translation.

Another strategy to improve systemic delivery involves the use of nanoconstructs as siRNA carriers^{137–142}. Molecularly defined nanoconstructs with appropriate shape and size can be designed to incorporate siRNA strands with high programmability. Most studies have used only unmodified or partially modified siRNA configurations, and in vivo characterization of these constructs is often less explored. Certain high-payload designs to incorporate large numbers of siRNAs may trigger undesired immune responses. Thus, to harness nanoconstructs for therapeutic applications in vivo, future exploratory efforts are needed to advance our understanding of how to avoid potential immunogenicity and improve payload release.

Local delivery

Local administration of optimized siRNAs into the CNS, eye, lung and skin has shown robust efficacy^{49,52,99,143,144}. Direct delivery of compounds to these relatively enclosed or easy-to-access tissues enhances retention and ensures an adequate supply of compounds for cellular uptake. As noted above, molecular characteristics of siRNAs – for example, hydrophobicity and molecular size – also reduce clearance and promote uptake. For example, conjugation of a C16 lipid chain to a 2'-ribose on the passenger strand (Fig. 4a) enables safe, potent and multi-month silencing in CNS, eye and lung of rodents and non-human primates (NHPs)¹⁴³. In practice, when siRNAs are administered locally, various designs can be tested to optimize scaffold configurations. Indeed, different scaffolds can generate distinct cell-type uptake profiles^{51,52,99,145}. Thus, determining where a particular scaffold accumulates can help to achieve selectivity, which in turn requires knowledge of the cell-type expression profiles of disease targets. Currently, there are numerous ongoing activities in the field that are expected to expand the utility of siRNA drugs through local delivery.

Central nervous system.—Delivering siRNA therapeutics to the CNS is feasible via direct injection into brain regions or infusion into the cerebrospinal fluid (CSF). The dosing regimen is an important consideration in delivering siRNA to the CNS; indeed, the brain and spinal cord have a diverse composition, and siRNA often accumulates unequally, perhaps related to the flow of CSF¹⁴⁶. For example, direct injection of divalent siRNA led to a highly local accumulation, which might prove useful for delivering antitumour therapies. Infusion of divalent siRNA into CSF supported widespread distribution and function, with high levels of accumulation in regions with high CSF exposure (for example, hippocampus, thalamus, cortex and spinal cord) and lower levels of accumulation in deeper regions (for example, caudate and putamen). Repetitive administration may help to reduce this unequal distribution. Thus CSF infusion of therapeutic siRNAs might be suitable to treat neurodegenerative disorders that affect cortex, hippocampus and spinal cord involvement, such as amyotrophic lateral sclerosis¹⁴⁷ and Alzheimer disease¹⁴⁸.

Skin.—Skin represents the largest and most accessible human organ for local siRNA delivery. Topical delivery of large molecules such as siRNAs across the outermost layer of the skin barrier (that is, the stratum corneum) is challenging. Although many earlier studies using various topical application methods have been conducted^{149–155}, their clinical translation is limited, particularly when complex formulation is involved. Intradermal injection – commonly used in the clinic to administer therapeutics for localized dermatological indications – is a viable method for siRNA delivery. Hydrophobic conjugates can significantly improve retention of siRNA in the skin after local injection¹⁰³ (Fig. 4b) and support functional modulation of disease pathways in rodent and ex vivo human skin^{99,156}. Future work to test intradermal injection of the optimized siRNA platforms in pig models, which better resemble human skin, may provide insights into siRNA PK–PD properties and facilitate clinical translation.

Ex vivo transplantation.—Delivery of siRNAs to ex vivo organs for transplantation is a potentially exciting application^{157,158}. Machine perfusion delivery of siRNAs that silence genes involved in ischaemia–reperfusion injury^{159–161} can help to preserve transplant quality. Delivery of siRNAs that silence immune factors may help to reduce fast graft rejection, but additional strategies are likely needed to prevent rejection in the long term. This is definitely an area to watch.

Clinical development considerations

SiRNA drug development differs significantly from that of traditional small molecules and biologics. The main difference is that the PK properties and target specificity of a siRNA can be independently optimized²¹. The PK properties are defined by the overall chemical scaffold and conjugation (dianophore), whereas the target specificity is primarily defined by the guide strand sequence (pharmacophore) (Fig. 2a). Therefore, the pipeline demands chemical optimization to improve delivery and bioinformatics to improve target specificity. Although computational design can profoundly accelerate siRNA drug discovery, as discussed, it sometimes faces challenges (Box 2). Notably, the success rate of design and development of a siRNA drug candidate towards clinical validation, on average, is

substantially higher than that achieved with conventional pipelines. Below, we highlight key considerations for siRNA drug development.

Clinical indication and target selection

The successful development of an siRNA drug for a clinical indication requires selection of a relevant target. The optimized siRNA delivery platform should align with the tissue in which the target is expressed (Fig. 5), and any potential on-target but off-tissue silencing should be safe. For example, when treating liver-related indications using GalNAc-conjugated siRNAs, the causative disease target should be expressed by hepatocytes. For neurological indications, several optimized platforms (for example, C16 and divalent siRNAs) can support a broad range of cell-type distribution in the CNS. Currently, multi-target modulation is gaining popularity, as many complex diseases are often caused by the dysregulation of more than one gene^{162–164} (Box 4).

Target selection and validation.—Definition of the underlying cause of a disease is essential for target selection. Although many genes are ‘identified’ as disease targets by analysing upregulated biomarkers in disease tissues or animal models, often, the knockdown or inhibition of these genes does not translate into therapeutic efficacy. This is either because the targets are irrelevant (bystanders in the dysregulated pathways) or compensating pathways exist for the pathology. Thus, the validity of a disease target must be substantiated by strong evidence for its role in driving the disease. Unfortunately, the molecular characteristics of many human diseases are challenging to model in animals. Target validation can benefit from more innovative approaches and better access to biopsy samples from human patients. Advances in genetics, multi-omics and artificial intelligence for target prediction, based on large numbers of complex human datasets, have shown strong promise^{165–168}.

The fundamental factor that governs target selection is the ability to deliver siRNA to the target tissue with robust efficacy and acceptable safety. As discussed, delivery to the liver is fully validated, and robust animal data exist for other tissues, such as CNS, lung, eye, muscle and skin. A second key factor is understanding the distribution and clearance profile. Although selective delivery and silencing has been achieved for the liver, selective delivery to other organs remains a challenge. For example, most muscle-targeting conjugates (for example, antibodies, DCA, and so forth) would also deliver to and induce target silencing in heart and liver. For therapeutic applications, therefore, developers should ensure the target is preferentially expressed in the intended tissue or that off-tissue modulation is benign.

If a disease target is highly upregulated, potentially surpassing the capacity of the RNAi machinery, an alternative strategy might be to target upstream signalling factors that interact with that target. Another approach might be to include additional targets using a multi-targeting siRNA, or a combination therapy with other drug modalities, to achieve potential synergistic effects. Simultaneously, target selection also requires consideration of the influence of mRNA turnover rates on siRNA efficacy. Studies show that mRNAs with high turnover rates can be more resistant to RNAi-mediated silencing¹⁶⁹.

The proliferation rate of target-expressing cell types can also dramatically affect siRNA potency and durability. Fast-dividing cells dilute the cellular concentration of internalized siRNAs over time. Thus, RNAi activity may be reduced in actively dividing cells (for example, in tumour cells¹⁷⁰ and epidermal keratinocytes¹⁷¹), requiring repetitive dosing to maintain silencing efficacy. In contrast, siRNA durability typically lasts longer in cell types in a non-dividing state, such as neurons¹⁷².

Another crucial consideration is clinical validity. For a long period of time, it was believed that siRNAs could not compete with small molecules and biologics; thus any targets for which there was already an effective drug were not considered. However, the potential for twice a year or yearly dosing frequency, which supports practical convenience and patient compliance, is now making siRNA a highly attractive alternative modality. The most obvious example is inclisiran, which targets proprotein convertase subtilisin/kexin type 9 (PCSK9) and lowers dysregulated cholesterol levels, although there are already several approved biologics on the market¹⁷³.

The PK–PD of siRNAs can offer advantages over small molecules. For example, mismatch repair factor MutS homologue 3 (MSH3) promotes somatic repeat expansion in the Huntington disease gene (*HTT*), encoding huntingtin¹⁷⁴, and silencing *MSH3* might offer a strategy to prevent expansion of *HTT* repeats. Silencing a factor required for maintenance of genomic stability raises concerns about potential genotoxicity and immunogenicity, but disease-causing *MSH3* mutations are mostly related to the carcinogenesis of colorectal cancers^{175,176}. The ability to deliver *MSH3*-silencing siRNAs to the CNS but not to the colon¹⁰⁰ provides a competitive advantage over small molecules, which would generally act in many tissues, including the colon. The mismatch repair pathway is most active in dividing cells¹⁷⁷, so silencing *MSH3* in the brain is less likely to be a problem in neurons, but it could be a problem in microglia. This hypothesis requires testing in future studies.

On-tissue target selectivity.—Selective delivery to target tissues through systemic administration is intrinsically difficult. Although mostly cleared in liver and kidney, many siRNA designs accumulate sufficiently in certain tissues to induce functional gene silencing. To avoid potential side effects from off-tissue silencing, a useful strategy is to focus on therapeutic targets that are preferentially expressed in one of the siRNA-deliverable tissues (see [The Human Protein Atlas database](#) (see Related links) for target expression information). For example, myostatin (encoded by *MSTN*) is a myokine that is primarily produced in skeletal muscle and acts on myocytes to inhibit muscle growth. Therapeutic modulation of myostatin may be beneficial for treating muscular dystrophies^{178–180}. An illustrative POC study is the use of a hydrophobic conjugate DCA to systemically deliver myostatin-targeting compounds to muscle to increase growth⁵⁰. The accumulation of DCA-conjugated siRNAs in multiple tissues is a minor concern¹⁰³, because myostatin is preferentially expressed in the muscle, with little or no expression in other tissues. In the case of universally expressed genes across multiple tissues, systemic targeting may be tolerable, but it requires careful evaluation through safety studies, as off-tissue target silencing could potentially carry liabilities.

In vitro screening and lead identification

Screening siRNAs in a disease-relevant cell model that expresses the target transcripts is ideal. However, difficulties often exist in practice, including the limited availability of suitable cell lines and the low expression level of the target. Additional challenges include handling cell lines that require complex procedures or are resistant to siRNA transfection and passive delivery methods. An alternative strategy is to use a reporter system to identify functional hits, followed by biological validation in a more complex model system^{52,121}. As discussed, reporter systems might not correlate with native targets and will require additional screening and validation.

Both the IC₅₀ values and the maximal silencing efficacy are important for lead selection from functional hits. Ideally, an optimal lead should demonstrate superior maximal silencing efficacy and a low IC₅₀ value. In cases where these two factors are not aligned, an evaluation is needed to find the compromise. In general, in vitro to in vivo translation is not absolute, thus several in vitro leads should be evaluated in vivo, but this requires more laborious work. Importantly, comparing IC₅₀ values determined under disparate experimental conditions is not advised for selecting the lead compound, as IC₅₀ values are significantly influenced by many factors, including cell line type and delivery method.

Lead optimization

Lead optimization is a crucial step for bridging in vitro screening and in vivo validation. Scaffold and nucleotide modifications (for example, 2'-OMe, 2'-F or PS backbone) can significantly affect siRNA activity, so any major changes during optimization must be tested to compare activity against the parental compound. The focus of lead optimization is mostly on conjugation, valency and the addition of extra stabilization modifications, to improve in vivo delivery, potency and durability. Typically, lead variants are systematically generated and evaluated in parallel to identify the best configuration for further development. Although this process can be carried out in vitro depending on the purpose (for example, RISC compatibility), in vivo evaluation is preferred to better understand the therapeutic behaviour in the complex biological context. Lead compounds should be meticulously purified and quality-controlled. A quick in vivo validation of the optimized lead to confirm target silencing would be helpful before committing to comprehensive and resource-intensive preclinical safety and efficacy studies.

Preclinical evaluation

Ideally, the efficacy of the lead compound should be evaluated in a relevant disease model. However, the lack or limited availability of animal models for many human diseases poses significant challenges. Additionally, the relevance of an animal model – that is, whether it accurately resembles the human disease characteristics^{181–183} – is essential, particularly for inflammatory disorders and cancers where the exact cause of the disease might be unknown. In the case of other diseases, such as Huntington disease^{184,185} and myotonic dystrophy¹⁸⁶, in which the underlying mechanisms are clearly defined at the genetic level, siRNA design and preclinical evaluation are more straightforward, and often more advantageous, than conventional therapeutic modalities, especially when humanized transgenic models are established. Whereas the efficacy and safety evaluation of many siRNA drug candidates

typically requires a distinct reiteration process, PK studies and dosing regimen optimization will generally follow a more standardized process.

Clinical validation

Significant progress has been made in the past two decades in understanding how to evolve drug-like properties of siRNA molecules, which has helped many preclinical programmes to succeed to clinical trials. Despite favourable preclinical safety profiles of siRNAs in rodents and NHPs¹⁸⁷, uncertainties may persist at clinical validation, as seen in the phase III trial of revusiran, which resulted in unexpected mortality¹². The exact cause (or causes) of failure remain unclear despite extensive investigations, as discussed in detail above^{34,39}, and emphasize the need for thorough safety evaluation at all stages of siRNA drug development.

Of note, the short timeline of siRNA lead identification and preclinical development supports high feasibility of clinical trials for individual patients with rare genetic diseases or advanced-stage cancers that fail to respond to existing treatments. These *N*-of-1 studies represent exceptional opportunities for fast-track clinical validation and development of siRNA drugs, thus benefiting the treatment of additional patients¹⁸⁸. This field is rapidly progressing, as shown by the recent efforts to advance ASO drug development¹⁸⁹ (see Related links '[FDA takes steps to provide clarity on developing new drug products in the age of individualized medicine](#)' and '[FDA takes new steps aimed at advancing development of individualized medicines to treat genetic diseases](#)').

Future directions

We are witnessing a notable shift of pharmaceutical development towards nucleic acid-based medicines. siRNAs stand out with distinct advantages over conventional drugs, including their ability to silence targets previously considered 'undruggable', their shorter development timeline and their prolonged clinical durability (Box 5).

As the chemical configurations to enable safe and effective tissue delivery are defined, the cost and time for the development of follow-up drugs that work in the same tissue will be profoundly reduced. We have seen this revolution happen in the development of siRNA drugs that are delivered to the liver. With several technology generations, the latest GalNAc-conjugated siRNAs now support up to 12 months of durability, with unprecedented efficacy and safety profiles. Validation of the tissue delivery platform enables development of a large variety of siRNA drugs, with limitations mostly driven by the biological understanding of the target involvement and disease relevance.

The natural evolution of siRNA drugs that are delivered to the liver included platform development, clinical exploration and target validation for genetically defined rare diseases, extending to highly prevalent disorders (for example, hypercholesterolaemia and hypertension) and many other targets¹³. Interestingly, RNAi-based drugs have the potential to replace certain small molecules and biologics, owing to their convenient, less-frequent dosing. Gene-editing approaches may eventually outcompete RNAi-based approaches for the treatment of genetically well-defined diseases for which reversal of the therapy would never be desired. Although next-generation gene-editing technologies may provide

a one-and-done treatment solution, the durability and safety of siRNA drugs could be a prerequisite for the clinical acceptance of gene-editing approaches. There are several unique advantages of RNAi-based therapies over conventional modalities, making them particularly promising in the following areas.

Isoform-specific modulation

Post-transcriptional processes such as alternative splicing are inherent cellular mechanisms that enable the production of multiple regulatory and coding RNA transcript isoforms from a single gene^{190,191}. This process may result in regulatory RNA and protein isoforms that differ in biological function and pathological roles, which have been suggested in certain cancers and neurodegenerative disorders^{192–196}. Owing to the lack of characterization of structural properties in RNA or protein isoforms, specific modulation of them through conventional small molecules and biologics is highly challenging. In contrast, oligonucleotide-based approaches offer solutions to precisely target disease-relevant isoforms on a post-transcriptional level, to achieve selectivity and improve safety^{121,195}.

The design of isoform-specific siRNAs requires that the target transcript possesses a unique sequence stretch that distinguishes it from non-targeted isoforms (Fig. 6a). Thus, validation of the disease-causative isoform and the difference in the use of alternative exons or UTRs for that specific isoform is needed. Although current genetic databases serve as a valuable starting point, using emerging and powerful tools such as long-read sequencing to ascertain the isoform expression profile in a disease state would greatly add confidence to the design¹⁹⁷. As this field is moving forwards, the ability of siRNA drugs to selectively modulate a disease-causative isoform would be unprecedented.

Viral infections

siRNAs could have an important role in rapid response to emerging viral pathogens and pandemics. The ability to rapidly develop antiviral drugs is an unmet need exemplified by the recent coronavirus disease 2019 (COVID-19) pandemic and its subsequent variants. The lung and liver are currently promising tissues for applying siRNAs, such as for the treatment of viral pneumonia and hepatitis¹⁹⁸. Indeed, multiple anti-hepatitis B siRNAs have advanced to clinical validation¹⁹⁹. siRNAs could be ideal therapeutic modalities for treating viral infectious diseases. First, siRNAs are designed exclusively against viral RNA components while minimizing off-target effects on host RNAs. Second, by targeting the conserved regions of viral genomes that are essential to maintain viability, siRNAs could cover multiple existing and future strains^{52,200} (Fig. 6b). Validation of the efficacy of antiviral siRNAs often faces challenges in establishing proper animal models. Although humanized rodent models are valuable for evaluating therapeutic outcomes, the virus infection mechanisms and antiviral responses in NHP models, although costly, closely resemble those of the human immune system, making them the most accurate model for this purpose²⁰¹.

Modulation of long non-coding RNAs

A large fraction of the human genome does not encode proteins but instead produces non-coding RNAs (ncRNAs) that have regulatory roles in gene expression and protein functions²⁰². Accumulating evidence reveals that ncRNAs, including long non-coding RNAs (lncRNAs), could serve as therapeutic targets for certain diseases^{203–208}. The existing definition of lncRNA includes a diverse group of RNAs, characterized by size in excess of 200 nucleotides. Given the diversity of lncRNAs and their various roles in regulation of biological functions, therapeutic modulation of lncRNAs represents a less-explored but opportunistic direction for drug developers. Although currently there are limited drugs targeting lncRNAs that have achieved clinical productivity, siRNAs and other oligonucleotide-based therapeutics are well suited for this purpose and have high potential to stand out in the future (Fig. 6c).

Combination therapy

Combining siRNAs with other drug modalities, such as small molecules or biologics, may synergistically improve clinical outcomes in treating complex diseases. Practically, combination therapy may also benefit patients by leveraging the advantages of each type of drug modality (Fig. 6d). An ongoing study is the combination of Alnylam's cemdisiran²⁰⁹ (ALN-CC5; siRNA that targets complement C5 component) with Regeneron's pozelimab (anti-C5 monoclonal antibody) for the treatment of paroxysmal nocturnal haemoglobinuria^{210,211}. The two companies also announced a broad collaboration (see Related links '[Regeneron and Alnylam announce a broad collaboration](#)') that might involve other development programmes. The concept of combination therapy is not entirely new, but often requires certain scientific and regulatory issues that may arise during the co-development of two or more therapeutic agents to be addressed, as per FDA requirements. The clinical success of combination therapies incorporating siRNAs is yet to be determined, but the future potential is easily foreseeable.

Conclusion

RNAi technologies have undergone a remarkable journey, spanning almost the first quarter of the twenty-first century, from RNAi mechanism discovery in *C. elegans*, demonstration of efficacy in mammalian cells, to the development of a new class of human medicines, especially to treat liver diseases. The current six approved products are based on rational design and entered the market within a relatively short time-frame, highlighting the exceptional productivity of RNAi drug development pipelines. Several advanced clinical programmes, using lipid and antibody–protein conjugates, have shown promising disease-modifying efficacy, which may lead to the next wave of siRNA drug approvals. As molecular design and extrahepatic delivery platforms continue to advance, we anticipate a major expansion in the utility of siRNA drugs across various indications in the near future.

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Related links

Nedosiran: https://www.accessdata.fda.gov/drugsatfda_docs/label/2023/215842s000lbl.pdf

Revusiran: https://www.alnylam.de/wp-content/uploads/2017/08/Revusiran-RNAi-Roundtable_FINAL2_08092017.pdf

A phase I/II study: https://www.aviditybiosciences.com/wp-content/uploads/2023/04/230330-AAN-2023-MARINA-Prelim-Data-Oral-Presentation_v6.0_FINAL.pdf

ABX1100: <https://www.arobiotx.com/aro-receives-fda-orphan-drug-designation-for-abx1100>

FDA Takes New Steps Aimed at Advancing Development of Individualized Medicines to Treat Genetic

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FDA Takes Steps to Provide Clarity on Developing New Drug Products in the Age of Individualized Medicine: <https://www.fda.gov/news-events/press-announcements/fda-takes-steps-provide-clarity-developing-new-drug-products-age-individualized-medicine>

Regeneron and Alnylam announce a broad collaboration: <https://investor.regeneron.com/news-releases/news-release-details/regeneron-and-alnylam-announce-broad-collaboration-discover>

The Alnylam GEMINI platform: https://capella.alnylam.com/wp-content/uploads/2022/05/Theile_TIDES-2022.pdf

The Human Protein Atlas database: <https://www.proteinatlas.org>

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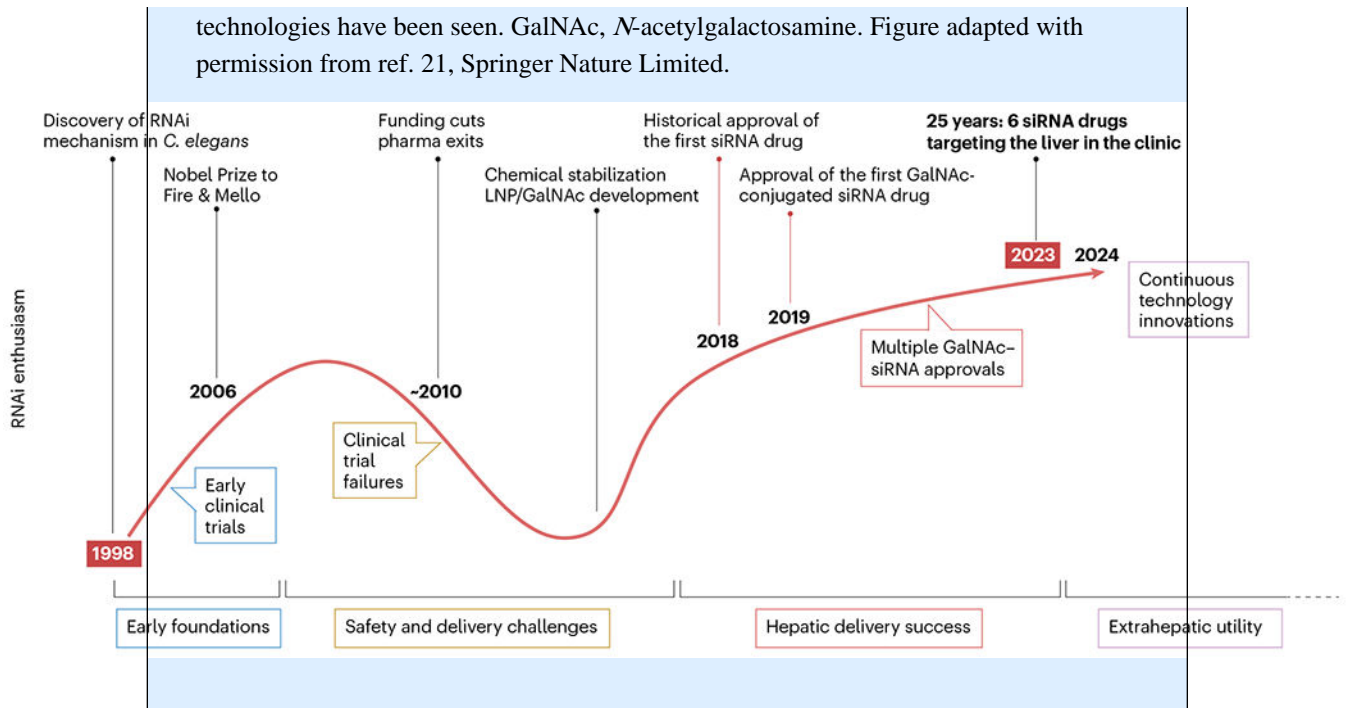
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Box 1**Twenty-five years of RNAi from discovery to clinic utility**

The discovery by Andrew Fire, Craig Mello and colleagues in 1998 that double-stranded RNAs can trigger RNAi to catalytically degrade complementary mRNA transcripts in *Caenorhabditis elegans*²¹² not only provided researchers with powerful tools but also sparked excitement for pharmaceutical development^{213–216}. RNAi was later demonstrated in mammalian cells by Elbashir et al.²¹⁷ and Caplen et al.²¹⁸ in 2001. Merely a year after, RNAi activity was shown in mice by McCaffrey et al.²¹⁹ using hydrodynamic delivery. Many pioneering works have greatly contributed to the advancement of therapeutic small interfering RNAs (siRNAs) towards in vivo implementation^{2,81,82,220–229}. These early efforts facilitated our understanding of the potential of RNAi in various applications, including cell-targeted delivery, disease allele gene-selective targeting and antiviral therapies.

The concept of applying RNAi-induced gene silencing for therapeutic development appeared simple and compelling — essentially, the ability to turn off any molecular target by simply rewriting the siRNA base sequence. Early clinical programmes proceeded rapidly, using unmodified or slightly modified siRNAs. The hope was that sufficient compound would be taken up by tissues of interest to provide therapeutic benefit. The evidence of RNAi in humans from systemic delivery was first reported in 2010 by Davis et al.¹⁰⁴. However, the first-generation compounds showed either marginal clinical efficacy or unacceptable toxicity. The realization that siRNAs faced serious hurdles, such as immunogenicity^{230–234} and off-targeting effects^{69,235,236}, quickly led to doubt in the technology, as pharmaceutical companies cut funding and abandoned their RNAi platforms^{237,238}. By the early 2010s, inflated expectations had dampened enthusiasm in the field of siRNA drug development.

Learning from failures, we now have a much clearer understanding of the underlying biological mechanisms at play and the necessity for significant chemical innovation to evolve siRNAs towards drug candidates with acceptable pharmacokinetic and pharmacodynamic (PK–PD) properties and disease-modifying potential. This knowledge has greatly facilitated the clinical implementation of siRNAs with favourable efficacy and safety profiles in the liver²³⁹. In 2018, a major milestone was reached as the FDA historically approved the first siRNA drug patisiran for the treatment of hereditary transthyretin amyloidosis^{9,105}. Today, siRNA drugs have entered the market^{14,240} and many others are in late-stage clinical validation, being tested to treat various diseases, including neurodegenerative, muscular and ocular disorders. The field is once again prospering, underscored by a notable shift in focus from rare genetic disorders to prevalent diseases. This is highlighted by the recent positive clinical results of zilebesiran in treating hypertension^{241,242}, as well as the approval of inclisiran (2020) in the European Union (EU) and (2021) in the USA for the treatment of hypercholesterolaemia — a major cause of atherosclerotic cardiovascular disease^{243,244}. Additional siRNA drugs for the liver and extrahepatic tissues are expected to be available soon, as robust results from advanced clinical programmes and progress in innovating delivery



Box 2**Challenges of computational design**

Functional small interfering RNA (siRNA) hits can be predicted from algorithms. However, for certain genes, the overall chance of identifying potent hits is significantly lower than for other genes. This gap between computational prediction and actual biological activity is mainly due to the complexity of target biology and cellular environments. Factors such as masking effects on the siRNA targeting sites owing to the presence of secondary or tertiary structures^{245,246} or RNA-binding proteins need to be considered, as these elements can profoundly limit the accessibility of siRNA for target site recognition.

With unmodified siRNA datasets, linear regression can provide reasonable predictive power and indeed many early works in the field used this methodology^{247–250}. The application of more advanced machine learning methods can slightly increase the predictive power, and among various decision trees, the random forest²⁵¹ is the commonly used approach to enhance predictive power⁹⁴. Although it is relatively easy to algorithmize the ability of the siRNA sequence to load into the RNA-induced silencing complex (RISC) and cleave the mRNA target in ideal circumstances, other target-specific factors such as the aforementioned masking effects from higher-order mRNA structures^{246,252,253} and interactions with RNA-binding proteins^{254,255} are hard to model. For example, only a small fraction of functional siRNA hits targeting viral sequences in a context of reporter-based validation (that is, viral genes constructed in an expression vector) are active in blocking the viral infection in natural conditions⁵².

The lack of perfect correlation between the ability of RISC entry and its activity in modulation of the native target is highly target related. For some targets, only a small fraction of RISC-competent compounds are active in the context of native mRNAs²⁵⁶. In contrast, for many targets, specifically for housekeeping genes, the subcellular localization and accessibility of transcripts are not significant factors affecting siRNA activity and typically there is a strong correlation between the readouts of reporter and native targeting. For genes with a significant fraction of the transcripts localized in the nucleus^{257,258}, especially those expressed in neurons^{259,260}, or targets involved in immune responses²⁶¹, cytoplasmic accessibility in specific cell types could be a major limiting factor. Novel methods, including semi-supervised feature extraction²⁶² and other artificial intelligence-type elements, may provide a viable path to improvement of the predictive power of the algorithms in these complex circumstances. Currently, the limited predictive power of informatics needs to be compensated by extensive experimental screening of a large panel of siRNA candidates.

Box 3**Multivalent scaffolds for local siRNA delivery**

Multivalent scaffolds, designed to covalently link two or more small interfering RNAs (siRNAs) that target the same gene, can be chemically made in a relatively straightforward synthetic scheme. In optimal designs, branched linkers are used to simultaneously elongate multiple passenger strands, followed by annealing of the guide strands. The direct impact of these scaffolds is the increase in molecular weight, which alters the pharmacokinetic properties of the siRNA construct. As discussed, when injected systemically, the high-valency nature of the siRNAs can help to reduce renal clearance and promote systemic distribution. When delivered locally, this property could also reduce clearance from systemic absorption, improve siRNA retention and enable widespread tissue distribution.

The effect of multi-valency (that is, di-, tri- and tetravalent scaffolds) on siRNA has been systematically evaluated in a recent study for lung delivery to treat severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) viral infection⁵². Intratracheal administration of the multivalent compounds showed robust tissue distribution and target modulation in a mouse model of SARS-CoV-2 infection. The optimized multivalent scaffolds could be reprogrammed to target other genes. As almost any siRNAs validated as functional could be formulated to generate aerosols for use with inhalers or nebulizers²⁶³, numerous opportunities exist to enable non-invasive lung delivery in the treatment of pulmonary diseases.

More recently, intravitreal administration of di-, tri- and tetravalent scaffolds into rodent and porcine eyes has been systematically studied¹⁴⁴. A single intravitreal injection of the tetravalent siRNA targeting photoreceptor cell-expressed genes demonstrated safe and multi-month durability. The technology platform may open up opportunities to treat various retinal diseases^{264–266} with a yearly or twice-a-year dosing frequency. The observed effects in lung and eyes are attributed to the increase in molecular weight of the high-valency designs. These designs could carry a payload of two to four siRNAs within a single molecular entity, thus dramatically reducing clearance and promoting distribution in the tested tissues. Although multivalent scaffolds have shown robust efficacy in the lung and eyes, their therapeutic potential has not been fully realized as these scaffolds could be conjugated to targeting entities to further manipulate the PK properties, which remains to be tested in future studies.

Box 4**Multi-targeting siRNA drugs**

Many complex diseases are often caused by the dysregulation of more than one gene^{162–164}. Targeting multiple genes is likely necessary for the treatment of these diseases, particularly cancers and neurological disorders^{196,267–269}. The ability to simultaneously modulate several genes with flexibility in target selection, including targeting those previously considered ‘undruggable’, is highly attractive. This can be achieved through unimolecular multi-targeting small interfering RNA (siRNA) designs capable of carrying different siRNA sequences^{86,270–272}. Such designs allow the modulation of genes from the same pathway or different pathways, potentially benefiting clinical treatment outcomes. In this direction, there are many opportunities to further advance multi-targeting siRNA designs, especially for those capable of targeting three or more genes and possessing drug-like properties suitable for clinical translation.

One of the key advantages of multi-targeting siRNA drugs over combination therapies is the predictable biodistribution of drug molecules targeting different genes as a whole. This advantage can greatly benefit preclinical development by simplifying the characterization of in vivo pharmacokinetic properties. Additionally, the unprecedented programmability of the unimolecular multi-targeting siRNA scaffolds allows accommodation of virtually any combination of targets of interest. The concept could be applied to treat neurodegenerative diseases, such as Alzheimer disease, that are associated with overactive neuroinflammation, as well as inflammatory diseases that involve dysregulation of multiple genes.

Currently, dual-gene targeting scaffolds have been developed and been demonstrated to have successful in vivo activity by several biopharmaceutical and academic laboratories, each using different designs^{86,273} (see Related links ‘[The Alnylam GEMINI platform](#)’). More complex scaffolds capable of incorporating three or more different siRNAs are under active exploration, with some successful outcomes that have not yet been published. The modulation of multiple genes using a single molecular construct might represent the next generation of innovations in siRNA drug development.

Box 5**Advantages of siRNAs over conventional drugs**

Certain proteins^{83,274,275}, protein isoforms (encoded by mRNA isoforms of the same gene)¹²¹ and regulatory RNAs²⁷⁶ were once considered ‘undruggable’ when there was a need for discriminative targeting in a disease state. These clinically meaningful targets offer great therapeutic opportunities but pose significant challenges for conventional drug modalities. This is mainly because many protein targets lack proper ligand binding sites or have limited properties for allosteric inhibition by small molecules, and intracellular targets are inefficient for therapeutic intervention using biologics. Additionally, modulation of regulatory RNAs is also challenging using small molecules and biologics. Thus, the ability of small interfering RNA (siRNA) drugs to expand the druggability of many well-defined targets at the RNA level through a simple base-pairing mechanism could profoundly influence the future of pharmaceutical development.

The ‘informational’ nature of RNAi-based drug design could significantly shorten the development timeline, especially with the streamlined platforms for high-throughput chemical synthesis, in vitro screening and in vivo validation. Typically, the identification of an siRNA drug lead candidate requires only 1–2 months, a time-frame rarely seen in the process of identification and medicinal chemistry optimization of small-molecule drug candidates. After establishing a tissue delivery platform, it becomes possible to develop a class of siRNA drugs that target many more genes within the same tissue. On average, the overall cost in an siRNA drug pipeline is significantly lower than in conventional drug pipelines when considering the short development timeline and high clinical translatability. In addition, the infrequent dosing regimen and multi-month durability of siRNA drugs will undoubtedly reduce the consumer pricing of future siRNA drugs, making them more affordable to patients.

Recent advances in nucleic acid chemistry have allowed further improvement in the durability of siRNA drugs. One or two yearly clinical visits would dramatically relieve the burden on the health-care system and encourage full patient compliance. Additional innovations to further enhance the clinical durability and potency of siRNA drugs are conceivable, considering that many advances used in the currently approved siRNA drugs, once thought difficult, were realized by applying only simple chemical modifications after understanding the fundamental principles at play. For treating many chronic diseases, including ageing-related disorders that once required lifelong repetitive dosing, the potential cumulative side effects from long-term medications may not be a significant concern with siRNA drugs.

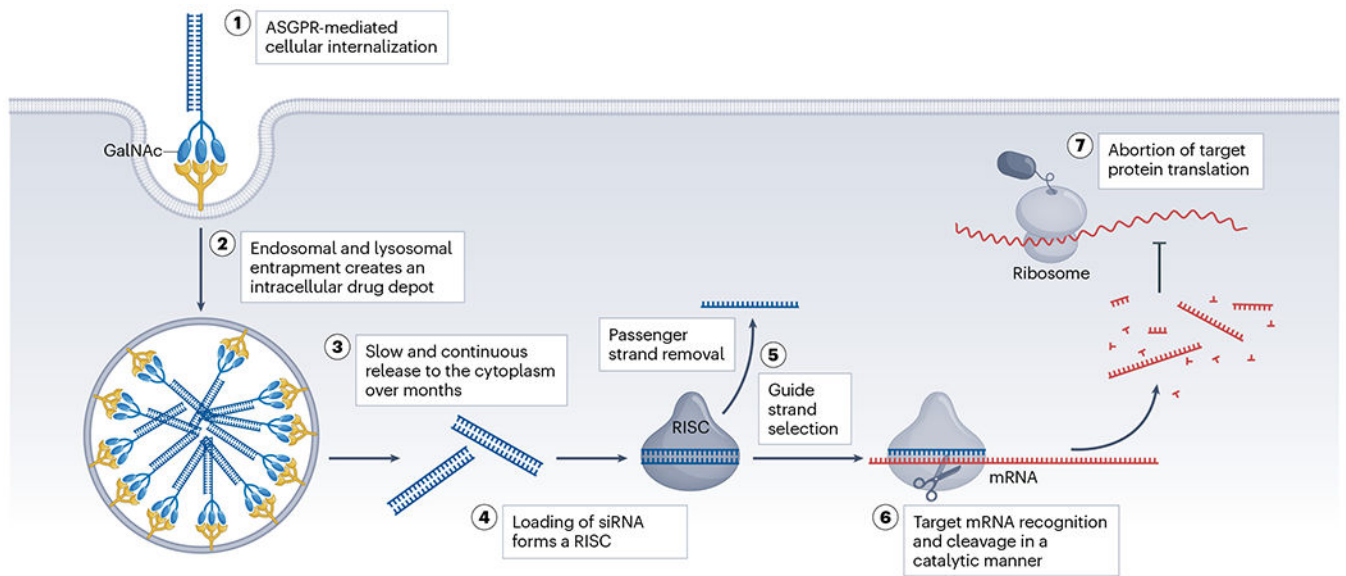


Fig. 1 | Mechanism of action of siRNA drugs.

Effective gene silencing of small interfering RNA (siRNA) drugs requires efficient cellular internalization, endosomal escape, RNA-induced silencing complex (RISC) loading, target recognition and cleavage. *N*-acetylgalactosamine (GalNAc)–siRNAs exhibit multi-month durability owing to the rapid asialoglycoprotein receptor (ASGPR)-mediated membrane endocytosis and slow release from the intracellular drug depot after internalization. ASGPRs are highly expressed in hepatocytes and have a high recycling rate (minutes) for GalNAc-conjugated oligonucleotide internalization. Entrapment of chemically stabilized siRNAs in endosomal and lysosomal compartments can serve as an intracellular drug depot to support long-term durability. After being released into the cytoplasm, the siRNA needs to be assembled into a RISC to enable guide strand selection and subsequent recognition and cleavage of complementary mRNA substrates. Argonaute 2 (Ago2) protein is the catalytic component of RISC with RNA-guided endonuclease activity to mediate target cleavage. Ab, antibody.

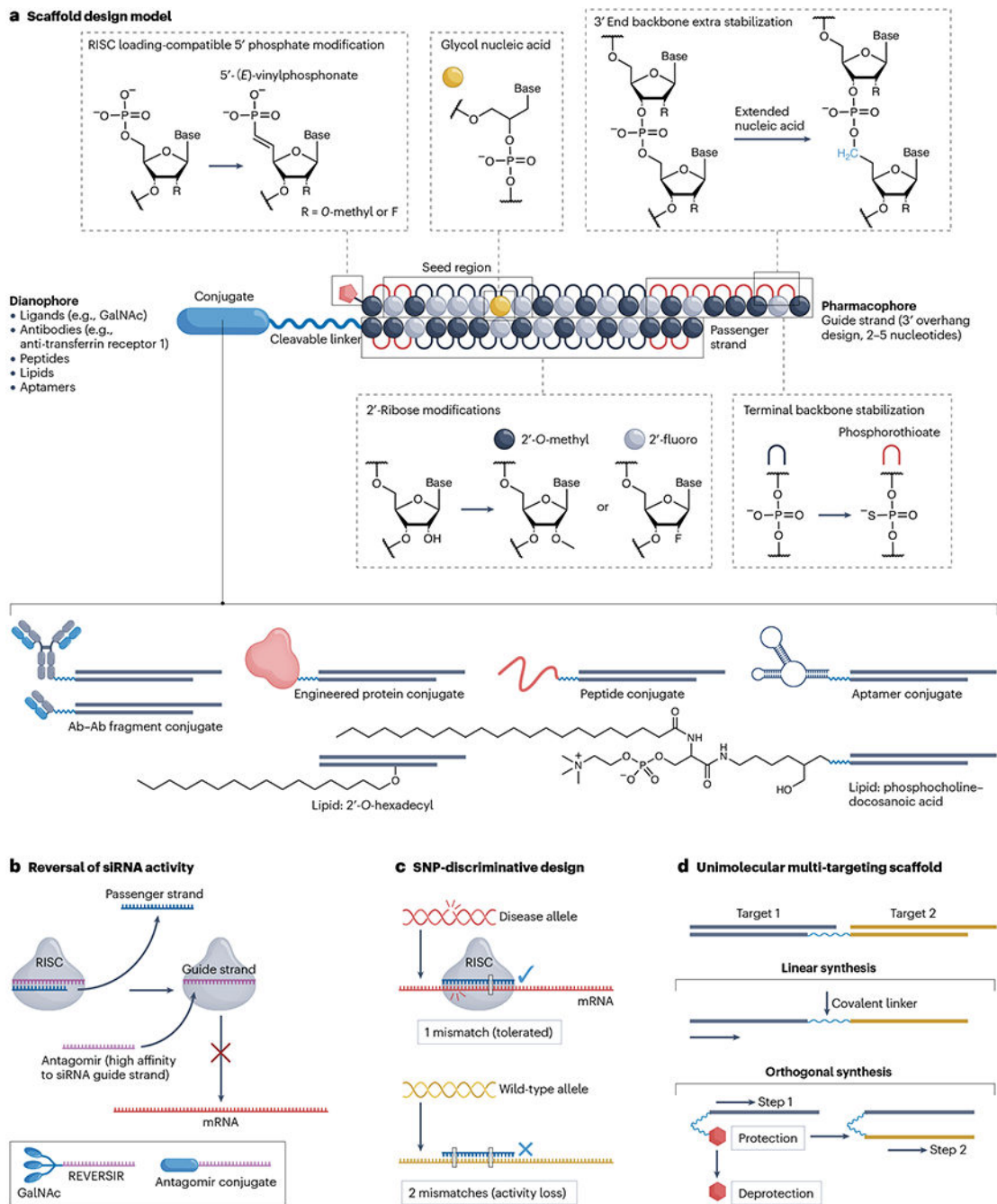


Fig. 2 | Medicinal chemistry of siRNA design.

A schematic of small interfering RNA (siRNA) nucleotide configuration, targeting entity conjugation, chemical modification and fine-tuning strategies for a specific therapeutic purpose. **a**, Scaffold design model for conjugated siRNAs. Pharmacophore: base sequence that determines siRNA specificity against target mRNA transcript; dianophore: targeting entities that dictate the tissue distribution profile of the siRNA; seed region: nucleotides 2–8 from the 5′-end of the guide strand; cleavable linker: nuclease-labile phosphodiester bonds or other designs. **b**, Antagomir (or anti-microRNA) design to reverse undesired

siRNA activity. An antagomir is a single-stranded oligonucleotide that typically uses an identical delivery strategy to its target siRNA to inactivate RNA-induced silencing complex (RISC)-loaded guide strand activity in the same tissue or cell type. **c**, Guide strand mismatch design to improve SNP-discriminative targeting on the disease-causing allele while reducing activity against the wild-type allele. **d**, Synthetic strategies for unimolecular siRNA scaffold to incorporate multiple targeting sequences (dual-targeting design shown). GalNAc, *N*-acetylgalactosamine. Part **a** adapted from ref. 156, CC BY 4.0 (<https://creativecommons.org/licenses/by/4.0/>).

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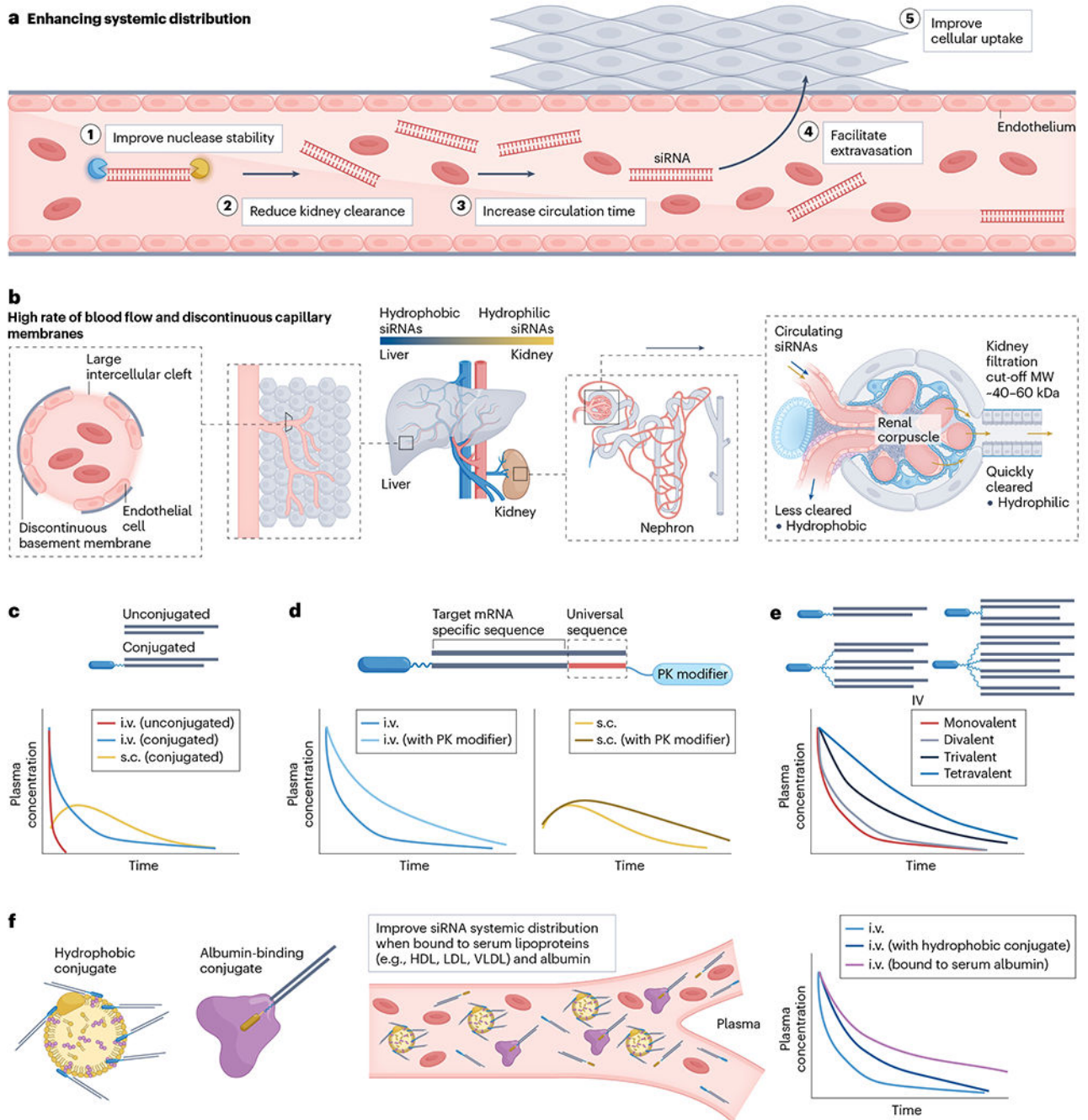


Fig. 3 | Strategies for altering pharmacokinetic profiles to improve siRNA systemic distribution and promote extrahepatic efficacy.

a. Systemic distribution is a multistep process that requires optimization of small interfering RNA (siRNA) molecular configuration to improve the efficiency. **b,** Liver and kidney are two major clearance organs that limit systemic distribution. High blood flow rate and discontinuous membrane of liver capillaries lead to a high level of accumulation of systemically injected compounds. Kidney filtration is a natural process of siRNA clearance with molecular size below the molecular weight threshold. The overall hydrophobicity and

hydrophilicity ratio of the siRNA scaffold has a significant impact on tissue clearance mode, with more hydrophobic siRNA being cleared in the liver and more hydrophilic siRNA being filtered by the kidney. **c**, Conjugation enables improvement of the area under the curve (AUC) of plasma concentration upon systemic administration. **d**, Pharmacokinetic (PK) modifiers increase the scaffold overall molecular size that drives AUC improvement. **e**, Valency of the siRNA has a significant impact on molecular size to reduce systemic clearance. **f**, Targeting entities that associate with serum lipoproteins and albumin during circulation can take advantage of the natural processes to improve the siRNA PK profile for systemic distribution. HDL, high-density lipoprotein; i.v., intravenous; LDL, low-density lipoprotein; MW, molecular weight; s.c., subcutaneous; VLDL, very low-density lipoprotein.

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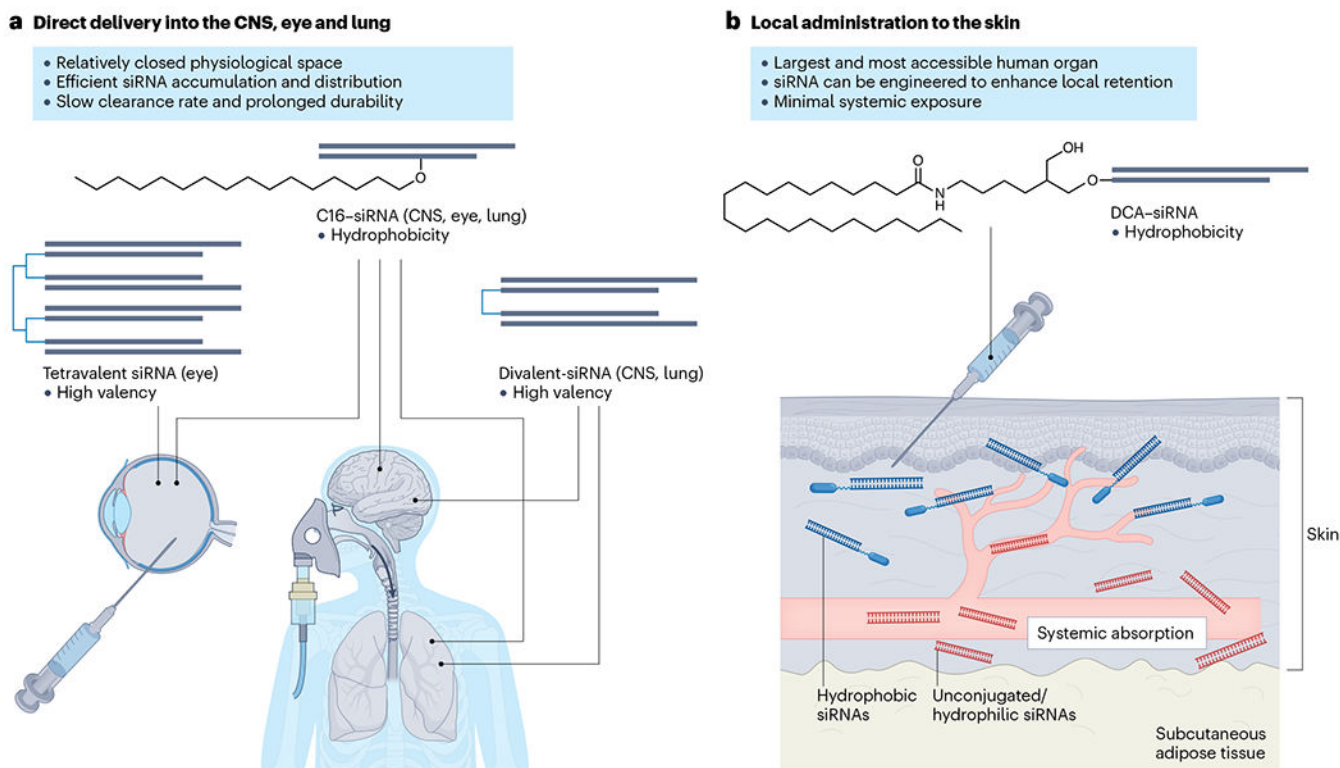


Fig. 4 |. Local delivery of siRNAs.

a, Representative examples of optimized small interfering RNA (siRNA) configurations for local delivery into accessible tissues including central nervous system (CNS), eye and lung. Lipophilic conjugates such as 2'-*O*-hexadecyl (C16) supports effective delivery into multiple tissues attributed to the increase in hydrophobicity of the siRNA. The high-valency design allows the manipulation of size and structure of the siRNA scaffold, which provides enhanced distribution and reduced clearance properties for certain in vivo applications.

b, Skin is the largest and most accessible human tissue for siRNA local administration (transdermal and intradermal). Systemic exposure of siRNA could be minimized through increasing the hydrophobicity (thus better skin retention) of the scaffold to treat localized skin diseases. Unconjugated or hydrophilic conjugates have limited local retention and cellular uptake, and thus lead to more systemic absorption. Topical application of siRNA molecules crossing the outermost skin layer (stratum corneum) poses inherent challenges that require innovations in delivery formulations. DCA, docosanoic acid.

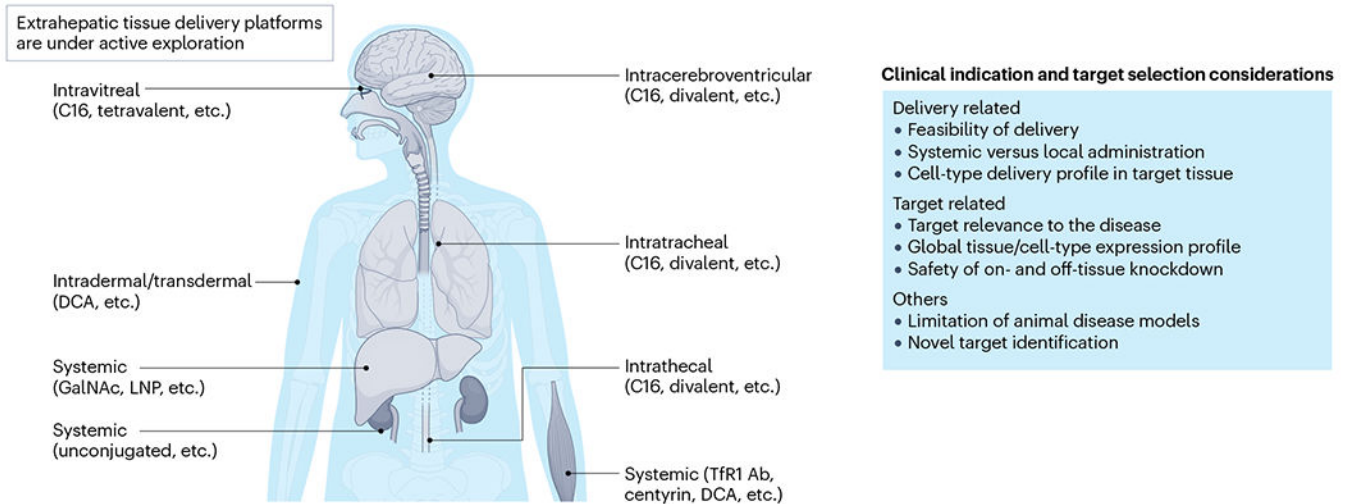


Fig. 5 |. Clinical indication and target selection for RNAi modulation.

Clinical indication and target selection should be aligned with the feasibility of current optimized delivery platforms (locally or systemically) to target tissues or cell types. The listed small interfering RNA (siRNA) conjugates or designs are approved or at least have shown robust animal or clinical proof of concept (discussed in the main text). Certain tissues such as muscle can be delivered to by either intramuscular or systemic injections depending on the platform. Systemic administration of unconjugated siRNAs to kidney is mainly through clearance mode mechanism, the compounds are mostly accumulated in proximal tubule epithelia; further technology innovation may expand the deliverable cell types in the kidney. Ab, antibody; C16, 2'-*O*-hexadecyl; DAC, docosanoic acid.

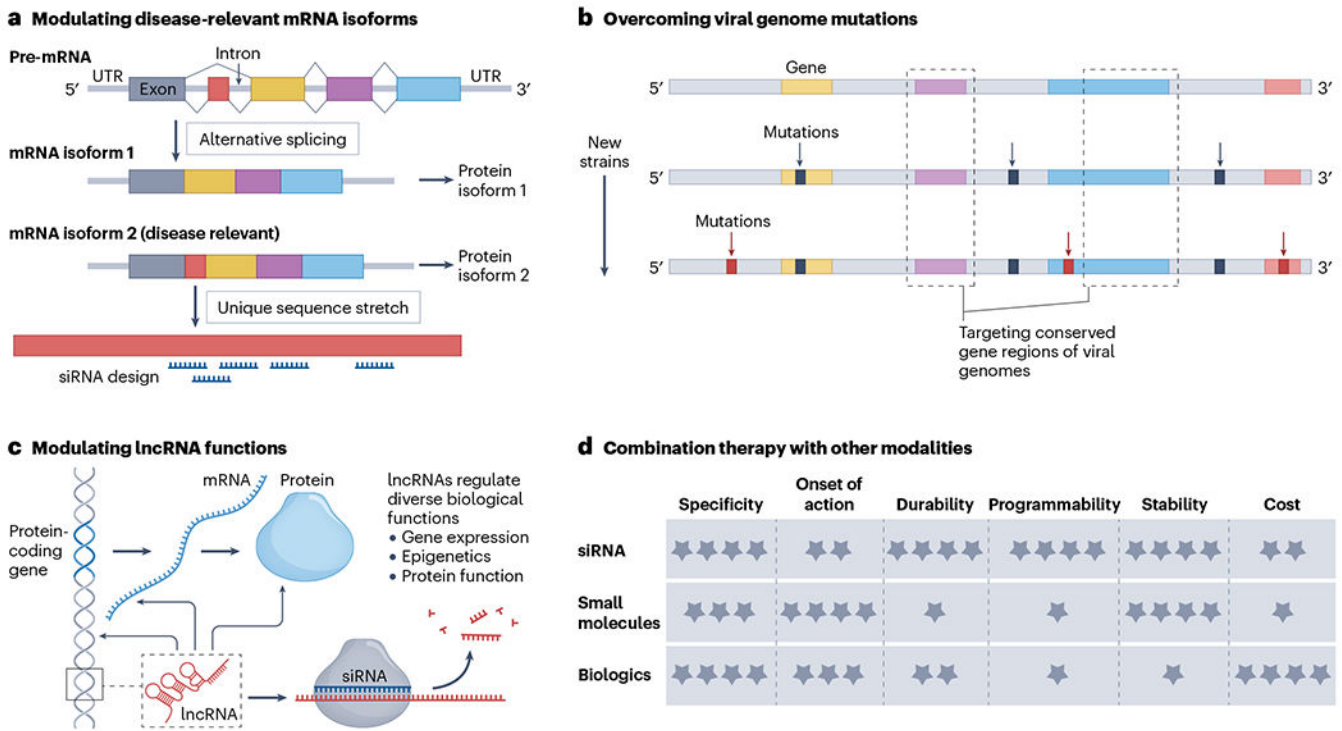


Fig. 6 | Promising future applications of siRNA drugs.

a, RNA splicing is a common post-transcriptional processing mechanism that may result in multiple regulatory or protein-coding transcript isoforms. The action of mechanism of RNAi potentiates the design of small interfering RNA (siRNA) drugs to precisely modulate transcript isoforms, particularly those that are disease relevant. **b**, Mutations in viral genomes impose substantial challenges for antiviral drugs and vaccines. The discovery pipeline of siRNA drugs not only enables targeting of conserved gene regions of viral genomes to overcome the challenges but also allows rapid design of new variants of antiviral drugs for future strains. **c**, The expanding knowledge of long non-coding RNAs (lncRNAs) indicate their role in regulation of diverse biological functions. siRNA drugs may be designed to modulate certain lncRNAs that are involved in disease progression. **d**, Combination of a siRNA drug with another drug modality can benefit the treatment outcome of certain diseases. This benefit could be from the perspectives of both target modulation and clinical practice to combine the advantages of each specific drug modality. UTR, untranslated region.

Table 1 |

Delivery technologies enabling therapeutic RNAi in various tissues

Technology	Platform	Tissue	Animal proof of concept	Clinical proof of concept	Date of approval/development stage
LNP	Formulation	Liver	~2000s: various LNP-mediated siRNA delivery platforms had been explored in vivo	2012–2013: phase I results of patisiran ALP-TTR01 (1st gen. LNP) and ALP-TTR02 (2nd gen. LNP) show effective serum reduction of TTR protein	Regulatory approval 2018: 1st drug: patisiran (ALP-TTR02, i.v. infusion) for polyneuropathy of hereditary TTR-mediated amyloidosis
GalNAc 1st gen. (STC)	Sugar conjugate	Liver	~2014: multivalent GalNAc-conjugated siRNA shows robust and prolonged gene silencing in mouse liver	2013–2016: revusiran elicits significant reduction of serum TTR	Phase III; revusiran toxicity (discontinued)
GalNAc 2nd gen. (ESC)	Sugar conjugate	Liver	2014 TIDES meeting: ESC-GalNAc improves potency and durability in NHP	ESC-GalNAc is applied in all advanced clinical programmes	Regulatory approval 2019: givosiran 2020: lumasiran 2020 (EU), 2021: inclisiran 2022: vuirsiran 2023: nedosiran (GalXC platform, stabilization similar to ESC)
GalNAc 3rd gen. (ESC+ with glycol nucleic acid modification)	Sugar conjugate	Liver	2022: local administration of C16-conjugated siRNAs enable potent and durable gene silencing in CNS, eye and lung in rodents and NHP	2023: phase I trial shows promising data for ALN-APP targeting APP for the treatment of Alzheimer disease and cerebral amyloid angiopathy	2023: phase II study of zilebesiran demonstrates encouraging efficacy and tolerability profile in adult patients with mild-to-moderate hypertension
C16	Lipophilic conjugate	CNS Eye Lung	2022: local administration of C16-conjugated siRNAs enable potent and durable gene silencing in CNS, eye and lung in rodents and NHP	2023: phase I trial shows promising data for ALN-APP targeting APP for the treatment of Alzheimer disease and cerebral amyloid angiopathy	Phase I
Cholesterol	Lipophilic conjugate	Placenta	2018: cholesterol-conjugated siRNA enables placental reduction of sFLIT in pregnant mice and NHP, ameliorating clinical signs of pre-eclampsia	NA	2018: preclinical (discontinued)
PC-DCA	Lipophilic conjugate	Placenta	2022: PC-DCA conjugation increases siRNA accumulation and silencing efficacy of sFLIT in placenta of pregnant mice	2023: PC-DCA-siRNA under phase I trial shows uneventful safety in healthy volunteers	Phase I
DCA	Lipophilic conjugate	Muscle Heart Skin	2019–2023: DCA conjugation allows enhanced siRNA local retention and systemic distribution to multiple extrahepatic tissues with functional gene silencing 2022–2023: DCA-siRNA offers enhanced local retention and functional gene silencing in rodent and pig skin	NA	Preclinical IND-enabling
Anti-TTR I antibody	Protein conjugate	Muscle	2021: AOC 1001 (anti-TTR I siRNA) effectively reduces levels of <i>DMPK</i> mRNA in muscle of NHP	2023: phase I/II trial of AOC 1001 demonstrates functional improvement, DMPK reduction, splicing improvements and safety	2024: phase III expected

Technology	Platform	Tissue	Animal proof of concept	Clinical proof of concept	Date of approval/development stage
Centyrins	Protein conjugate	Muscle	2022: CD71 centyrin-siRNA shows robust muscle gene modulation in Pompe disease mouse model	NA	2023: phase I trial of ABX1100 initiated for the treatment of Pompe disease
Centyrins	Protein conjugate	Tumour	2021: EGFR centyrin-siRNA conjugates show effective tumour delivery in mouse models	NA	Preclinical
Divalent scaffold	Size/structure	CNS lung	2019: intracerebroventricular injection of divalent siRNA supports potent and sustained gene silencing in the brain of mice and NHP for months 2022: intratracheal delivery of divalent siRNA enables effective suppression of SARS-CoV-2 in mouse model of pulmonary infection	NA	Preclinical
Tetravalent scaffold	Size/structure	Eye	2023: intravitreal injection induces potent and multi-month gene silencing in rodent and pig eyes	NA	Preclinical

Representative siRNA delivery platforms that show promising in vivo proof of concept (POC). The timeline of major POC events reflects the most recognized research publications or conference reports that aim to provide a general overview of the technology progress. All events listed are discussed in the main text with references cited. APP, amyloid precursor protein; C16, 2'-*O*-hexadecyl; CNS, central nervous system; DCA, docosanoic acid; DMPK, myotonin-protein kinase; ESC, enhanced stabilization chemistry; EU, European Union; GalNAc, *N*-acetylgalactosamine; gen., generation; i.v., intravenous; LNP, lipid nanoparticle; NA, not applicable; NHP, non-human primate; PC, phosphocholine; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; siRNA, small interfering RNA; STC, standard template chemistry; TIR1, transferrin receptor 1; TTR, transthyretin.