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Author manuscript

*Nat Biotechnol*. Author manuscript; available in PMC 2017 September 01.

Published in final edited form as:

*Nat Biotechnol*. 2017 March ; 35(3): 238–248. doi:10.1038/nbt.3765.

# The chemical evolution of oligonucleotide therapies of clinical utility

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## Abstract

After nearly 40 years of development, oligonucleotide therapeutics are nearing meaningful clinical productivity. One of the key advantages of oligonucleotide drugs is that their delivery and potency properties are derived primarily from the chemical structure of the oligonucleotide, while their target is defined by the base sequence. Thus, as oligonucleotides with a particular chemical design demonstrate appropriate distribution and safety profiles for clinical gene silencing in a particular tissue, this will open the door to the rapid development of additional drugs targeting other disease-associated genes in the same tissue. To achieve clinical productivity, the chemical architecture of the oligonucleotide needs to be optimized as a whole, using a combination of sugar, backbone, nucleobase and 3′/5′-terminal modifications. A portfolio of chemistries can be used to confer drug like properties onto the oligonucleotide as a whole, with minor chemical changes often translating into major improvements in clinical efficacy. Outstanding challenges in oligonucleotide chemical development include optimization of chemical architectures to ensure long-term safety and to enable robust clinical activity beyond the liver.

The informational nature of oligonucleotide drugs[1] (i.e., drug design based on sequence information) promised to lend itself well to the post-genomic era of medicine. Researchers were drawn by the promise of rapid and rational design of drugs against virtually any genetic target. However, it has taken over three decades for these therapies to reach clinical maturity

As with any therapeutic modality, the success of an oligonucleotide drug is defined both by its ability to affect its target and by its pharmacokinetic behavior, including absorption, distribution, metabolism, and excretion (ADME). Oligonucleotide therapeutics comprise a diverse class of drugs, including small-interfering RNAs (siRNAs)[2], antisense oligonucleotides (ASOs)[3], microRNAs[4], aptamers[5], and others[6]. As these all work by different mechanisms, the activity and pharmacokinetic properties can be, to some extent[7], be independently optimized (Fig. 1). In contrast, for traditional small-molecule drugs these are inseparable, necessitating a unique, iterative process of optimization for each drug.

The pharmacokinetic properties of a drug depend on a set of molecular features we refer to as the dianophore, from the Greek ‘dianomi’ for distribution or delivery. For oligonucleotide

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drugs, the dianophore is largely defined by chemical and structural architecture, such as chemical modifications of sugars, bases, and phosphate backbone, single strand or duplex structure, and the presence or absence of a targeting ligand. In contrast, the pharmacophore (the ensemble of molecular features that determine target regulation) is defined by its nucleotide sequence.

Although base sequence and the precise pattern of chemical modifications can affect the global properties of an oligonucleotide and can affect its trafficking, cellular uptake, and other behaviors[7], the ability to separately optimize the pharmacophore and dianophore, at least to some extent, is a key advantage of oligonucleotide drugs. Development of an optimized dianophore, a chemical architecture enabling effective delivery to a certain tissue, enables rapid progression of multiple drugs with a predictable ADME profile for multiple indications, as long as the same tissue and cell type is being involved in disease progression (e.g., siRNAs formulated in lipid nanoparticles for the liver or *N*-acetylgalactosamine (GalNAc)-conjugated ASOs and siRNAs for hepatocytes).

Early on, unmodified or minimally modified compounds were rushed to the clinic without conjugates or delivery vehicles. Massive dose requirements and limited clinical efficacy created a dramatically negative view of the technology, damaging the reputation of the field of oligonucleotide therapeutics for years. A consequent decrease in available funding delayed progress. But advances in oligonucleotide chemistry and an understanding of fundamental principles that define the *in vivo* behavior of oligonucleotides have enabled oligonucleotide therapeutics to approach clinical productivity (at least in some tissues).

As a result, the current pipeline of oligonucleotide drugs is broad, including varied molecules with different mechanisms of action. In hepatitis B virus (HBV) treatment, for example, four oligonucleotide drugs are currently undergoing human testing. Two are siRNAs (Vancouver, British Columbia-based Arbutus is using a lipid nanoparticle (LNP) and Cambridge, Mass.-headquartered Alnylam a GalNAc conjugate) whereas Ionis (Carlsbad, CA) is developing both naked and GalNAc-conjugated ASOs. The fact that four platforms are simultaneously being tested allows several shots on goal, and the clinical comparison of these four platforms, for the same tissue and disease, will surely inform the direction of future clinical development of oligonucleotide drugs in the liver.

In this review, we describe current aspects of the evolution of the chemistry of both antisense oligonucleotides and siRNAs that have opened the way for clinical utility. We place particular emphasis on ASO and siRNA conjugates currently in human testing. Advances in nucleic acid chemistry that are earlier in the preclinical pipeline have been reviewed elsewhere [8-11].

## Chemical evolution of ASOs

In 1978, Zamecnik and Stephenson demonstrated that an oligonucleotide ‘antisense’ (i.e., complementary) to a viral RNA could reduce protein translation and viral replication[12, 13]. It is now clear that ASOs can make use of multiple mechanisms to reduce or modulate gene

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expression[14]. Nonetheless, all ASOs require chemical modification to be sufficiently active *in vivo*.

The first chemical modification applied to antisense technology is still the most widely used: the phosphorothioate backbone (Fig. 2).[15] Although originally incorporated to provide nuclease stability, the major impact of phosphorothioate modification has been on oligonucleotide trafficking and uptake[15-18]. ASOs bearing phosphorothioate linkages are compatible with recruitment of RNase H, which cleaves the targets of ASOs.

Although they improve oligonucleotide stability, phosphorothioates alone do not fully protect ASOs from nucleases and the *in vivo* efficacy of first-generation ASOs (which comprised fully PS DNA; Fig. 4) required repeated administration at high doses. Moreover, phosphorothioates reduce the binding affinity of an oligonucleotide toward its RNA target. Improved stability and increased affinity have been achieved using nucleotides with sugar modifications, including 2′-modified and conformationally constrained nucleotides (Fig. 2).

The 2′-*O*-methyl modification of RNA (2′OMe-RNA), which occurs in nature, improves binding affinity and nuclease resistance[19-21] and reduces immune stimulation[22]. Using 2′-*O*-methyl as a starting point, medicinal chemists worked to find an ideal 2′-*O*-alkyl substituent[23-27]. Among dozens of variants tested, 2′-methoxyethyl (MOE)[28] emerged as one of the most useful analogs, providing a further increase in nuclease resistance and a jump in binding affinity of $T_m$ 0.9°C to 1.7°C per modified nucleotide. The approved antisense drug mipomersen, as well as numerous oligonucleotide drugs currently in clinical trials, carry the 2′-MOE modification. ASO affinity can also be increased with 2′-fluoro modification of RNA (2′F-RNA, $T_m$ ~2.5°C per modified nucleotide).

Reducing the conformational flexibility of nucleotides can increase their binding affinity[29, 30] Locked nucleic acid (LNA), which links the 2′ oxygen and 4′ carbon of ribose, show unprecedented increases in binding affinity ( $T_m$ 4°C to 8°C per modification when binding RNA[31-33]). The very high binding affinity of LNA and its methylated analog, known as ‘constrained ethyl’ or cEt (Fig. 2), have opened entirely new doors in nucleic acid chemical biology and therapeutics (Fig. 3)[34]. Tricyclo DNA (tcDNA) is another constrained nucleotide based on a very different three-ring scaffold[35]. Its binding affinity ( $T_m$ ~2°C ) is smaller than that of LNA, but it has shown much promise in splice-switching applications, for reasons that are not fully understood[36].

This variety of sugar modifications can be used to make chimeric oligonucleotides with very high binding affinities or to help offset negative effects caused by another modification. For example, fully LNA-modified oligomers longer than approximately eight nucleotides tend to aggregate, so LNA and cEt modifications are often used in chimeric oligonucleotides containing multiple types of modified nucleotides (e.g., mixtures of LNA/DNA or LNA/2′OMe/MOE-RNA). Although MOE and tcDNA have lower binding affinities per modification than LNA, they can both be used to make longer, fully modified oligomers.

An ASO that simply binds and blocks its RNA target requires relatively few constraints on chemistry besides nuclease resistance and high binding affinity. If an enzyme is required,

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such as RNase H or Argonaute, the constraints on chemical modification are more complex. Below, we describe the two most common categories of ASO in turn.

## RNase H-dependent ASOs

RNase H cleaves the RNA strand of a DNA:RNA hybrid; as such, the sugar-modified RNA-like nucleotides described above do not elicit RNase H cleavage of complementary RNA. The most common solution, called a ‘gapmer’ ASO, consists of a central window (i.e., a gap) of PS DNA, which recruits RNase H, flanked by modified RNA-like nucleotides (Fig. 2).

There are no hard and fast rules about gapmer symmetry. Asymmetric ASOs with the high-affinity modifications on one end of the oligonucleotide can also be used, sometimes with a cap or ligand on the other end to help prevent nucleolytic decay[37]. The overall affinity of an oligomer for its target needs to be high enough to displace RNA secondary structure or compete with RNA-binding proteins. But cleaved target RNA fragments must be released before an ASO can find, bind and cleave the next target, so overemphasis on a molecule's target affinity can reduce potency *in vivo*[38].

Short (12 to 15 nucleotide) gapmer ASOs built with LNA and cEt nucleotides tend to be more potent than longer oligonucleotides built with lower-affinity chemistry[39, 40]. Thus, the high binding affinity of the constrained ribose allows shorter oligomers to bind their RNA targets with sufficient affinity to be functional. The improved potency translates to a wider range of tissues than can be accessed by systemic administration of naked ASOs[17].

LNA and cEt ASOs have been associated with liver toxicity[41]. The risk of toxicity seems to apply equally to LNA and cEt, despite previous reports to the contrary, and is sequence-dependent. In the past year, three groups independently demonstrated that LNA and cEt gapmer ASOs induce liver toxicity by directing off-target RNase H cleavage of mismatched transcripts, particularly within introns[42-44]. Armed with this information, computational methods can be used to select ASOs with minimal complementarity to off-target transcripts (including introns).

Chemistry can be used to improve ASO specificity. Gapmer ASOs that are highly selective for single-nucleotide polymorphisms (SNPs) have been developed using combinations of modifications—including 2-thiothymidine, 3′-fluorohexitol nucleic acid (FHNA), cEt, a 5-modified pyrimidine base, and an analog called α,β constrained nucleic acid (α,β–CNA) in which the phosphate is included in a ring structure (Fig. 2)—in combination with shorter gaps[45–46]. These gapmers minimize the region that can be cleaved by RNase H without reducing cleavage of the desired site (e.g., a disease allele), but a mismatch near the desired cleavage site (i.e., normal allele) incurs a major loss of cleavage activity[47]. SNP-selective ASOs to treat Huntington's disease are expected to be the first to enter the clinic. It remains to be seen how readily the principles used for SNP selectivity can be applied to the more general problem of target selectivity.

As an alternative to the gapmer approach, modifications that adopt a DNA-like conformation can also be used to improve affinity and stability of RNase H compatible ASOs.

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Fluoroarabinonucleic acid (2′F-ANA) is the paradigmatic example of this approach[48, 49]. Although 2′F-ANA modification at every position of an ASO increases stability and affinity, the RNase H cleavage rate drops substantially. But rapid kinetics of cleavage can be restored by combining 2′F-ANA with DNA[50, 51]. 2′F-ANA and other DNA mimics are thus valuable tools for tuning the thermodynamic properties of RNase H-dependent ASOs.

## Steric blocker ASOs

The second major class of ASOs does not seek to recruit RNase H, and therefore a DNA-like gap in the oligonucleotide is unnecessary. This class of ASOs has seen two major clinical uses to date: Splice switching and microRNA (miRNA) inhibition.

In the past year, two splice switching oligonucleotides have achieved clinical success. Last August, the US Food and Drug Administration (FDA; Rockville, MD) approved eteplirsen (Sarepta), a 30-mer phosphorodiamidate morpholino oligomer (PMO; Fig. 2) for treatment of Duchenne muscular dystrophy[52]. The molecule was approved, despite controversy over the levels of eteplirsen that actually reached muscle tissue and the degree of splice switching attained. Four months later, nusinersen (Spinraza), a fully MOE-modified 18-mer ASO that redirects the splicing of *SMN2* gene[53], was approved for treatment of spinal muscular atrophy[54].

Several chemical approaches have been used for oligomer-mediated miRNA inhibition[55]. A direct comparison of anti-miRNAs (anti-mIRs) showed that chimeric LNA/2′OMe-RNA oligomers with phosphorothioate backbones are the most potent[56]. Researchers generally design anti-miRs to be complementary to the mature miRNA sequence and thereby inhibit them directly, but in some cases, anti-miRs can also target or disrupt the precursor miRNA structures and inhibit miRNA maturation[57]. A family of miRNAs that shares a common seed sequence can be inhibited by a single, short (8-nucleotide) oligomer that is fully modified with LNA[58]. These ultra-short oligomers sometimes show enhanced distribution in some tissues compared with longer anti-miRs.

## Other ASO developments

The length of an ASO contributes to its dianophore, affecting distribution and tissue uptake. Shorter ASOs tend to distribute more to the kidney, and longer oligomers to the liver[59]. Shorter ASOs bind plasma protein poorly, and consequently have a short half-life in plasma, but they can be assembled into multimers using cleavable linkers[60].

Idera Pharmaceuticals (Cambridge, MA) has found that connecting two first-generation phosphorothioate-modified ASOs by their 5′ ends (leaving the 3′ends exposed) substantially increases the potency of gene silencing and reduces innate immune activation[61]. This approach may provide an independent way to increase potency and specificity.

The phosphorothioate linkage introduces a stereocenter at phosphorus, and oligonucleotides are normally a mixture of $2^{n-1}$ diastereomers (e.g., an 18-mer phosphorothioate oligonucleotide has $2^{17}$ diastereomers). The $S_p$ and $R_p$ diastereomeric linkages have different properties: the $R_p$ diastereomer is less resistant to nucleases than the $S_p$

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diastereomer, but it binds with higher affinity and elicits RNase H more effectively[62-64]. Overall, uniformly stereopure phosphorothioate ASOs (i.e., all-$S_p$ or all-$R_p$) are inferior to the stereorandom phosphorothioate ASOs. Precise patterns of alternating stereochemistry at phosphorus (e.g., $R_pR_pS_p$ and $S_pS_pR_p$) may improve mismatch discrimination and RNase H activity compared with stereorandom or stereopure oligonucletides[65]. Based on this principle, WaVe Life Sciences (Singapore) is planning to advance a stereo-defined SNP-selective ASO drug to treat Huntington's disease to clinical trials. Because specificity and mismatch discrimination are becoming increasingly important in ASO therapeutics, the increased specificity of stereoselective PS ASOs may find wide application in improving other drug candidates.

## Chemical evolution of siRNAs

RNAi was discovered in 1998 (ref. 66), and the demonstration that RNAi silences gene expression in mammalian cells in 2001 (ref. 67)—which roughly coincided with completion of the human genome sequence. This resulted in an explosion of interest in, and funding for, RNAi. The original hope was that siRNAs (the double-stranded oligonucleotide triggers of RNAi) could be used to silence any gene in any cell. Several biotech companies, including the flagship RNAi company Alynlam (Cambridge, MA) and many major pharmaceutical companies entered the fray (Fig. 1b). Confident in the power of RNAi, in which an siRNA becomes associated with Argonaute and other proteins to form the RNA-induced silencing complex (RISC) and cleave complementary RNA, programs moved rapidly toward the clinic, mostly using local delivery by eye injection or intranasal spray[68,69].

In many of these early programs, completely unmodified or slightly modified compounds were administered in the hope that a small but sufficient amount of oligonucleotide would be taken up by the appropriate cells and silence the target. Ultimately, most of these attempts showed limited clinical efficacy and unacceptable toxicity, primarily from induction of the innate immune response by non-modified duplex RNAs. Thus, chemical modification of siRNA is absolutely necessary to achieve clinical utility.

The significant legacy of nucleic acid chemistry developed for ASO therapeutics sped up the evolution of RNAi technology tremendously[70]. Nevertheless, the molecular requirements for effective recruitment of the RNAi enzymatic machinery and the double-stranded nature of RNAi imposed a unique set of limitations on the chemical modification of siRNAs, which took years of investigation to overcome.

### Metabolic stabilization

When injected into the bloodstream, naked siRNAs are degraded within minutes[71]. Studies quickly revealed, however, that relatively few chemical modifications are sufficient to increase stability, prevent innate immune activation[72] and reduce off-target effects[73]. Extensive modification of siRNAs (~50% of nucleotides) doesn't significantly increase the duration of silencing *in vivo*, when siRNAs are delivered by lipid nanoparticles or hydrodynamic injection[71,74]. Moreover, the RNAi machinery can efficiently bind heavily modified siRNAs (i.e., most or all ribose content removed)[75-78], but extensive modification can negatively impact efficacy. Consequently, the idea that a minimal number of

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modifications could improve stability and activity *in vivo* was viewed as a key advantage of RNAi technology over antisense for years. (This minimal modification has more recently proven inadequate for conjugate-mediated delivery; see below).

Initial siRNA compounds were therefore modified at only a few positions. Many different chemical configurations have been used to stabilize siRNAs, particularly combinations of 2′OMe, 2′F, and phosphorothioate[72, 79, 80]. Modifications that increase or decrease sugar flexibility have also been explored, including LNA and unlocked nucleic acid (UNA)[81], but they are mainly used to introduce chemical asymmetry into duplex siRNAs. That is, they block passenger strand entry and promote RISC loading of the guide strand, which can also be easily achieved by 2′OMe modification of the two nucleotides at the 5′ end of the passenger strand[73].

The most common configurations included modification of terminal nucleotides[82], of every second sugar with 2′OMe[83], or of all pyrimidines. The popularity of the last stemmed from the high cost and low availability of 2′F-modified purines, which only recently became widely accessible. The guide strand must bind efficiently to the RNAi machinery, and is therefore more sensitive to chemical modification. 2′-F, which is the best mimic of the 2′-OH group by size and charge, is generally well tolerated and has been used extensively as a primary guide strand modification[84]. Often, the guide strand is modified with 2′F and sense strand with 2′OMe[85].

Modifications typically interfere with silencing activity by making the duplex too stable, which prevents removal of the passenger strand and interferes with proper loading of guide strand, or by forcing the nucleic acid into a suboptimal geometry[86]. The 2′-F and 2′-OMe modifications favor the C3′-endo ribose conformation and support the A-form helical structure of the guide strand, which positions the target mRNA into the cleavage center of RISC[87]. But both modifications introduce slight structural distortions. 2′F-RNA slightly overwinds the duplex (more stacking, higher $T_m$) and 2′OMe-RNA slightly underwinds the duplex (less stacking). Either modification is tolerated in any individual position of an siRNA[76], but a fully modified 2′OMe guide strand is completely inactive, and a fully modified 2′F guide strand often has substantially reduced activity[78]. When 2′OMe and 2′F modifications are alternated, however, the combination creates a compound ideally suited for RISC assembly and function[75].

Thermodynamic or structural tuning[88] may further enhance the efficacy of modified siRNAs. Many of the advanced clinical compounds carry additional stretches of 2′OMe/2′F (e.g., three in a row[89]) in the context of the alternating 2′F/ 2′OMe-RNA pattern (Fig. 3). The pattern was designed to chemically mimic the sinusoidal thermodynamic stability described for highly functional siRNAs[90]. An ideal guide strand has: a more flexible 5′ end, which can be easily introduced by structural and chemical modifications[73]; a high affinity ‘seed’ region, which drives the initial base pairing between the guide strand and target; and a lower affinity 3′-region required for product release. This profile was initially derived by comparing active and non-active siRNAs[90], but recent single-molecule RISC studies provide a clear mechanistic explanation[91]. Structures of fully modified siRNAs bound to Ago2 will

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also enable more precise tuning of modification patterns to optimize RISC binding and activity[92].

Additional nuclease stability is conferred by backbone modifications[14]. Limited phosphorothioates are tolerated by Ago2, and phosphorothioate modifications at both ends of both strands of an siRNA duplex are incorporated into many of the leading clinical candidates. This simple combination of backbone and sugar modification provides additional resistance to exonucleases—the primary effectors of RNA degradation—and an order of magnitude increase in oligonucleotide accumulation *in vivo*. Methylation of the 5′ carbon to give (*S*)-5′-*C*-methyl-RNA[93] has also been used to enhance 3′-exonuclease resistance.

## 5′-phosphate stabilization

The 5′-phosphate of a siRNA guide strand is essential for recognition by RISC[94-96]. siRNAs with a 5′-hydroxyl are efficiently phosphorylated and loaded onto Ago2 inside cells[97]. Blocking phosphorylation of the 5′-hydroxyl in siRNA prevents RISC loading and activity[98]. Chemical modification (e.g., 2′OMe or 2′F) of the 5′-ribose of the guide strand can interfere with intracellular phosphorylation but the activity of these 5′-modified guide strands can be restored if a 5′-phosphate is introduced chemically[99,75]. Chemical phosphorylation does not significantly increase the cost or complexity of chemical synthesis, and most commercial sources of modified siRNAs add a 5′-phosphate chemically. However, when dosed systemically, the 5′-phosphate is quickly removed by phosphatases, resulting in an accumulation of biologically inactive siRNAs. Within two hours after intravenous administration, at least 90% of fully modified siRNAs are dephosphorylated, and within 24 hours the phosphorylated guide strand is essentially undetectable (R. Haraszti, L. Roux, and A. Khvorova, unpublished data).

Phosphatase-resistant analogues of the 5′-phosphate can improve *in vivo* efficacy[100]. Ionis modified the 5′end of single-stranded siRNA (ss-siRNAs) with *E*-vinyl phosphonate (5′-*E*-VP), which substitutes the bridging oxygen with carbon in the context of a double bond (Fig. 2)[101, 102]. The 5′-*E*-VP is in a suitable conformation for RISC binding, whereas the other stereoisomer (5′-*Z*-VP) shows reduced activity due to inappropriate positioning of the phosphonate[92, 103]. In this context, 5′ chemical stabilization was absolutely essential for the *in vivo* efficacy of ss-siRNAs[36, 102].

5′-*E*-VP has a major impact on the *in vivo* efficacy of GalNAc-conjugated siRNAs[100], discussed below. The effect is not specific to GalNAc: phosphate stabilization of hydrophobically modified siRNAs significantly enhances the distribution, accumulation, and retention of intact oligonucleotide in primary and secondary tissues, and extends the duration-of-effect beyond a month after injection (R. Haraszti, L. Roux, and A. Khvorova, unpublished data). In the absence of lipid formulation, therefore, metabolic stabilization of the 5′-phosphate is essential for stability, biodistribution, activity and duration-of-effect of therapeutic siRNAs *in vivo.* Notably, phosphate stabilization also increases the accumulation of guide strand in tissues, probably because it provides additional protection from XRN1-mediated hydrolysis. XRN1 is the primary cellular nuclease that rapidly degrades 5′-

phosphorylated RNA and DNA, but it does not recognize metabolically stable 5′-phosphate analogs (R. Haraszti, L. Roux, and A. Khvorova, unpublished).

Chemical stabilization of the 5′-phosphate without interfering with RISC recognition can be accomplished in multiple ways (Fig. 2). Though 5′-*E*-VP has been explored extensively, 5′-methyl phosphonate, 5′-*C*-methyl analog, and phosphorothioate all increase siRNA stability and are well tolerated by RISC[104], similar to 5′-*E*-VP[36]. Many are simpler modifications from a synthetic chemistry perspective, and it remains to be seen which approach will gain wide acceptance.

## Conjugate mediated delivery

The *in vivo* efficacy of oligonucleotides is defined by blood flow, tissue structure, receptor-mediated cellular uptake, and endosomal escape. It is not surprising therefore that simple injection of a large amount of non-modified or partially modified siRNA was so ineffective. Lipid formulation of siRNAs has been a mainstay of siRNA delivery since the first demonstration of RNAi in human cells[105,106], and advances in lipid chemistry have substantially enhanced the efficacy and therapeutic index of formulated siRNAs (reviewed in refs 66,104,105). Indeed, several lipid-formulated siRNAs have moved ahead clinically, including patisiran (Alnylam), which targets the *TTR* gene and is in a phase 3 clinical trial to treat hereditary transthyretin amyloidosis.

Apart from lipids, conjugate-mediated delivery is also emerging as an important component of the delivery toolbox[107]. Indeed, the development of oligonucleotide drugs conjugated to GalNAc can be applied to all types of oligonucleotide therapeutics to treat liver diseases (Table 1). GalNAc is the ligand for the asialoglycoprotein receptor (ASGPR), which is very abundant in hepatocytes (~0.5- to 1-million copies per cell) and quickly recycled (15 minutes). The concept of using trivalent-GalNAc clusters for drug delivery to hepatocytes was first shown in 1987 (ref. 107) and for oligonucleotide delivery in 1995 (ref. 108), but it took almost two decades of development for GalNAc-conjugated oligonucleotides to reach the current level of clinical excitement[108].

For best results, GalNAc conjugation requires a metabolically stable oligonucleotide scaffold; that is, modification of every nucleotide to remove all ribose moieties and metabolic stabilization of the 5′-phosphate[109]. The resulting GalNAc-conjugated siRNA and ASO compounds show exceptional stability and duration-of-effect, allowing monthly or even semiannual subcutaneous injections.

GalNAc modification underscores the important role of interplay between ligand and oligonucleotide backbone. In the context of metabolically stabilized siRNAs, GalNAc preferentially delivers to liver. In the context of fully phosphorothioate ASOs, the GalNAc conjugate enhances delivery to, and efficacy in, liver but a significant fraction distributes to kidneys as well, this latter uptake mediated by the PS oligonucleotide backbone rather than the GalNAc moiety. Tuning the number of GalNAc moieties per oligonucleotide influences this distribution (i.e. greater than three GalNAc molecules per ASO drives preferential delivery to liver[110]). Interestingly, the presence of phosphorothioate bonds enhances the



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potency of GalNAc-delivered siRNAs[109]. Thus, tuning chemistry and structure is therefore a complex and multi-dimensional process.

The most clinically advanced GalNAc–siRNA conjugate, revusiran, had limited metabolic stability and was withdrawn from clinical development in October 2016. The drug was in phase 3 clinical trials for transthyretin amyloidosis with cardiomyopathy, and the data monitoring committee indicated that "the benefit-risk profile for revusiran no longer supported continued dosing. Will this setback affect other GalNAc conjugates in the pipeline? It is too early to say, but the use of a conjugate with limited metabolic stability and the focus on patients with highly advanced disease were likely the two major contributing factors for revusiran's failure. Revusiran was given at high doses (~2 g loading dose followed by 400 mg per week, corresponding to a yearly exposure of 20–25 g). In contrast, siRNA conjugates based on next-generation technology are more extensively stabilized; for example, recent data from inclisiran (an siRNA targeting *PCSK9*) shows 6–9 month clinical efficacy with a single injection of 300mg[111]. In addition, inclisiran has approximately two-fold lower 2′F-RNA content than revusiran, which might reduce exposure to potentially toxic 2′-fluororibonucleotide metabolites. There is some evidence that phosphorothioate 2′F-RNA-modified oligonucleotides might cause non-sequence-specific loss of some cellular proteins,[112] though the extent of in vitro toxicity is heavily dependent on structure (double-stranded vs single stranded) and method of delivery[113].

The development of GalNAc siRNA[108, 114-116] and ASO[117-119] conjugates may play a big role in defining a useful dianophore for therapeutic oligonucleotides for silencing in hepatocytes. The combination of blood flow to the liver, discontinuous endothelium, and high receptor expression level, all work together to achieve sufficient uptake and to support multi-month efficacy with a single injection. In the long term, trivalent-GalNAc conjugates will likely be the clinically dominant approach for delivery to hepatocytes, with its wide therapeutic index and excellent safety profile. It is possible that monthly or quarterly subcutaneous injections of oligonucleotides might be preferred over daily oral regimen, an unforeseeable concept only a decade ago.

## Beyond the liver

Hydrophobic modification of siRNAs with fatty acids or cholesterol has been explored as a delivery strategy. Cholesterol conjugated to partially modified siRNAs supports only marginal systemic efficacy (>100 mg/kg)[82120]. When combined with asymmetric siRNAs structure (see below), the hybrid compounds induce potent gene silencing *in vitro* in many cell types, and support robust efficacy *in vivo* by local injection[121, 122]. One of these compounds, RXI-109 which targets connective tissue growth factor (CTGF), has progressed towards phase 2 clinical trials for the treatment of hypertrophic scarring (rxipharma.com).

Whereas partially modified siRNAs with hydrophobic conjugates show limited systemic efficacy, fully metabolically stabilized compounds show robust systemic distribution (M. Hassler, A. Turanov, J. Alterman, A. Coles and A. Khvorova, unpublished data). Modulating the identity of the hydrophobic conjugate can be used to alter the tissue distribution profile or modulation of diffusion for the site of injection[123]. Notably, changing the hydrophobic

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moiety to a polyunsaturated fatty acid derivative supports a wider therapeutic index, thus enabling another direction in systemic conjugate-based gene modulation[123].

Kidney will likely be the next tissue clinically targetable by systemically delivered RNAi. Like liver, spleen, and bone marrow, kidney has a discontinuous endothelium and natural filtration function, which is being exploited in an ongoing clinical trial involving Quark Pharmaceuticals' (Ness Ziona, Israel) QPI-1002, a partially 2′OMe-modified siRNA that targets P53, is cleared rapidly[124], but retains sufficient clinical efficacy to justify moving to phase 3 clinical trials. The conjugation of polyunsaturated fatty acids to fully metabolically stable siRNAs further supports delivery to kidney (M. Hassler, A. Turanov, J. Alterman, A. Coles and A. Khvorova, unpublished data) and potent and persistent efficacy *in vivo*, which may make the kidney accessible to robust gene silencing. Conjugate-mediated delivery of oligonucleotides to non-primary tissues, including heart, pancreas, lung, and tumor will require further advances in chemistry to take advantage of mechanisms driving oligonucleotide clearance, tissue distribution, cellular uptake and endosomal escape.

## Different designs for different tasks

It is tempting to think of the different families of oligonucleotides as redundant or parallel options for gene silencing. The reality is more complex.

The first parameter to consider is related to the different biophysical properties of single-stranded and double-stranded oligonucleotides. The flexible, amphiphilic nature of single-stranded oligonucleotides favors binding to a range of proteins because the bases and phosphates can flex and align with appropriate amino acids. Heparin-binding proteins are one of the highest affinity targets for phosphorothioate oligonucleotides[125]. Serum proteins, including albumin and cell surface proteins, including trafficking proteins and scavenger receptors, promote the effective cellular uptake of single-stranded oligomers.

The different biophysical properties of single-stranded and double-stranded oligonucleotides have consequences in terms of the clinical pipeline. Both single-stranded and double-stranded oligomers can be effectively targeted to the liver by GalNAc modification, and both are actively in development (Table 1). But for other tissues (e.g., the brain or spinal cord), single-stranded character provides a big delivery advantage[122, 123] with three ASOs in clinical trials for central nervous system (CNS) indications (see p. **WahlestedtXXX** in this issue).

The pharmacologic properties of ssRNAs are similar to those of ssASOs, but ssiRNAs in general are at least an order of magnitude less effective in RISC engagement than conventional siRNAs. We and others have been exploring the use of partially double-stranded, or asymmetric siRNAs, with a 19- to 21-nucleotide guide strand that is duplexed to an 11- to 15-nucleotide sense strand. These asymmetric compounds are as effective in RISC loading as duplex siRNAs[121, 122]. The single-stranded fully-phosphorothioate tail resembles ssASOs and in part confers PK/PD behavior characteristic of conventional ASOs. The fully phosphorothioate single-stranded region works in combination with different conjugates to

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enhance *in vivo* delivery and cellular uptake, demonstrating properties that cannot be achieved with the conjugate alone, including promising activity in CNS tissues[122, 123].

Oligonucleotide duplexes may allow a more complete separation of the optimization of pharmacophore and dianophore relative to single-stranded oligonucleotides. This is because RNA duplexes consistently adopt an A-form helix with a relatively small range of structures and protein targets. In contrast, single-stranded oligonucleotides can adopt a much larger variety of structures, including partially self-complementary and aptameric structures, and their inherent flexibility and amphiphilicity allow them to bind a much larger variety of proteins. Thus, even in the context of a robust dianophore, the distribution profile of an ASO will maintain a certain dependence on the base sequence.

The second parameter to consider is mechanism of action. What cellular factors partner with oligonucleotides to carry out the desired activity? The RNAi machinery, RNase H or no protein at all? In some cases, the choice is simple (e.g., for miRNA inhibition, most researchers use single-stranded steric blocker oligonucleotides). For long non-coding RNA (lncRNA) inhibition, the choice is more complex. For silencing nuclear transcripts, ASOs that recruit RNase H are a safe option, whereas predominantly cytoplasmic transcripts tend to be more readily targeted by siRNAs[126]. For mRNA and cytoplasmic ncRNA silencing, making use of the RNAi pathway often provides increased potency and duration of effect because association with RISC protects the siRNA guide strand from degradation. In some cases, there are other advantages from using one pathway over another; for example, greater selectivity in inhibiting the expanded CAG repeats characteristic of Huntington's disease can be achieved using molecules that engage the RNAi pathway than simple steric blocker ASOs[102, 127].

An advantage of RNAi is that it invokes a natural pathway, in which RISC binds the guide strand, protects it from nucleases, unwinds self-structure in the target RNA, and helps scan for target sites[91]. For these reasons, as few as 100 to 500 loaded RISC complexes per cell are believed to be sufficient for potent, durable silencing[128,129]. But the chemical modification of siRNA must maintain an A-form helix, and of course be compatible with RISC loading and target recognition and cleavage.

Continued progress in understanding the interplay between chemical architecture and oligonucleotide properties enables a constantly expanding spectrum of applications. For example, gene silencing remains a mainstay of the clinical oligonucleotide pipeline, but gene activation is also an increasingly attractive possibility. Gene activation can be achieved either by miRNA inhibition[55] or using ASOs/siRNAs to bind[130], cleave[131], or sterically block[132] lncRNAs. Recently, researchers have also used ASOs to activate gene expression by disrupting R-loop formation[133], inhibiting nonsense-mediated decay[134], or blocking an upstream open reading frame[135].

## Conclusions

Ionis CEO Stan Crooke called the FDA approval of mipomersen (Kynamro) in 2013 “the end of the beginning” for antisense[136]. Although mipomersen failed to become a major

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commercial success, we are indeed witnessing the end of the beginning for the broader field of oligonucleotide therapeutics. Chemistry and delivery technologies have yet to reach maturity, and many of the most promising approaches in early clinical or pre-clinical development are showing substantially improved clinical performance relative to their predecessors.

Several promising dianophores are now in clinical trials, including naked ASOs for targets in the CNS and GalNAc-conjugated oligomers for targets in liver. Additional clinical data on these and other approaches will confirm which dianophores lead to robust clinical results across multiple sequences. At that point, the long-term goals of reducing the time and cost of drug development, and tackling targets and diseases once seen as undruggable or impractical, may be within reach.

## Acknowledgments

We would like to acknowledge the oligonucleotide research community who contributed to the evolution of this field, including those whose excellent work we could not mention for lack of space. We thank Darryl Conte for significant help with manuscript preparation. This work was supported by the RNA Therapeutics Institute of UMass Medical School, the CHDI Foundation, RO1GM1088030181, R01 HD086111, and UH3TR000888.

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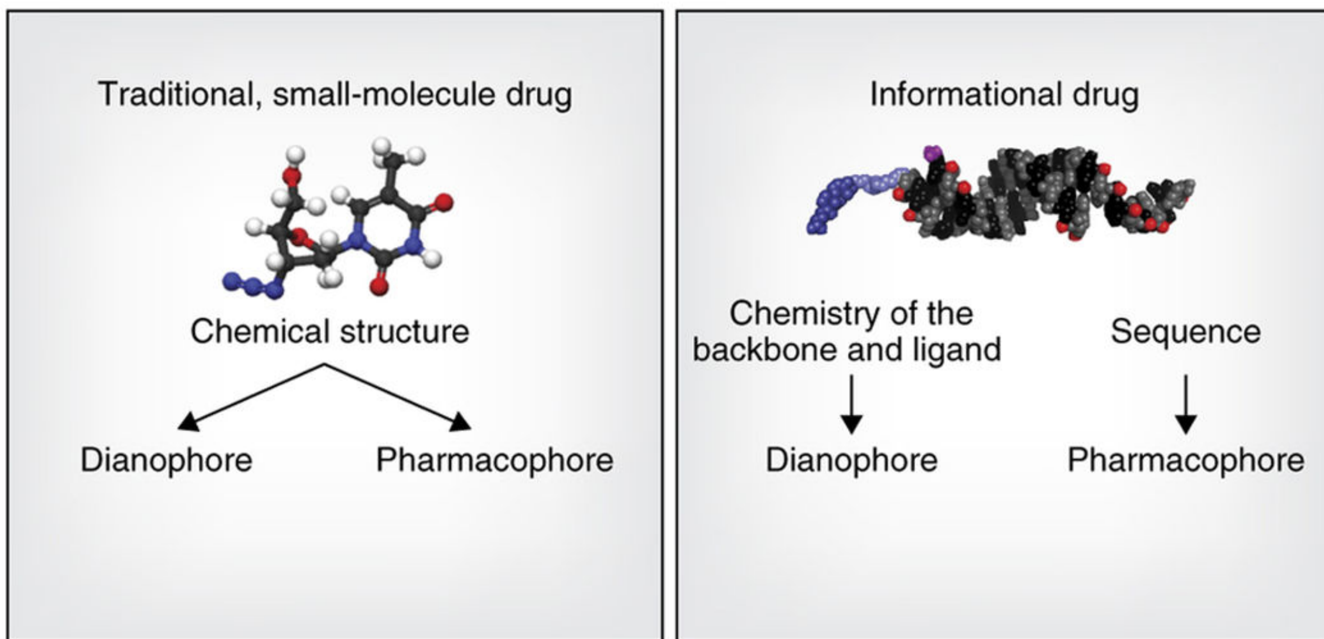


**Figure 1. The key advantage of an informational drug**
is that the *pharmacophore* (molecular features that determine target specificity) and the *dianophore* (molecular features that determine tissue distribution and metabolism) can be optimized separately. When a dianophore for a particular tissue or cell type is defined, it can be applied to a range of pharmacophores that are rationally designed based on sequence information.

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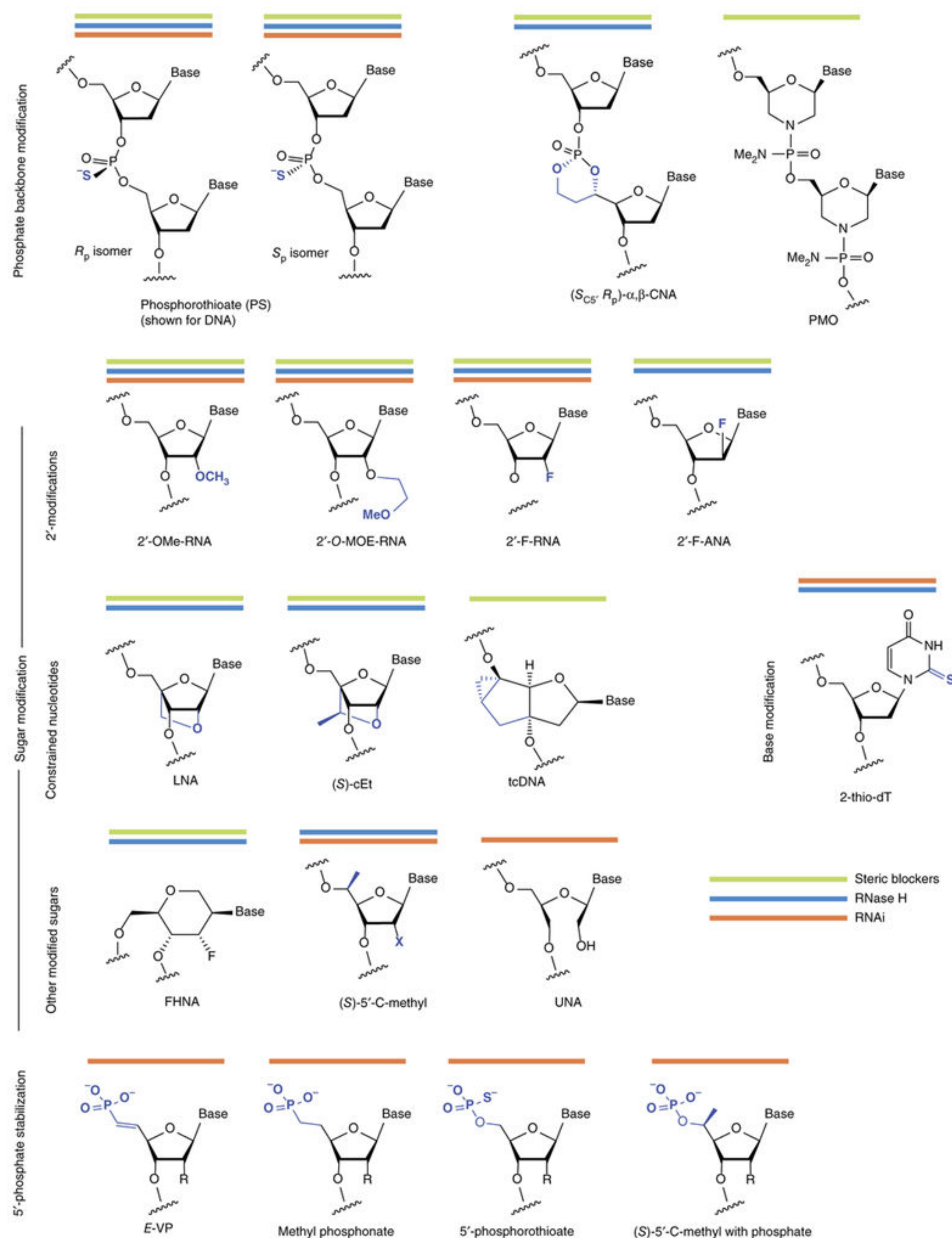


**Figure 2. Structures of chemical modifications discussed in this review**

Combining modifications of the oligonucleotide backbone, sugars, bases and the 5′-phosphate are necessary to develop compounds with optimal activity. Some modifications are used for oligonucleotides that work by different mechanisms: steric blockers, green; RNase H, blue; RNAi, orange lines.

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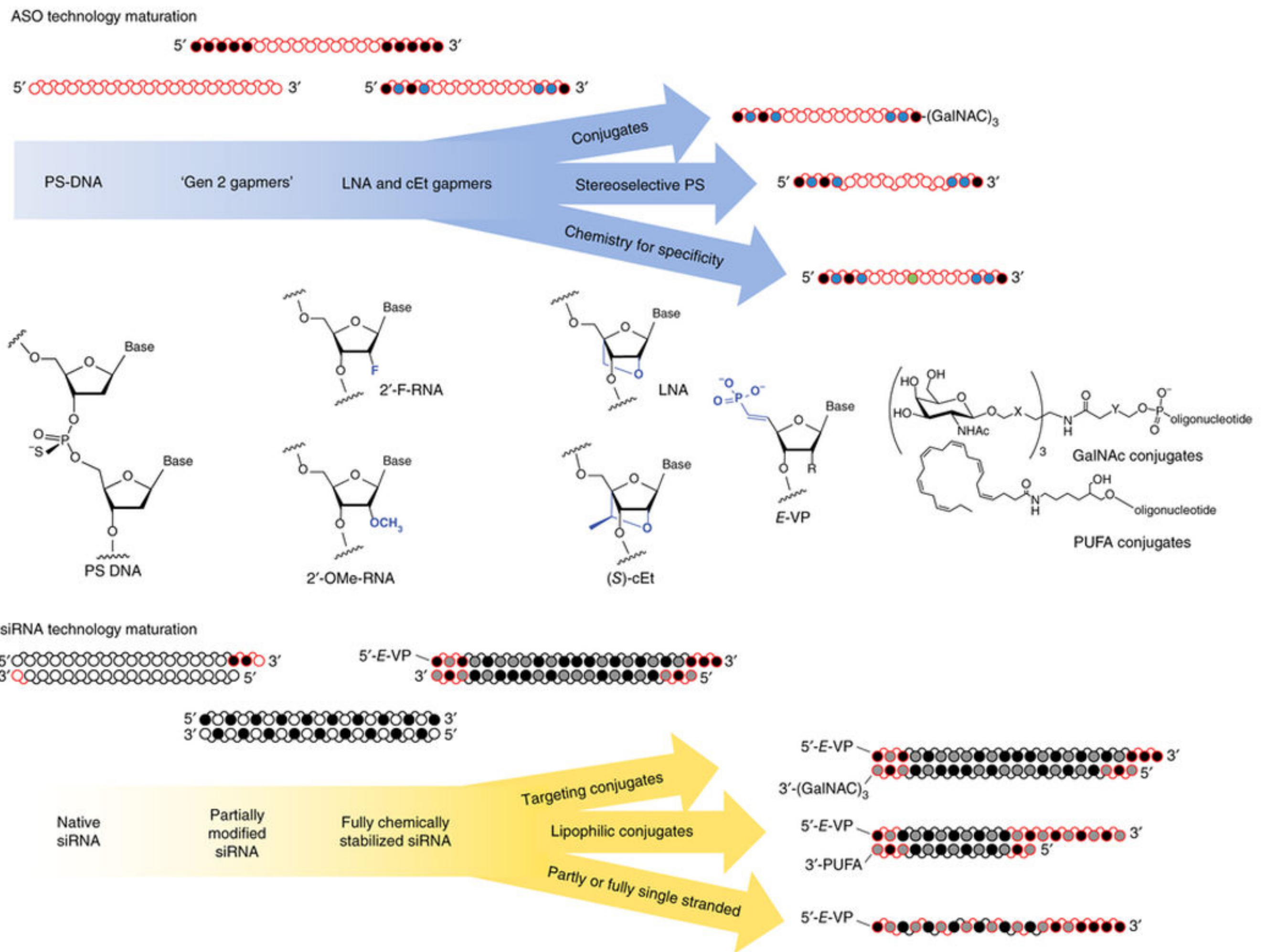


**Figure 3. The evolution of RNase H antisense and RNAi technologies, including key chemical modifications and structural configurations that have enabled major advances toward clinical efficacy**

○ White circles, 2′-OH (RNA), or 2′-H (DNA); ● Gray, 2′-F; ● Black, 2′-OMe or 2′-MOE; ● Blue, LNA or cEt, ● Green, specificity enhancing modification; **red,** phosphorothioate backbone modification (direction of the bond indicates positional stereopurity $R_p$ or $S_p$). PUFA, polyunsaturated fatty acids; gen 2, second generation.

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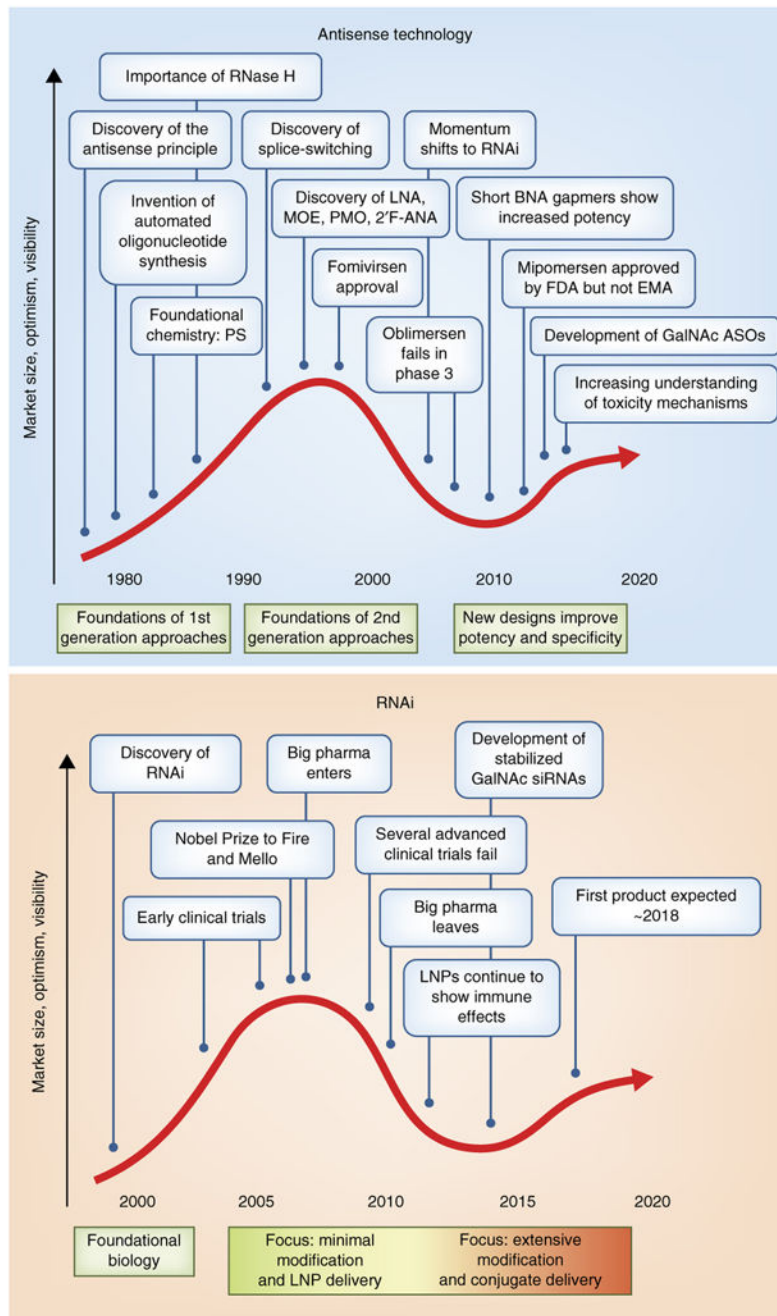


**Figure 4. Key events in antisense and RNAi therapeutics mapped to the Technology Curve**

Both antisense (a) and RNAi (b) approaches have passed through the stages of novel technology trigger, peak of inflated expectations, and trough of disillusionment and are now approaching the plateau of productivity.

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## Table 1

Clinical programs based on GalNAc conjugates

| Drug | Company | Mechanism and chemistry | Target gene | Disease | Development |
|---|---|---|---|---|---|
| Revusiran | Alnylam | siRNA [i] | Transthyretin (mutant and wild type) | Hereditary ATTR amyloidosis | Withdrawn |
| Fitusiran | Alnylam | siRNA [ii] | Antithrombin | Hemophilia | Phase 2 |
| Inclisiran | Alnylam | siRNA [ii] | PCSK9 | Hypercholesterolemia | Phase 2 |
| IONIS-APO(a)-$L_{Rx}$ | Ionis | ASO [iii] | Apolipoprotein A | Very high apolipoprotein a | Phase 2 |
| IONIS-ANGPTL3-$L_{Rx}$ | Ionis | ASO [iii] | Angiopoietin-like 3 ANGPTL3 | Mixed dyslipidemias | Phase 2 |
| RG-101 | Regulus (Carlsbad, CA) | anti-miR [iv] | miR-122 | Hepatitis C virus infection | Phase 2 |
| ALN-CC5 | Alnylam | siRNA [ii] | Complement component C5 | Complement-mediated diseases | Phase 1/2 |
| ALN-AS1 | Alnylam | siRNA [ii] | Aminolevulinic acid synthase | Hepatic porphyrias, including acute intermittent porphyria | Phase 1 |
| IONIS-HBV-$L_{Rx}$ | Ionis | ASO [iii] | HBV genome | HBV infection | Phase 1 |
| RG-125 | Regulus | anti-miR [iv] | miR-103/107 | Non-alcoholic steatohepatitis (NASH); type 2 diabetes / Pre-diabetes | Phase 1 |

[i] – GalNAc-conjugated “standard template chemistry” siRNA

[ii] – GalNAc-conjugated “enhanced stabilization chemistry” siRNA. The main difference between “standard template chemistry” and “enhanced stabilization chemistry” as used by Alnylam appears to be the inclusion of additional phosphorothioate linkages on the 5′-termini of both strands. Inclisiran includes 73% of 2′-O-Me RNA and 25% of 2′F-RNA, while fitusiran and revusiran both contain about 50% of each of those two modifications[137]. See also ref [138].

[iii] – GalNAc-conjugated MOE gapmer ASO

[iv] – GalNAc-conjugated anti-miR (Regulus backbone chemistry is based on combinations of MOE and cEt)