

Medicinal Chemistry of Oligonucleotide Drugs - Milestones of the Past and Visions for the Future

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Abstract: The oligonucleotide therapeutics field has blossomed in recent years, with thirteen approved drugs today and the promise of accelerated growth in coming years. Much of the progress in this field is due to advances in the medicinal chemistry of oligonucleotides, combined with a judicious choice of molecular targets and disease areas. In this perspective, we describe the growth of this new class of drugs highlighting selected milestones in oligonucleotide medicinal chemistry.

Keywords: Antisense · Gapmer · Oligonucleotide · Small interfering RNA



The co-authors are doctoral students with chemistry and biology backgrounds in the group of Jonathan Hall that are working to help bring new RNA-targeting ligands and new RNA drug targets into mainstream drug discovery and development.

1. Past and Present of Oligonucleotide Drugs

The field of oligonucleotide therapeutics arguably began 45 years ago with work from the laboratory of Stephenson and Zamecnik,^[1] who showed that in a cell-free system, translation of the Rous sarcoma virus could be blocked using a 13 nucleotide (nt)-long (13-mer) oligoribonucleotide complementary to the viral RNA. These findings introduced the antisense concept: a means to inhibit cellular protein synthesis with a ‘hybridon’ (oligonucleotide) of sufficient length to act sequence selectively. The route from concept-prototype-drug took 20 years.

When asked in the eighties about the likelihood that oligonucleotides could be developed as therapeutics, most chemists were skeptical and it was even difficult to win research funding for such an idea:^[2] i) the manufacturing of such constructs appeared beyond the capabilities of conventional chemistry, ii) textbook knowledge at the time suggested oligonucleotides did not possess suitable pharmacokinetics properties for use *in vivo* and, iii) it was difficult for industry to make a business case for the necessary research, given the projected costs. Nevertheless, today, thanks to the perseverance of chemists, biologists, pharmacologists, and entrepreneurs, there are 13 approved oligonucleotide drugs with the prospect of accelerated growth in coming years.^[3]

Much of the success of RNA-based drugs is due to medicinal chemists, who systematically tweaked the ribonucleotide structure

to endow oligonucleotides with stability against nucleases *in vivo* and the means to enter cells, whilst maintaining a capacity for: i) Watson-Crick-based target recognition, ii) recruiting cellular effector enzymes and iii) an economically viable synthesis. Given such hurdles, it is unsurprising that the advent of RNA drugs took longer than that of therapeutic antibodies, a modality with which they are often compared. In this perspective, we describe growth of the oligonucleotide field with selected milestones in the medicinal chemistry of oligonucleotides.

1.1 Oligonucleotide Synthesis – The Heart of the Matter

In the eyes of a chemist, oligonucleotides sit midway between conventional small-molecule drugs and biologicals. Although a semi-synthetic preparation of oligonucleotides might have been viable, the repeating units of an oligonucleotide suggested a solid-phase synthesis in analogy to that of peptides. The earliest syntheses of oligonucleotides date to the mid-1950’s when Michelson and Todd prepared a dinucleotide by coupling a 5’ protected thymidine with the 5’ hydroxyl of a 3’ protected thymidine (Fig. 1a; **(I)**).^[4] In his remarkable synthesis of a 72-mer tRNA gene, Khorana prepared di-, tri- and tetranucleotides which were then condensed into 8-12 mer DNA fragments before enzymatic ligation.^[8] A, G and C nucleobases were protected as amides and the dimethoxytrityl group was introduced for the protection of the 5’ hydroxyl group (Fig. 1a; **(II)**).^[9] Its stability, its easy removal and its high hydrophobicity led to it becoming a cornerstone of oligonucleotide synthesis. In the 1970’s, Letsinger brought oligonucleotide synthesis to the solid phase using the phosphotriester method.^[10] He also introduced the β-cyanoethyl group as a ubiquitous protecting group for oligonucleotides, still in use today.^[11] The switch to P(III) reagents was a breakthrough due to their fast, quantitative reactions.^[12] Letsinger’s nucleoside phosphomono-chloridite intermediates proved too reactive, and thus Caruthers exchanged the chloride for an amine to provide the phosphoramidite reagents of today^[7] (Fig. 1a; **(III)**). Another critical element of oligonucleotide synthesis was the development of inorganic matrices as the solid phase,^[10,13] in particular, controlled pore glass (CPG). Together, these advances yielded the modern oligonucleotide synthesis cycle (Fig. 1b).

These milestones were accompanied by engineering advances in DNA synthesizer machines. Applied Biosystems released the Model 380A in the 1980s,^[14] which became a workhorse. As the

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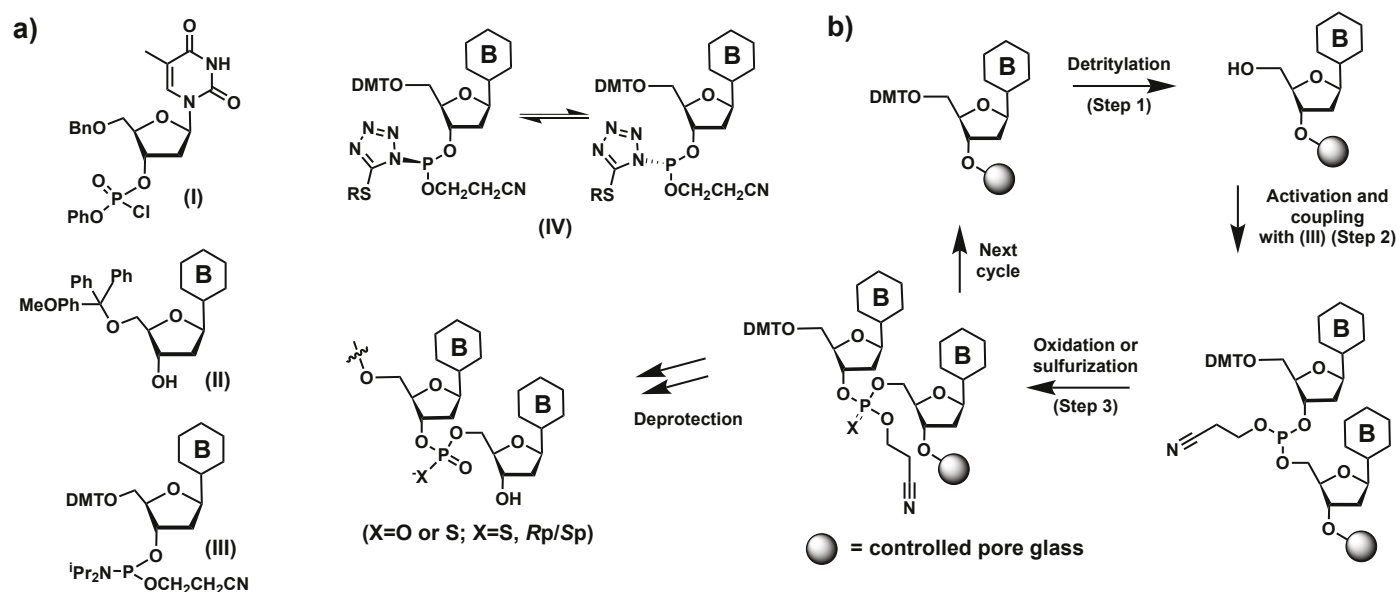


Fig. 1. Oligonucleotide synthesis: **a)** Building blocks in historical development of oligonucleotide synthesis from Todd^[4] (I), Khorana^[5]/Letsinger^[6] (II) and Caruthers^[7] (III). **b)** Oligonucleotide synthesis cycle; (IV) is the tetrazolidine intermediate from activation of (III) (DMT is dimethoxytrityl; B is nucleobase; R is alkyl or aryl).

field developed, ever larger capacity machines were introduced including the OligoProcessTM, which yields up to 15 kg of oligonucleotide in single runs. There is still room for innovation in oligonucleotide synthesis; *e.g.* improving the low atom-economy process due to multiple protecting groups, reducing solvent consumption and raising loading of solid supports.

1.2 Fomivirsen – The Proof of Concept

The phosphodiester of DNA and RNA require protection against nucleases for use *in vivo*. However, even minor changes to the backbone can adversely affect the ability of the oligonucleotide to hybridize with a complementary target. In the 1980s, the challenge of finding a replacement for phosphodiester groups appeared daunting. The phosphorothioate (PS) linkage satisfied the requirements^[15] and was easily adapted to solid phase protocols, by replacing aqueous iodine-mediated oxygenation with sulfuration to P(v). Two unexpected benefits to the PS-linkage were that: i) the sulfur groups facilitate cell entry of PS-oligonucleotides and, ii) they bind weakly to serum proteins that helps them distribute to tissues and organs. These advantages did not extend to double-stranded small interfering RNAs (siRNAs). In 1998, **Fomivirsen** became the first antisense oligonucleotide (ASO) drug to be approved by the FDA (Table 1).^[16] The drug is a 21-mer PS DNA (Fig. 2a). Treatment involved injection of 165–330 micrograms of fomivirsen directly into the eye, to counter CMV infections in AIDS patients. The local delivery meant only small amounts of the drug were needed and eye injections were well tolerated. Fomivirsen binds the viral mRNA, which inhibits synthesis of the CMV IE2 protein, however it is likely that some of fomivirsen's activity derives from off-target effects and immune stimulation (see ref. [3]).

Fomivirsen was a commercial failure, at least partly due to competition with small-molecule antivirals. However, it was a proof-of-concept for oligonucleotide drugs. The field would wait another 15 years for the next approval.

1.3 Eteplirsen, Mipomersen, Miravirsen – Second Generation Chemistries and New Modes of Action

Mipomersen was approved by the FDA in 2013 for the treatment of the rare disease homozygous familial hypercholesterolemia (FH). Mipomersen inhibits the synthesis of apolipoprotein

B (ApoB100), which transports cholesterol in the blood stream. It was the first chimeric PS ‘gapmer’ to reach the market (Table 1). Mipomersen incorporates five 2'-O-methoxyethyl (MOE)-modified riboses (Fig. 2a) flanking a 10-mer DNA stretch. The MOE groups resist nuclease degradation. They also bring increased binding affinity for target RNAs by imposing a *gauche* effect in the sugar 2'-3' torsional axis. This favors a C3'-endo conformation in the oligonucleotide, which pre-organizes it for duplex formation.^[18] The MOE modification is today the most widely used modification in clinically relevant oligonucleotide drugs.^[3] The central DNA ‘gap’ in mipomersen recruits RNase H1 in the nucleus,^[19] which catalyzes cleavage of the target mRNA (Mechanism A; Fig. 2b), thereby halting protein synthesis. Mipomersen also carries 5-methylcytosines (5-MeC), which are commonly used in oligonucleotides to raise binding affinity and minimize proinflammatory effects.^[20]

In patients with FH, mipomersen reduces blood-ApoB100, -cholesterol and -lipoprotein levels after weekly subcutaneous (SC) injections of 200 mg.^[21] Despite its benefits, however, mipomersen had little commercial success, due to its limited approval, some hepatotoxicity and the availability of alternative therapies with fewer side effects.^[22]

The splice-switching oligonucleotide (SSO) **Eteplirsen** was approved in 2016 (Table 1). Its target is the pre-mRNA of dystrophin, to which it binds and alters splicing. Eteplirsen is a 30-mer phosphorodiamidate morpholino oligonucleotide (PMO) (Fig. 2a). PMO chemistry was first described in the 1990s.^[23] Its elegant synthesis comprises periodate-mediated opening of the ribose and reductive amination to give a morpholine. The morpholines are linked by a phosphorodiamidate backbone which retains neutral charge at pH 7.^[24] The PMO is resistant to nucleases and does not elicit enzymatic degradation of its target RNA. Furthermore, PMOs have excellent safety profiles after intravenous (IV) injections.^[25]

Mutations in the dystrophin gene can lead to loss-of-function of the protein, causing Duchenne muscular dystrophy (DMD) primarily in boys who lack the compensatory allele. DMD patients suffer from progressive muscle degeneration and die in their mid-twenties. The most prominent mutation in exon 51 introduces a frame shift in the dystrophin mRNA, leading to a loss of protein synthesis.^[26] Eteplirsen enters the nucleus of muscle cells and

Table 1. Sequence, chemistries and mechanisms of action of oligonucleotides tested in humans; n indicates modified building blocks; - indicates PS linkage; - indicates PO linkage; ^ indicates Sp PS linkage; ASO: antisense oligonucleotide; SSO: splice-switching oligonucleotide; siRNA: small interfering RNA (upper: guide; lower: sense strands); LNP: lipid nanoparticle; gal: tri-antennary N-acetylgalactosamine ligand; (for a comprehensive list of oligonucleotides in trials see ref. [3]).

Drug	Sequence (5'-3')	Chemistry (Fig. 2a)
fomivirsin ^[17]	G-C-G-T-T-T-G-C-T-C-T-T-C-T-T-G-C-G	N=DNA (ASO)
mipomersen ^[17]	g-c-c-t-c-A-G-T-C-T-G-C-T-T-C-g-c-a-c-c	n=MOE; N=DNA (gapmer)
etelplirsin ^[17]	ctccaacatcaaggaagatggcattct	n=morpholino (SSO)
miravirsin ^[17]	c-C-a-T-T-g-t-C-A-c-A-c-T-c-c	n=LNA; N=DNA(anti-miR)
danvatirsin ^[17]	c-t-a-T-T-T-G-G-A-T-G-T-C-a-g-c	n= cEt; N=DNA (gapmer)
nusinersen ^[17]	u-c-a-c-u-u-u-c-a-u-a-a-u-g-c-u-g-g	n=MOE (SSO)
patisiran ^[17]	A-U-G-G-A-A-uM-A-C-U-C-U-U-G-G-U-uM-A-C-dT-dT	N=RNA; n _M =2'-OMe;
	G-uM-A-A-cM-cM-A-A-G-A-G-uM-A-uM-uM-cM-cM-A-uM-dT-dT	(siRNA-LNP)
givosiran ^[17]	u _M -a _F -a _F -g _F -a _M -u _F -g _M -a _F -g _M -a _F -c _M -a _F -c _M -u _F -u _M -u _F -c _M -u _F -g _M -g _M -u _M	n _F =2'-F; n _M =2'-OMe
	c _M -a _M -g _M -a _M -a _M -a _M -g _F -a _M -g _F -u _M -g _F -u _M -c _F -u _M -c _F -a _M -u _M -c _M -u _M -u _M -a _M :gal	(siRNA-GalNAc)
inclisiran ^[17]	a _M -c _F -a _M -a _F -a _F -a _F -g _M -c _F -a _M -a _F -a _M -a _M -c _M -a _F -g _M -g _F -u _M -c _F -u _M -a _M -g _M -a _M -a _M	n _F =2'-F; n _M =2'-OMe
	c _M -u _M -a _M -g _M -a _M -c _M -c _F -u _M -g _F -u _M -T-u _M -u _M -g _M -c _M -u _M -u _M -u _M -u _M -g _M -u _M :gal	(siRNA-GalNAc)
suvodirsin ^[17]	u _F [^] c _F [^] a _F [^] a _F [^] g _F [^] g _F [^] a _M [^] a _F [^] g _M [^] a _M [^] u _F [^] g _M [^] g _M [^] -c _F [^] a _F [^] u _F [^] u _F [^] c _F [^] u _F [^]	n _M =2'-OMe; n _F =2'-F (SSO)
milasen	a-a-u-g-u-u-a-g-u-g-c-u-u-g-u-u-g-a-g-g-g-c	n=MOE (SSO)

binds to exon 51, altering splicing so as to prevent inclusion of this exon into mature mRNA (Fig. 2b). This results in synthesis of a truncated but partially functional protein. Eteplirsin was the first oligonucleotide to be approved that operates by a splice-switching mechanism, correcting a loss-of-function phenotype. It was evaluated in patients by a 6-minute walk test, and its approval was highly controversial.^[27] Nevertheless, new PMO drugs to address other disease-causing mutations in the gene, have been developed, including **goldirsin** and **vitolarsen** (exon 53) and **casimersen** (exon 45).^[28]

The locked nucleic acid (LNA) modification (Fig. 2a) of **miravirsin** (Table 1) was a milestone of oligonucleotide chemistry for its hybridization properties and also for its complex multi-step synthesis. LNA nucleotides carry a bridge connecting the 2' oxygen with C4 of the ribose.^[29] The nucleotide is locked in a 3' *endo* conformation, which endows the oligonucleotide with very high hybridization affinity. LNA oligonucleotides longer than 7-mers tend to self-aggregate,^[30] which can be prevented by adding deoxynucleotides in the sequence.

The introduction of LNAs coincided with increasing research on a newly discovered class of RNAs - microRNAs (miRNA). One particular miRNA, miR-122, is highly expressed in hepatocytes, where it regulates fatty acid metabolism.^[31] Surprisingly, miR-122 was identified as a key factor in mediating hepatitis C viral (HCV) replication, due to its binding to the viral RNA.^[32] These observations led to the design of **miravirsin**, the first oligonucleotide drug candidate to sterically block the action of a miRNA.^[33] Miravirsin is a 15-mer PS mixmer consisting of eight LNA and seven DNA nucleotides. Miravirsin is complementary to the mature miR-122 sequence, but also allows for binding to the precursor versions of miR-122.^[34] By sterically blocking the binding of miR-122 to HCV RNA, miravirsin terminates viral replication.^[35]

Miravirsin was administered to patients by once-weekly SC injections at doses of 3-7 mg/kg.^[36] As with other PS drugs, miravirsin accumulates in the liver. Data from phase 1 trials showed that it elicited a dose-dependent reduction in virus load, and hence it advanced to phase 2 trials. However, after initial successes in viral suppression, follow up studies have revealed issues with ap-

pearance of escape mutants.^[37] Although miravirsin's development has stalled, the LNA remains a modification of high potential for oligonucleotide drugs.

LNA served as the precursor of the constrained ethyl (cEt) modification (Fig. 2a), which carries an *S*-methyl group on the ribose bridge. Thus, the cEt bicycle constrains the ribose to a favorable conformation for binding to RNA.^[38] Curiously, cEt ASOs have reportedly lower incidences of hepatotoxicity compared to LNA ASOs.^[39] **Danvatirsin** is one of several cEt ASOs that is currently tested in clinic trials. It is a 16-mer PS ASO gapmer consisting of six cEt and ten DNA units. This sequence is complementary to a region in the mRNA of STAT3, which is an oncogene responsible for cancer cell growth and survival. Hybridization of **danvatirsin** to STAT3 mRNA results in repression of STAT3 protein, which in turn is responsible for drug efficacy of long-term partial remission in cancer patients.^[39]

1.4 Nusinersen – into the Central Nervous System

In 2016, the FDA approved the SSO **nusinersen** for the treatment of spinal muscular atrophy (SMA) (Table 1).^[41] Nusinersen was the first therapy for this rare, autosomal recessive neuromuscular disease. The cause of SMA is homozygous loss of the *SMN1* gene, which encodes the survival of motor neuron (SMN) protein. In humans, however, this protein is also produced in limited amounts from the redundant *SMN2* gene. Normally, the expression of *SMN2* is suppressed by a splicing enhancer sequence which leads to exclusion of exon 7. This results in the synthesis of an unstable truncated SMN protein. Nusinersen is a fully PS-MOE-modified 18-mer, that binds to *SMN2* pre-mRNA and alters its splicing, so as to include exon 7^[42] and restore the loss of SMN. Nusinersen was the first approved oligonucleotide to target neurons of the nervous system, although it was established in the mid-1990s that intrathecal delivery of oligonucleotides was a viable means to use oligonucleotides in the CNS.^[43] Nusinersen is administered every four months and can even be administered pre-symptomatically to prevent disease manifestation.^[44] The drug is safe and leads to improved motor function for all types of SMA. Since its approval, alternative SMA treatments have emerged, namely the gene therapy *zolgensma* and the small molecule drug

risdiplam.^[45] It will be interesting to watch nusinersen compete with these new modalities.

1.5 Patisiran – The First of the siRNAs

Patisiran was a milestone in the oligonucleotide field, as the first siRNA approved by the FDA in 2018, approx. 20 years after the discovery of RNA interference.^[46]

Conventional siRNAs are 21-23 nt duplexes with overhanging nucleotides at the 3' end.^[47] Once in the cytoplasm, the siRNA guide strand is loaded into the RNA-induced silencing complex (RISC) (Mechanism C; Fig. 2b). The siRNA then activates RISC to cleave the target mRNA, halting protein synthesis (Fig. 2b).^[47] Although researchers heralded this innate silencing mechanism as a potential new source of RNA therapeutics,^[48] others were skeptical: ASOs were not yet successful and an siRNA molecule is twice the size of an ASO!

Patisiran is encapsulated into lipid nanoparticles (LNP), which protect the siRNA from nuclease degradation. The siRNA bears eleven 2'-OMe groups (Fig. 2a)^[49] and two deoxythymidine residues in the 3' overhangs of the RNAs. In second-generation siRNAs only the antisense guide strand carries an overhang.^[50] Patisiran is administered intravenously to treat polyneuropathy caused by hereditary transthyretin-mediated amyloidosis (hATTR). This rare disease is due to mutations in the transthyretin (TTR) gene, leading to accumulation of misfolded TTR protein. Patisiran prevents TTR protein synthesis in hepatocytes by binding to the 3' untranslated region of wild-type and mutant TTR mRNAs. In phase 3 trials, TTR levels in the circulation of patients treated with 0.3 mg/kg of drug were lowered by >70%.^[51]

The LNP of patisiran comprises cholesterol, a pegylated lipid, an ionizable cationic lipid and an amphiphilic phospholipid. In circulation, the lipids are replaced by the apolipoprotein E, which aids LNP uptake into hepatocytes via receptor-mediated endocytosis.^[52] The cholesterol and the phospholipid stabilize the encapsulation of the siRNA in the LNP.^[53] More than 300 lipids were investigated to identify one with an optimal pKa value (6.2-6.5).^[54] Ionizable cationic lipids can also promote endosomal escape of the siRNA inside cells. The acidic environment of the endosomes regenerates the cationic amino lipids, leading to endosome disassembly and siRNA release.^[55] LNPs are an efficient delivery vehicle for nucleic acids but their development is challenging and they are not used in newer siRNA drugs (*vide infra*).

1.6 Givosiran - The Rise of the GalNAc Conjugate

Givosiran and inclisiran are second generation siRNA drugs (Table 1) and are milestones for their innovative chemistry and a ground-breaking conjugate-mediated targeted delivery into cells. Remarkably, the second generation siRNAs do not contain any natural ribonucleotides; they comprise 2'-O-methyl riboses interspersed with tactically placed 2'-fluoro modifications, capped by two PS linkages at the RNA termini (Fig. 2a). The placement of the modifications was optimized to balance nuclease stability and RNAi activity.^[56] The 3'-end of the siRNA passenger strand is covalently linked to three N-acetylgalactosamine (GalNAc) moieties on short spacers (Fig. 2a).^[57] This ligand exhibits high affinity for asialoglycoprotein receptors (ASGPRs), that are expressed almost exclusively in hepatic tissues. Upon binding, the receptors undergo endocytosis delivering the siRNA cargo into

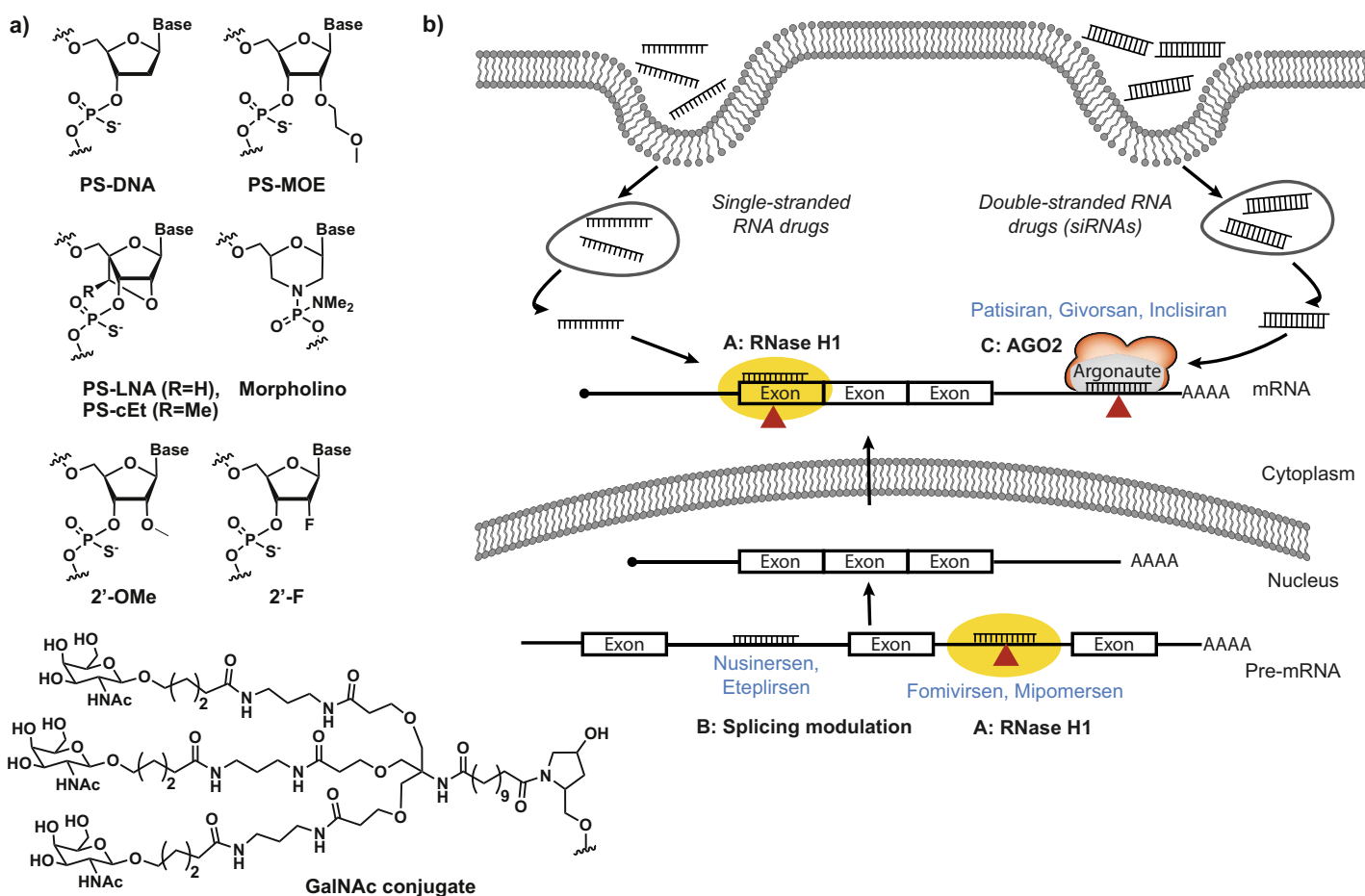


Fig. 2. a) Structural modifications in clinically approved oligonucleotide drugs. The PS-DNA building block was used in the first generation fomivirsen, and is present in the gap of second generation ASOs comprising the modifications on the 2'-O of the ribose groups (MOE, LNA and cEt); the morpholino chemistry is present in the SSOs used to treat DMD; 2'-O methyl and 2'-F PS groups are used in siRNAs. b) Mechanisms of approved oligonucleotide drugs in cells (A: RNaseH-mediated pre- and mRNA cleavage; B: splicing modulation of pre-mRNA; C: Ago2-mediated mRNA cleavage).

cells.^[58] Strikingly, the GalNAc group increases hepatotropism to such a degree that drug dosing in the clinic could be decreased by 10-30-fold compared to non-conjugated analogues.^[59] Thanks to this success, use of the GalNAc ligand has been extended to ASO gapmers such as **olezarsen**.^[60]

Givosiran targets hepatic delta-aminolevulinic acid synthase 1 (ALAS1) as a treatment for the rare inherited metabolic disorder acute hepatic porphyria (AHP). Patients suffering from this defect in heme biosynthesis will accumulate δ -aminolevulinic acid (ALA) and porphobilinogen (PBG) leading to porphyria attacks.^[61] Givosiran is administered as an SC injection on a monthly basis at 2.5 mg/kg.^[62]

Approved in 2021, Inclisiran is a milestone in the field because it is the first RNA drug to treat a common disease - atherosclerotic cardiovascular disease (ASCVD).^[63] ASCVD affects millions of Americans, suggesting that a significant large scale manufacturing campaign will be needed. Inclisiran targets the mRNA encoding the PCSK9 enzyme: While this regulatory protein is widely expressed in tissues, reduction of hepatic PCSK9 levels leads to an increase in recycling of low-density lipoprotein receptors (LDLRs) onto the cell surface of liver cells.^[64] This is associated with increased LDL uptake into the liver and thereby reduction of the undesired free LDL cholesterol in blood.^[65]

1.7 Suvodirsen – The First Stereopure PS RNA Drug

The substitution of sulfur for oxygen in a non-bridging phosphodiester creates *Rp* and *Sp* diastereoisomeric phosphorothioate linkages (Fig. 3; (I)-(II)). Thus, nusinersen contains 2¹⁷=131'072 possible diastereoisomers, each with its own distinct biochemical properties. The earliest efforts of stereopure PS-oligonucleotide synthesis used P(V) chemistry^[66] (Fig. 3; (III)) which led to the synthesis of short oligodeoxynucleotides. These sufficed to show that *Sp* deoxynucleotides are more resistant to nucleases than their *Rp* analogues, while the latter bind to complementary RNA with higher affinity.^[67]

For PS oligonucleotides, it is important to demonstrate *Rp/Sp* ratio reproducibility during manufacturing, since changes in this ratio can substantially affect pharmacological activity, as shown for siRNAs.^[68] Using conventional phosphoramidites (e.g. (III), Fig. 1a), the nucleotide coupling reaction is unselective on the solid phase, yielding *Rp/Sp* ratios of approximately 1:1.^[69,70] The reaction of the 5'-hydroxyl group with the phosphoramidite is catalyzed by tetrazole activators, that act as an acid to protonate the di-isopropylamino leaving group, then as a nucleophile that epimerizes the phosphorus center prior to displacement by the hydroxyl group ((IV); Fig. 1b).^[71]

The difficulty in stereopure synthesis of PS oligonucleotides has been to find a phosphoramidite that reacts stereospecifically and quantitatively and which is compatible with solid phase synthesis. A breakthrough came with the introduction of the bicyclic P(III) chiral auxiliaries, (Fig. 3; (IV),^[72] R¹=Ph, R²=R³=cyclopentyl). Stereopure PS oligonucleotides bearing modifications at the 2' position were synthesized, albeit in poor yields and limited in lengths.^[69,72,73] These building blocks permitted the synthesis of partially stereopure MOE and cEt gapmer ASOs which demonstrated that all-*Rp* DNA linkages in the gap activate target cleavage more efficiently than their *Sp* counterparts.^[74] However, overall *in vivo* activity of these sequences was lower due to their increased susceptibility to nucleases. The first study of all-*Rp* and all-*Sp* PS-MOE SSOs in cells - made from phosphoramidite (V) and its diastereoisomers (Fig. 3) - showed that an *Rp* SSO was more potent than the *Sp* homolog in correcting the splicing of an mRNA encoded by ferrochelatase gene, but not better than the stereorandom version.^[75] Later reports described how PS stereochemistry affects RNase H1 cleavage, protein localization and toxicity in cells and mice,^[74,76] with gapmers and with siRNAs.^[68b,77] Together these findings suggested that a mixture

of *Rp* and *Sp* stereochemistry is required for an optimal balance across these pharmacological parameters.

In 2017, **suvodirsen** entered clinical trials to treat DMD patients. Suvodirsen bears stereopure 2'-OMe and 2'-F ribose modifications and binds at the same binding site as eteplirsen (Table 1). However, the drug failed to show efficacy and it was terminated. Two other stereopure PS gapmers for treatment of Huntington's disease (**rovanersen**, **lexanersen**) were also stopped in 2020 for lack of efficacy. The consequences of these failures on the future of stereopure RNA drugs remain unclear.

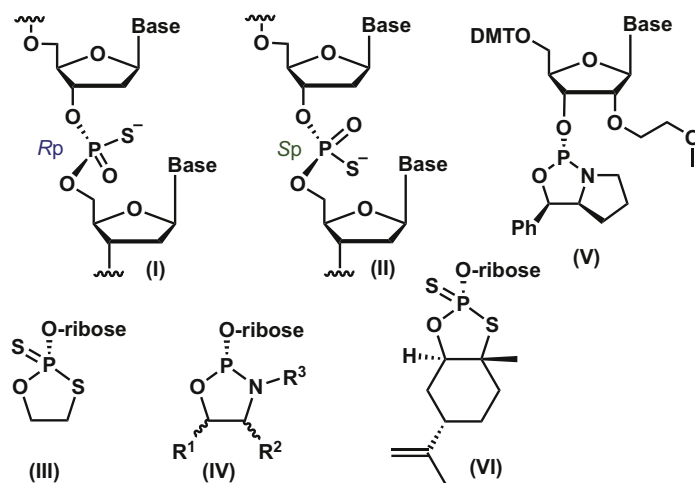


Fig. 3. Chiral PS oligonucleotides. Fig. 3. Chiral PS oligonucleotides. Fig. 3. Chiral PS oligonucleotides. Fig. 3. Chiral PS oligonucleotides.

1.8 Milasen – A New Personalized Medicine

With **milasen**, a new milestone for RNA therapeutics was achieved. Milasen was the first oligonucleotide of the N-of-1 initiative, which provides life-long oligonucleotide treatments for patients with ultrarare diseases, i.e. where 'N' may be one.^[78] At six years of age, Mila was diagnosed with Batten's disease - a fatal neurodegenerative disease. Whole genome sequencing revealed that she carried a mutation in the *MFSD8* gene, which likely caused mis-splicing and loss of MFSD8 synthesis.^[79] Hence, scientists and clinicians set out to identify an oligonucleotide capable of correcting this splicing defect in patient-derived fibroblasts. This yielded **milasen**, a PS-MOE 22-mer (Table 1), which was developed in only one year. Intrathecal administration of **milasen** resulted in patient stabilisation without serious adverse events,^[79] however the drug could not cure Mila and she passed away. This In spite of this, the N-of-1 approach is a unique platform for a breakthrough type of personalized treatments.^[78-80]

2. Future Developments of Oligonucleotide Drugs

Progress in oligonucleotide chemistry over four decades has brought RNA therapeutics into mainstream drug discovery. Where is further innovation needed to advance the field?

New types of targeting ligands that emulate the GalNAc and provide access to extra-hepatic tissues is an urgent need. This is especially relevant for siRNAs, which today are constrained to liver diseases, although early work suggests that siRNA can be used in the CNS without conjugated groups.^[81] Peptide^[82] and lipid conjugates^[83] offer new opportunities, as do small molecule ligands.^[84]

As the RNA therapeutics expand into mainstream diseases, so the pressure to find alternatives to solid phase synthesis will grow. Interest in solution-phase synthesis has increased in recent

years (see refs in [85]). One exciting recent report described the synthesis of a 18-mer PS MOE gapmer prepared in solution using phosphoramidite chemistry.^[85] The final sequence was assembled *via* the convergent coupling of tetramer and pentamer oligonucleotides.

Clearly, chemistry continues to play a central role for the field of RNA drugs, also with the advent of therapeutic mRNAs and CRISPR-Cas systems. The search for new nucleotide modifications continues, arguably with less urgency, since free access to MOE or LNA chemistry today suffices to develop a drug. Nevertheless, some new modifications are well advanced, *e.g.* tri-cyclo DNAs,^[86] achiral thiophosphates^[87] and phosphoryl guanidynyl backbones,^[88] although these chemistries may not be widely accessible for reasons of intellectual property. The march towards stereopure PS drugs will go on, buoyed by concerns of stereoreproducibility in drug manufacturing.^[89] In this context, a return to P(v) reagents offers refreshing new possibilities with building blocks such as ((VI); Fig. 3a).^[90] However, new ways to identify the optimal arrangement of Rp and Sp stereocenters at all positions of a stereopure PS oligonucleotide an oligonucleotide will then be needed.

The rare disease area for RNA drugs has been enabling, at least partly because competition from small molecules and biologicals is limited, and premium pricing policies are possible. It is interesting to note an emerging trend in which companies with distinct oligonucleotide chemistries work on the same targets for the same diseases (dystrophin, transthyretin, PCSK9 etc).

As for the tantalizing possibility of oral availability for an oligonucleotide? Seems unlikely, but maybe not!^[91]

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