BIOMOLECULES & THERAPEUTICS

Review
Biomol Ther 31(3), 241-252 (2023)

Drug Discovery Perspectives of Antisense Oligonucleotides

Yeonjoon Kim*

Qmine Co., Ltd., Seoul 03759, Republic of Korea

Abstract

The era of innovative RNA therapies using antisense oligonucleotides (ASOs), siRNAs, and mRNAs is beginning. Since the emergence of the concept of ASOs in 1978, it took more than 20 years before they were developed into drugs for commercial use. Nine ASO drugs have been approved to date. However, they target only rare genetic diseases, and the number of chemistries and mechanisms of action of ASOs are limited. Nevertheless, ASOs are accepted as a powerful modality for next-generation medicines as they can theoretically targ*et al*l disease-related RNAs, including (undruggable) protein-coding RNAs and non-coding RNAs. In addition, ASOs can not only downregulate but also upregulate gene expression through diverse mechanisms of action. This review summarizes the achievements in medicinal chemistry that enabled the translation of the ASO concept into real drugs, the molecular mechanisms of action of ASOs, the structure-activity relationship of ASO-protein binding, and the pharmacology, pharmacokinetics, and toxicology of ASOs. In addition, it discusses recent advances in medicinal chemistry in improving the therapeutic potential of ASOs by reducing their toxicity and enhancing their cellular uptake.

Key Words: Antisense oligonucleotide, ASO, Medicinal chemistry, Chemical modification, ASO-protein interaction, Structure-activity relationship

INTRODUCTION

Since the antisense oligonucleotide (ASO) concept (Zamecnik and Stephenson, 1978) and RNA interference mechanism (Fire *et al*., 1998) were disclosed, it took 20 years to introduce RNA therapeutics into the clinic. The first ASO drug, formivirsen, and the first small interfering (si)RNA drug, patisiran, were approved by the U.S. Food & Drug Administration in 1998 and 2018, respectively. The antisense concept could be translated into drugs owing to advances in many fields, including chemistry (medicinal, analytical, organic, and process), biology (RNA, genomics), and pharmacology. Specifically, innovations in medicinal chemistry, including endowing oligonucleotides with drug-likeness and sufficient binding affinity while maintaining target RNA degradation activity via natural enzymatic cleavage, were key. In addition to the several RNA-targeted drugs, including nine ASO drugs (Table 1) and five siRNA drugs, the two mRNA vaccines for COVID-19 approved in 2021 advanced the era of RNA therapy.

RNA-targeted drugs bind to the complementary sequences of their target RNAs through Watson–Crick hybridization; therefore, they can modulate target RNA function very specifically, with few off-target effects, and they can be rationally designed based on sequence information. In addition, they are ideal for precision medicine or personalized medicine. For example, milasen was developed for a single patient named Mila (Kim *et al*., 2019). Most importantly, with RNA-targeted drugs, nearly all disease-related targets that are "undruggable" or "undrugged" by conventional medicinal approaches can theoretically be controlled. Only approximately 15% of all proteins are considered "druggable" (Makley and Gestwicki, 2013); therefore, 85% of proteins have not been targeted by small-molecule drugs or biologics and can be targeted by RNA therapeutics. Only 2% of human DNA is translated into proteins, while 90% of the genome is transcribed into RNAs, most of which are non-protein-coding (Djebali *et al*., 2012). Non-coding RNAs are also considered to be potential targets of RNA therapeutics.

RNA-targeted oligonucleotide drugs are either single-stranded or double-stranded. In contrast to double-stranded siRNAs, which can only degrade a target RNA to downregulate gene expression, ASOs possess more versatility as they can not only degrade RNA but also upregulate gene expression or manipulate mRNAs through diverse modes of action. Furthermore, ASOs can undergo more chemical modifications than siRNAs as the chemical modification of the latter is only

Open Access **https://doi.org/10.4062/biomolther.2023.001**

Received Jan 3, 2023 **Revised** Feb 12, 2023 **Accepted** Feb 13, 2023
Published Online Mar 2, 2023

***Corresponding Author**
E-mail: yeonjoonkim@gmail.com
Tel: +82-70-8670-8277, Fax: +82-2-6499-2888

Table 1. Approved ASO drugs

Drug	Approval year	Target indication	Target gene	Mode of action	Chemistry
Formivirsen	1998	CMV retinitis	CMV	RNase H1	PS-ODN
Mipomersen	2013	HoFH	ApoB-100	RNase H1	PS-MOE gapmer
Nusinersen	2016	SMA	SMN2 intron 7	Exon inclusion	PS-MOE
Eteplirsen	2016	DMD	Dystrophin exon 51	Exon skipping	PMO
Inotersen	2018	hATTR	TTR	RNase H1	PS-MOE gapmer
Golodirsen	2019	DMD	Dystrophin exon 53	Exon skipping	PMO
Volanesorsen	2019	FCS	ApoC-III	RNase H1	PS-MOE gapmer
Viltolarsen	2020	DMD	Dystrophin exon 53	Exon skipping	PMO
Casimersen	2021	DMD	Dystrophin exon 45	Exon skipping	PMO

ApoB-100, apolipoprotein B-100; ApoC-III, apolipoprotein C-III; CMV, cytomegalovirus; DMD, Duchenne muscular dystrophy; hATTR, hereditary transthyretin-mediated amyloidosis; FCS, familial chylomicronemia syndrome; HoFH, homozygous familial hypercholesterolemia; MOE, 2′-methoxyethyl; ODN, oligodeoxynucleotide; PMO, phosphorodiamidate morpholino oligonucleotide; PS, phosphorothioate; SMA, spinal muscular atrophy; TTR, transthyretin. For details on chemistries, refer to the figures below.

limited to AGO2 enzyme substrates. Therefore, this review focuses on ASOs. It summarizes the past, present, and future of the medicinal chemistry of ASOs, including how to endow oligonucleotide derivatives with drug-likeness to allow them to overcome evolutionary defense systems that act against invading RNAs; such systems include lipid bilayers, RNases, the reticuloendothelial system, immunogenicity, and endocytosis (Dowdy, 2017). In addition, it discusses discoveries regarding the molecular mechanisms underlying the pharmacology, pharmacokinetics, and toxicology of ASOs, and perspectives for improving the therapeutic potential of ASOs by enhancing their therapeutic index and resolving the issue of tissue/cell-specific delivery.

CHEMICAL MODIFICATIONS AND DESIGNS TO ENDOW ASOS WITH DRUG-LIKENESS

Short DNA/RNA-like oligonucleotides with phosphodiester bonds are not drug-like because they are not able to cross the cell membrane, are rapidly degraded by nucleases and cleared by the kidneys through glomerular filtration, and activate innate immune responses. Even as they are taken up into the cells via endocytosis, they remain trapped inside the lipid bilayer of the endosome and cannot access their target RNAs in the cytosol or nucleus to exert pharmacological effects (Dowdy, 2017).

During the last decades, to endow ASOs with drug-likeness, various chemical modifications have been made and tested. Chemical modifications can be made in various sites of nucleotides, including the linkers, sugars, and nucleobases. Fig. 1 summarizes key chemical modifications that have already been applied in commercial products or are considered promising. Fig. 2 shows representative non-ribose sugar modifications, such as phosphorodiamidate morpholino oligomers (PMOs) and peptide nucleic acids (PNAs).

Among them, however, only four chemical classes, including phosphorothioate (PS) oligodeoxynucleotides (ODNs), PS-2′-*O*-methoxyethyl (MOE) gapmers, and occupancy-only oligonucleotides such as PS-MOEs and PMOs, have been clinically accepted, as shown in Table 1 and Fig. 3. Therefore, in this section, the chemistries, designs, and pharmacological properties of these classes of ASOs will be described.

Nucleobase modifications

While numerous nucleobase modifications have been attempted to date, the only clinically accepted and commonly used modification to avoid CpG immune stimulation and enhance nuclease stability and binding affinity is 5-methyl cytosine (MeC, Fig. 1).

Linker modifications

The first breakthrough innovation for ASO drug-likeness was the introduction of a PS linkage (Fig. 1) (Stec *et al*., 1984). In this linker, one non-bridging oxygen of phosphodiester is replaced with a sulfur atom, which greatly alters the physicochemical and pharmacological properties of the resulting ASO (Sands *et al*., 1994; Eckstein, 2000). The PS modification greatly enhances nuclease resistance. Another key benefit of the PS linkage is that the negative charge in PS is more widely distributed than that in a phosphodiester (PO) bond, increasing lipophilicity. This prevents rapid renal clearance through stronger binding with plasma proteins, and enhances cellular uptake through stronger binding with cell surface proteins such as stabilin-1 and 2 (Miller *et al*., 2016). However, its stronger binding to immune receptors results in non-specific pro-inflammatory toxicity (Yanai *et al*., 2011), and the PS modification reduces ASO binding affinity to its complementary nucleic acids by –0.5°C per modification (Freier and Altmann, 1997). The lower potency and pro-inflammatory effect of PS-ODNs (Fig. 3) limit their clinical application and therefore, PS-ODNs require additional chemical modifications.

Sugar modifications

Sugar modification with 2′-alkyl substitution has proven to be a useful strategy to overcome the drawbacks of the PS modification. The most widely used modification, 2′-MOE (Fig. 1), has several advantages, including increased potency resulting from a higher binding affinity (T_m increase of 1.5°C per modification) (Freier and Altmann, 1997), enhanced nuclease resistance, decreased immune-stimulating effect (Henry *et al*., 2000), and prolonged tissue elimination half-life (Geary *et al*., 1997, 2001). The bulky alkoxy group at the 2′ position drives the conformational equilibrium of the furanose ring toward the RNA-like C3′-*endo* conformation (Plavec *et al*., 1993), which has a higher binding affinity for target RNA than the DNA-like C2′-*endo* conformation due to the *gauche* effect. Crystal struc-

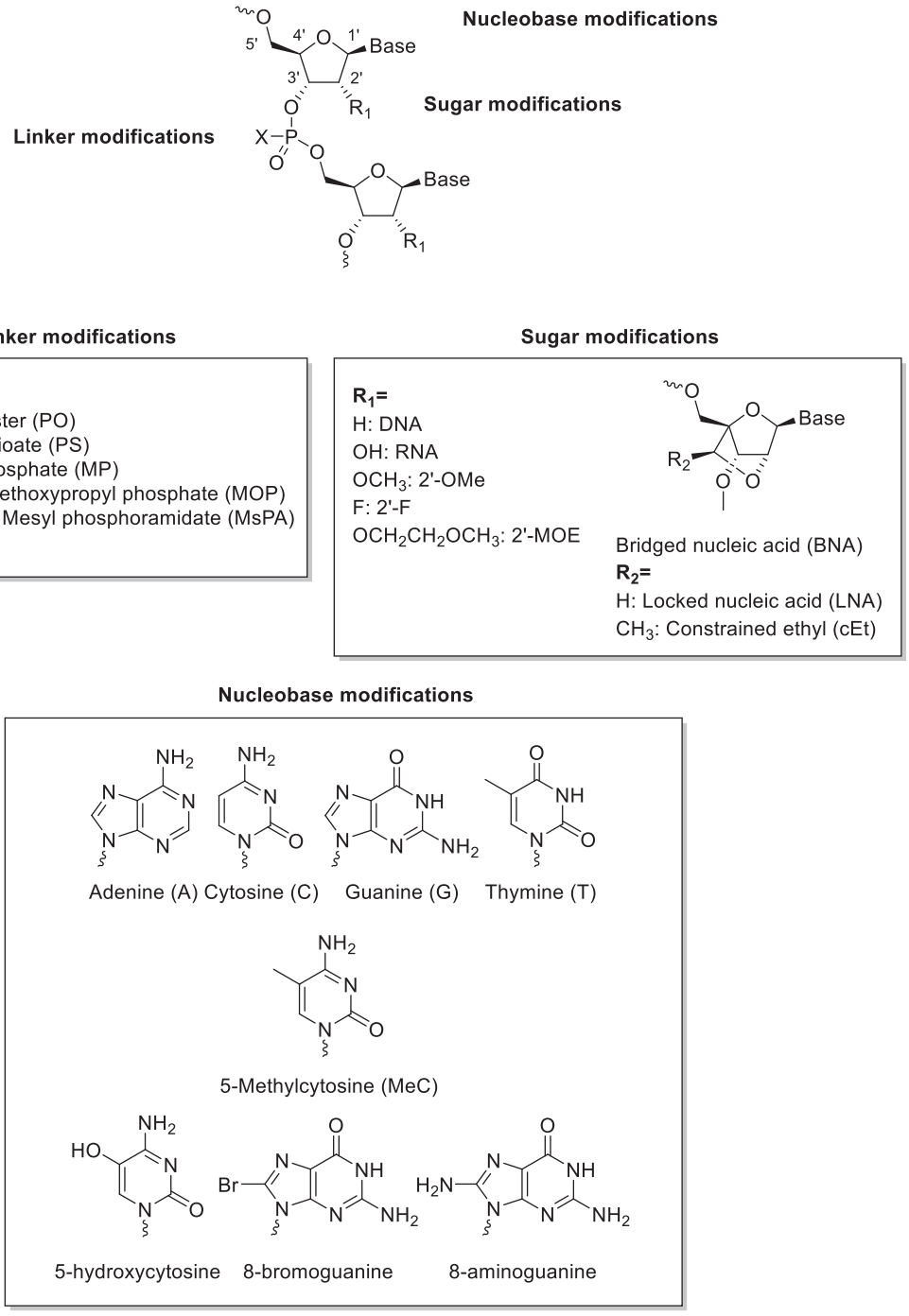

Fig. 1. Chemical modifications in ASOs.

ture analysis supported this conformation and revealed that hydration of MOE and phosphate oxygen plays a key role in overall stability (Teplova *et al*., 1999). As it does not support RNase H-mediated degradation, it has been used for occupancy-only oligonucleotides or as flanks or wings of RNase H-active MOE gapmers (Fig. 3).

As mentioned above, the C3′-*endo* conformation is preferred for strong binding with the target RNA. This conformation is fixed by creating a covalent tether between the 2′ and 4′ positions, leading to a 2′-*O*,4′-C-methylene-bridged nucleic acid (Fig. 1) or locked nucleic acid (LNA) (Obika *et al*., 1997; Koshkin *et al*., 1998) to afford unprecedentedly strong binding affinity for nucleic acids (T_m increase of ~5°C per modification). Owing to the high binding affinity, LNA gapmers (Fig. 3) show strongly improved potency, but are associated with an increased risk of hepatotoxicity, which may depend on ASO

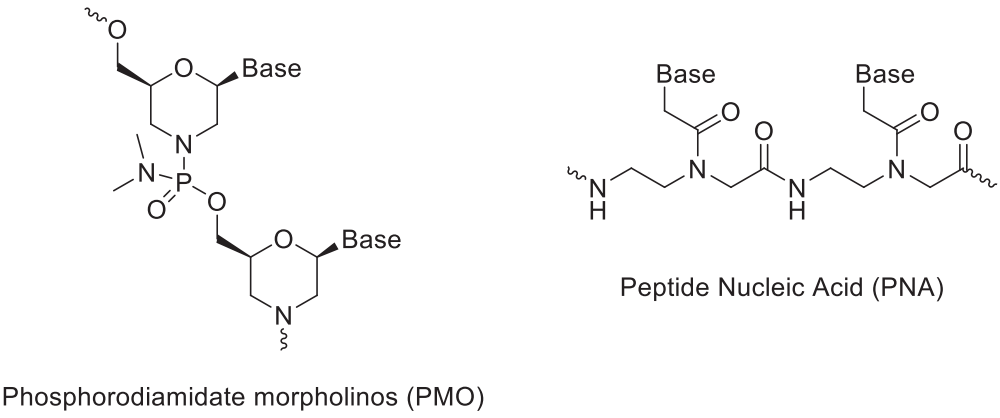

Fig. 2. Non-ribose sugar modifications in ASOs.

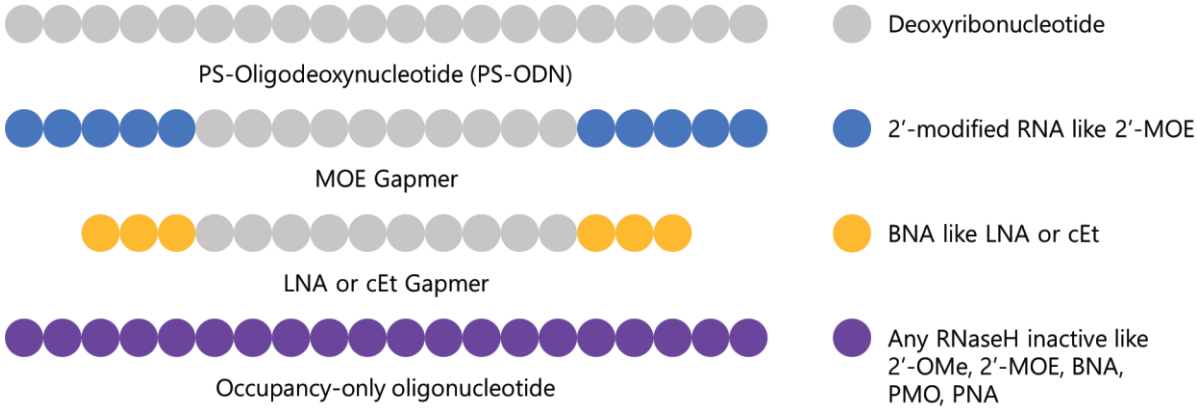

Fig. 3. ASO designs. BNA, bridged nucleic acid; cEt, constrained ethyl; LNA, locked nucleic acid; MOE, methoxyethyl; PMO, phosphorodiamidate morpholino oligonucleotide; PNA, peptide nucleic acid; PS, phosphorothioate.

sequence and design (Swayze *et al*., 2007; Hagedorn *et al*., 2013). Interestingly, the addition of a methyl group to the LNA structure to form 2′,4′-constrained ethyl (cEt, Fig. 1) dramatically reduced hepatotoxicity without a loss in potency as evident in the cEt gapmer (Fig. 3) (Seth *et al*., 2009b). Although ASOs with such modifications (LNA/cEt gapmers) have not been approved for commercial use, numerous candidates are in clinical trials.

Non-ribose sugar modifications

PMOs (Fig. 2) are a neutral class of oligonucleotide analogs in which a morpholino ring replaces the ribofuranose and a phosphorodiamidate linker replaces the PO (Summerton and Weller, 1997). As they also do not support RNase H activity, they have been used as occupancy-only oligonucleotides for exon skipping and successfully marketed for the treatment of Duchenne muscular dystrophy (DMD). PMOs have the advantage of skeletal muscle-selective distribution, which makes them more efficacious for DMD treatment than other RNase H-inactive structures, such as 2′-MOE or cEt (Sheng *et al*., 2020).

PNAs (Fig. 2) are another neutral class of oligonucleotide analogs comprising a peptide scaffold and nucleobases for more efficient Watson–Crick hybridization with complementary nucleic acids. PNAs also do not support RNase H-mediated degradation and act as steric blockers or splicing modulators. However, they have low water solubility and poor cell permeability, limiting their application to molecular genetic diagnostics. There are no successful therapeutic PNAs as yet, despite several efforts to overcome the drawbacks, including structural modifications of the PNA backbone (Gupta *et al*., 2017), conjugation with cell-penetrating peptides (Taylor and Zahid, 2020) or N-acetylgalactosamine (GalNAc) (Bhingardeve *et al*., 2020), or lipid nanoparticle formulation (Gupta *et al*., 2016). The diverse efforts for PNA delivery have been reviewed elsewhere (Volpi *et al*., 2020).

ASO design and main mechanisms of action

All marketed ASO drugs can be classified in two groups based on their mechanisms of action: RNase H-mediated degradation for PS-ODN (formivirsen) and MOE gapmers (mipomersen, inotersen, and volanesorsen), or alternative splicing (exon inclusion or skipping) for occupancy-only oligonucleotides (PS-MOEs such as nusinersen and PMOs including eteplirsen, golodirsen, viltolarsen, and casimersen).

In RNase H-mediated degradation, the RNase H1 enzyme specifically recognizes and cleaves RNA-DNA-like heteroduplexes (Wu *et al*., 2004; Cerritelli and Crouch, 2009). For the

Table 2. Modes of action and relevant chemistry designs of ASOs

	Enzymatic degradation	Occupancy-only	
		Alternative splicing	Steric blocking
Downregulation	RNase H-mediated	NMD	NGD
	AGO2-mediated		Translation arrest
			5′-cap inhibition
			Altering the polyadenylation site
Upregulation		Exon inclusion	miRNA function inhibition
		Exon skipping	Modulation of uORF/TIE
			Inhibition of NMD
Chemistry	RNase H: DNA (w/wo PS linkage) or gapmer	Fully 2′-modified RNA	
	AGO2: dsRNA (siRNA w/wo 2′- F/OMe) or ssRNA	PMO	
	(5′-phosphate)	PNA	

AGO2, Argonaute 2; NGD, no-go decay; NMD, nonsense-mediated decay; uORF, upstream open reading frame; TIE, translation inhibitory element.

ASO in an RNA-ASO duplex to be RNase H-active, at least five (Monia *et al*., 1993), but optimally, eight to ten contiguous deoxynucleotides are required. In addition, PS linkages can activate RNase H1 as PO linkages. Therefore, the central PS-ODN region of 8-10 nucleotides (gap) is flanked by various 3-5-nt 2′-modified nucleotides (wings) such as MOE, LNA, or cEt, which contribute to increased stability and affinity, to generate a gapmer structure (Fig. 3).

Since all 2′ modifications and non-ribose sugar modifications result in loss of RNase H activity, they result in occupancy-only oligonucleotides unless RNase H substrate ODNs are included (Fig. 3). The occupancy-only oligonucleotides bind to the target pre-mRNA and sterically block the spliceosome, leading to alternative splicing in the form of exon inclusion or skipping (nusinersen and four PMO drugs); as a result, they can be used in the treatment of spinal muscular atrophy (Garber, 2016) or DMD (Echevarría *et al*., 2018).

Spinal muscular atrophy is caused by homozygous loss of function of the survival of motor neuron 1 gene (*SMN1*). Although the redundant *SMN2* gene produces limited amounts of full-length SMN protein, it frequently produces short and unstable protein because of exon 7 skipping during pre-mRNA splicing. Nusinersen is an 18-mer fully 2′-MOE-modified PS ASO that binds to and modifies the splicing of *SMN2* pre-mRNA to include exon 7, thus restoring full-length SMN expression (Hua *et al*., 2007).

DMD is a rapidly progressing neuromuscular disorder. Mutations in the *DMD* gene that disrupt its reading frame introduce premature stop codons, halting gene expression. Eteplirsen repairs the reading frame by binding to exon 51 in the pre-mRNA, which is then skipped in translation, producing a shortened, but functional dystrophin (McDonald *et al*., 2021). Similarly, golodirsen (Servais *et al*., 2022) and viltolarsen (Clemens *et al*., 2020) adjust the splicing pattern of mutant *DMD* pre-mRNA by binding to exon 53. The absence of exon 53 in the mature mRNA restores dystrophin expression. Casimersen (Wagner *et al*., 2021) binds to and promotes the skipping of exon 45.

Additional molecular mechanisms for gene expression regulation by ASOs

In addition to the RNase H-mediated degradation or alternative splicing described above, ASOs have several other mechanisms to downregulate or upregulate gene expression, enhancing their versatility. Evolved RNA biology has identified diverse post-RNA-hybridization events of ASOs that are broadly classified into two mechanisms: enzymatic degradation and occupancy-only, as summarized in Table 2 (Crooke *et al*., 2021). Two enzymes degrade RNA-oligonucleotide duplexes. In addition to RNase H discussed above, ASO-RNA hybrids can recruit Argonaute 2 (AGO2) to degrade the target RNA. Occupancy-only can be further classified into alternative splicing and steric blocking. Alternative splicing of pre-mRNA leads to exon inclusion or skipping, as explained above, or it leads to nonsense-mediated decay (NMD) if the resulting mRNA contains a premature termination codon. The effects of steric blocking on the regulation of gene expression are known to vary based on the ASO binding site. The detailed mechanisms and structure-activity relationships in terms of the endpoint of gene regulation, i.e., downregulation or upregulation, are discussed below.

Downregulation of gene expression: AGO2-mediated degradation is the main mechanism by which miRNA/siRNA cleaves its target RNA (Song *et al*., 2004). However, single-stranded siRNA stabilized with 2′-modification and a 5′-(E)-vinylphosphonate, which mimics the 5′-phosphate required for AGO2 activation, can be a substrate (guide strand) of AGO2 (Lima *et al*., 2012).

There are two RNA degradation mechanisms other than degradation by RNase H or AGO2: NMD and no-go decay (NGD). NMD of mRNA is initiated when ASO triggers splicing modulation of pre-mRNA to generate an mRNA that contains premature termination codons, which are NMD targets (Ward *et al*., 2014). Fully modified 2′-MOE ASOs that bind to the 3′ terminus of the open reading frame (ORF) degrade target RNA via NGD (Liang *et al*., 2019b). Fully modified cEt ASOs, which have a higher binding affinity to RNA are known to have a lower efficacy, which may be a result of the stronger binding to proteins. 2′-MOE ASOs with only one cEt have a similar efficacy, while less affined 2′-OMe ASOs have a lower efficacy.

A few reports have disclosed steric blocking as a minor mechanism of downregulation. ASOs can sterically block the translation of mature mRNAs (Melton, 1985; Iversen *et al*., 2003) and guide the cleavage of 5′-cap structures, inhibiting

translation (Baker *et al*., 1999). Finally, ASOs can modulate the use of polyadenylation sites by binding to pre-mRNA sequences involved in cleavage and polyadenylation (Vickers *et al*., 2001).

Upregulation of gene expression: While miRNAs generally downregulate gene expression, ASOs can upregulate gene expression through the inhibition of miRNA function by binding to miRNAs or miRNA-binding sites (Ørom *et al*., 2006; De Santi *et al*., 2020)

Upstream ORFs in the 5′-UTR are sequences that are defined by an initiation codon in frame with a termination codon located upstream or downstream of the main AUG. They are present in approximately half of the human transcripts and inhibit protein expression (Calvo *et al*., 2009). A fully 2′-modified PO or PS-ASO targeting an upstream ORF dose-dependently increased protein expression by 30-150% (Liang *et al*., 2016b). Eighteen-mer PS-MOE, 12-16-mer PS-2′-OMe, and 16-mer PO-2′-OMe ASOs have been suggested as optimal lengths and chemistries for gene upregulation.

Translation was enhanced approximately 3-fold when the recruitment of translation initiation factors to the target mRNA was improved through the binding of fully 2′-modified 16-mer PS-cEt to translation inhibitory elements in 5′-UTRs (Liang *et al*., 2017).

As NMD regulates the normal expression of many genes, ASOs can upregulate gene expression by inhibiting NMD either through the degradation of NMD factors (Huang *et al*., 2018) or through targeting the binding site of the exon junction complex in an mRNA (Nomakuchi *et al*., 2016; Kim *et al*., 2022). Splicing modulation of an ASO preventing non-productive (NMD-inducing) alternative splicing upregulated gene expression (Lim *et al*., 2020).

ASO-PROTEIN INTERACTION: STRUCTURE-ACTIVITY RELATIONSHIP

Many years of research on the mechanisms underlying the potency, pharmacokinetics, and toxicity of ASOs have revealed that ASO-protein interaction is a key factor (Crooke *et al*., 2020a, 2020b). In general, the binding affinity of ASOs for several proteins depends on the PS linkage, gapmer design, and sequence (Vickers and Crooke, 2016). In addition, more hydrophobic 2′ modifications, such as fluorine (F), LNA, or cEt, tend to enhance protein binding compared to more hydrophilic 2′ modifications, such as MOE or OMe, leading to an approximate 10-fold difference in binding affinity for diverse proteins. Many proteins prefer binding to the 5′-wing and the ~6 nucleotides of the 5′-gap position, which contain more hydrophobic 2′ modifications (Shen *et al*., 2019). This section discusses the structure-activity relationship of ASO-protein interaction and its effects on ASO properties, such as nuclease resistance, pharmacokinetic profile, and cellular uptake and endosomal escape capabilities.

Nucleases

Ubiquitous nucleases cleave the PO bonds of unmodified DNA-type oligonucleotides, which therefore have a half-life of only minutes. PS linkages stabilize ASOs against nuclease activity, increasing their plasma half-life to up to 1 h. Chemical modification of the 2′ position with MOE, cEt, or LNA eliminates exonuclease activity, extending tissue elimination half-life to four weeks. MOE gapmers are highly resistant to exonucleases, but are slowly metabolized by endonucleases at the DNA gap site to yield short metabolites of DNA ends, which are degraded further by exonucleases and/or excreted in the urine due to weak plasma protein binding.

Plasma proteins

Plasma protein binding is the main factor for improving absorption and distribution by suppressing renal clearance (Gaus *et al*., 2019). As mentioned above, PS linkages are the key structure for protein binding. As effective binding requires at least 10-12 PS moieties, metabolites from endonucleases and exonucleases that have less than 10 PS groups are cleared from tissues and excreted in the urine via glomerular filtration (Geary *et al*., 2015). As albumin is the most abundant protein in plasma (600 μM) and its binding affinity to a PS-MOE gapmer has a K_d of 12.7 μM (Gaus *et al*., 2019), it plays a major role in the plasma protein binding of PS-ASOs. The binding affinity is sufficiently high to effectively inhibit renal clearance, but sufficiently low to facilitate tissue uptake. In addition, the binding affinity depends on the chemical structure and sequence of the ASO. For example, occupancy-only full PS-MOE has a K_d of 26.9 μM, and T20 PS-ODN has a K_d of 0.94 μM, whereas A20 PS-ODN has a K_d of 204.5 μM.

Cell surface proteins

ASOs are taken up into cells via endocytosis (Doherty and McMahon, 2009). Non-productive uptake results in the accumulation of ASOs in late endosomes and lysosomes, whereas productive uptake involves clathrin- and caveolin-independent endocytosis (Koller *et al*., 2011). Among the numerous cell surface proteins, stabilin-1 and 2 receptors are responsible for ASO cellular uptake and internalization (Miller *et al*., 2016). A binding affinity study (Gaus *et al*., 2018) revealed that PS-ODN had a similar affinity to full PS-MOE, PS-MOE gapmer (5-10-5), and PS-cEt gapmer (5-10-5), which implied that 2′ modifications do not strongly contribute to the binding affinity. The binding affinity decreased as the number of PS linkages decreased (15 PS has a K_d of 83.9 μM, whereas 10 PS and PO-MOE gapmer (5-10-5) have a K_d of >1,000 μM), which indicated that PS linkage is the major contributor to stabilin-2 binding and that at least 15 PS linkages are required for appropriate cellular uptake.

Asialoglycoprotein receptor is highly expressed on hepatocytes (Stockert, 1995). GalNAc is a ligand of this receptor and has been conjugated to an ASO (Prakash *et al*., 2014) as well as an siRNA (Zimmermann *et al*., 2017) for liver/hepatocyte-specific delivery. ASOs can be selectively delivered to pancreatic β-cells via conjugation with glucagon-like peptide 1 receptor agonist (Ämmälä *et al*., 2018; Knerr *et al*., 2021).

Intracellular proteins

After cellular internalization, ASOs are trafficked through multiple membrane-bound intracellular compartments. They then slowly escape endosomes into the cytoplasm and nucleus, where they bind to their target RNAs. The interaction between ASOs and diverse intracellular proteins impacts their intracellular location, including intracellular trafficking, endosomal escape, and transport to the nucleus, affecting their pharmacological effects, such as potency and toxicity.

PS-ASOs interact with more than 50 intracellular proteins, such as P54nrb, which have RNA-recognition motifs, as well

as chaperone proteins, such as HSP90, which lack RNA- or DNA-binding domains, and other proteins (Liang *et al*., 2015, 2016a). Some proteins, including La/SSB, NPM1, ANXA2, VARS, and PC4, appear to enhance ASO activity, likely through mechanisms related to subcellular distribution. VARS and ANXA2, which are co-localized with ASOs in endocytic organelles, may facilitate ASO release from the endosomes. La and NPM1 may play a role in ASO nuclear accumulation. In contrast, Ku70 and Ku80 proteins inhibit ASO activity, most likely by competing with RNase H1 for ASO/RNA duplex binding. Thus, PS-ASOs bind a set of cellular proteins that affect ASO activity via different mechanisms.

Endosomal escape is the rate-limiting step for ASO action. Several studies have suggested that intermediate endosomal compartments, such as multivesicular bodies and late endosomes, are key sites for productive ASO release into the cytosol (Crooke *et al*., 2017b; Juliano, 2018). The detailed mechanism has not yet been fully unraveled, but recent studies have revealed that proteins, such as M6PR (Liang *et al*., 2019a), and COPII vesicles (Liang *et al*., 2018) may play key roles in this process. No chemical modifications or conjugates for improving endosomal escape of ASOs have been discovered yet, although some small-molecular additives (Yang *et al*., 2015; Wang *et al*., 2017; Juliano *et al*., 2018), conjugation with synthetic endosomal escape domains (Lönn *et al*., 2016), and diverse cell-penetrating peptides (Kondow-McConaghy *et al*., 2020) have been evaluated. As it is estimated that the endosomal escape rate is quite low (<1%), ASO potency would be dramatically improved if the endosomal escape issue were to be resolved.

TOXICITY OF ASOS: STRUCTURE-ACTIVITY RELATIONSHIP

The toxicity of ASOs can be classified into sequence-dependent and sequence-independent toxicity. Sequence-dependent toxicity stems from the hybridization of ASOs with unintended targets, such as long pre-mRNA transcripts (Burel *et al*., 2016) or long introns (Kamola *et al*., 2015); however, it occurs less frequently than once thought (Lima *et al*., 2014). Sequence-independent toxicity stems from the interaction of ASOs with several proteins, such as P54nrb and RNase H1 (Vickers *et al*., 2019), which have been suggested to be key proteins. Toxic ASOs bind to paraspeckle proteins and RNase H1 to mislocalize them to the nucleolus and inhibit nucleolar RNA transcription and processing, which leads to nucleolar stress and apoptosis (Shen *et al*., 2019). The hepatotoxicity of ASOs may originate from sequence motifs, such as TCC/TGC, that typically bind to hepatocellular proteins, increasing the activity of the P53 and nuclear factor erythroid-2-related factor 2 stress pathways (Burdick *et al*., 2014).

MOE gapmer ASOs

Integrated safety assessment of MOE gapmer ASOs for kidney and liver function, hematology, and complement activation in non-human primates and humans has revealed that this chemical class is well tolerated in humans compared to the toxicities, such as complement activation and effects on platelets, found in non-human primates (Crooke *et al*., 2016). Although systemically administered MOE gapmer ASOs are distributed mainly to the kidneys and metabolites are cleared in the urine, no marked renal toxicity has been observed in numerous clinical trials using up to >175 mg/week dose and 52-week treatment (Crooke *et al*., 2018). After 4.6 years of treatment with up to 624 mg/week, MOE gapmer ASOs had no negative impact on platelet count (Crooke *et al*., 2017a).

Compared to PS-ODNs, MOE gapmers are substantially less immunostimulatory. In addition, immunotoxicity is dose-dependent; therefore, more potent MOE or cEt gapmer ASOs for which the therapeutic dose is lower have a lower risk of immunotoxicity. The CpG motif stimulates the innate immune response; however, 5-methyl cytosine replacement reduced this side effect.

A few MOE gapmers, including ISIS 147420, has been known to induce severe inflammatory response in mice. The adverse effects are characterized by the induction of interferon-β, followed by acute transaminitis and extensive hepatocyte apoptosis and necrosis (Burel *et al*., 2012). A specific sequence motif in viral DNA or RNA may trigger this acute innate immune response; thus, a single-nucleotide change can abolish this toxicity.

LNA/cEt gapmer ASOs

As mentioned above, LNA gapmers have higher risk of hepatotoxicity than MOE or cEt gapmers. The hepatotoxicity depends on a number of parameters, such as their length, gapmer design, and sequence. Shorter LNA gapmers have lower hepatotoxicity (Seth *et al*., 2009a). Regardless of 2′ modifications, toxic ASOs are able to bind to paraspeckle proteins, such as P54nrb and PSF, as well as RNase H1, delocalizing them to the nucleoli, which leads to nucleolar stress, P53 activation, and apoptosis (Shen *et al*., 2019). It has been reported that more toxic ASOs bind more proteins than non-toxic ones, and 5′-MOE-DNA-3′-cEt gapmer (mixmer) is less toxic than cEt gapmer or 5′-cEt-DNA-3′-MOE gapmer (mixmer), which implies that the 5′-side and more hydrophobic residues are preferred for protein binding. The introduction of a 2′-OMe at gap position 2 (Fig. 4) mitigated cytotoxicity by suppressing protein binding. Several chemical modifications with the aim of reducing toxicity and improving the therapeutic potential of ASOs are discussed in more detail below.

CHEMICAL MODIFICATIONS FOR IMPROVING THE THERAPEUTIC POTENTIAL OF ASO

As LNA and cEt gapmers with high affinity and potency have the issue of hepatotoxicity, diverse chemical modifications, including site-specific ones, have been developed, usually focused on the 5′ side of the gap, to mitigate toxicity (Fig. 4).

Nucleobase modifications

Recently, Yoshida *et al*. (2022) reported that some nucleobase modifications, such as 5-hydroxycytosine, 8-bromo, and amine guanine (Fig. 1), reduce the hepatotoxicity of LNA gapmers.

Linkage modifications

Alkylphosphonates: While the methyl phosphonate linkage (Fig. 1) was introduced long ago (Miller *et al*., 1979) and despite its advantages, such as having a neutral charge and nuclease resistance, it was not widely used due to RNase H

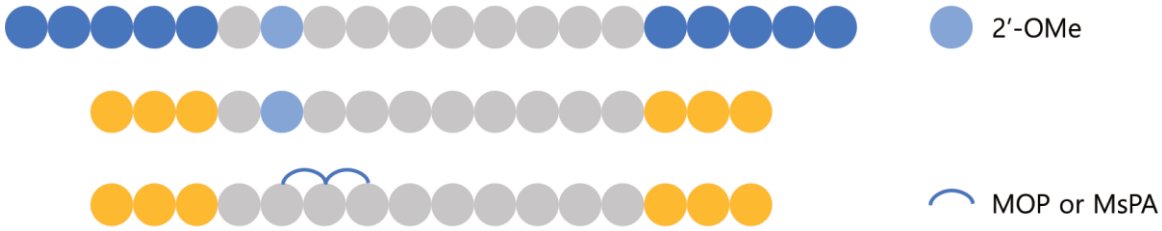

Fig. 4. Site-specific modifications to mitigate toxicity. MOP, methoxy propyl phosphonate; MsPA, mesyl phosphoramidate.

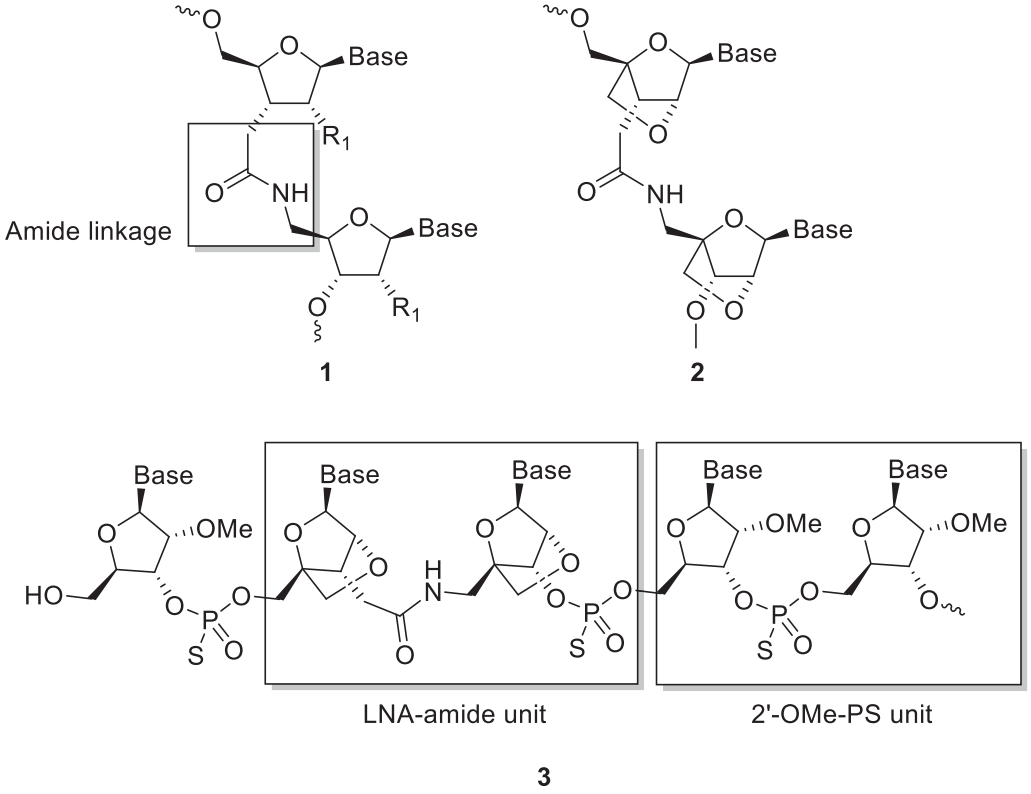

Fig. 5. Amide linkages and ASOs containing them. **1**. Amide linkage **2**. LNA-Amide linkage **3**. Combination of LNA-amide linkage and 2-OMe-PS linkage.

inactivity, a relatively low affinity for RNA, and chemical instability under the basic condition required for the cleavage/deprotection step of oligonucleotide synthesis on a solid support. Recently, site-specific replacement of PS with alkyl phosphonates, such as methyl phosphonate or methoxy propyl phosphonate (Fig. 1), was reported to reduce toxicity by modulating the interactions with intracellular proteins (Migawa *et al*., 2019). For example, a 3-10-3 cEt gapmer (Fig. 3) in which one or two PS linkages at position 2 or 3 from the 5′-gap end were replaced with methyl phosphonate or methoxy propyl phosphonate (Fig. 4) showed comparable potency and substantially reduced hepatotoxicity, resulting in a higher therapeutic index than that of the parent ASO. The reduction in toxicity was accompanied by the absence of nucleolar mislocalization of paraspeckle protein P54nrb, ablation of P21 mRNA elevation, and caspase activation, which are thought to be key mechanisms of hepatotoxicity of PS-ASOs.

Mesyl phosphoramidate (MsPA): Oligonucleotide derivatives with an MsPA (Fig. 1) linkage, the synthetic method of which was recently developed (Chelobanov *et al*., 2017), have been demonstrated to have RNase H-activating ability, nuclease resistance, and anti-miRNA activity (Miroshnichenko *et al*., 2019). Their superior *in vivo* efficacy over PS (Patutina *et al*., 2020) and potential application in splice-switching (Hammond *et al*., 2021) have been demonstrated. A comprehensive study by (Anderson *et al*., 2021) on the impact of MsPA linkages on therapeutic properties showed that replacing up to 5 PS linkages in the gap with MsPA (Fig. 4) was well tolerated, and replacing specific PS linkages at appropriate locations greatly improved nuclease stability and potency and reduced both immune stimulation and cytotoxicity, leading to an improved therapeutic index, reduced pro-inflammatory effects,

and extended effect duration.

Amides: Following the early attempts to replace PO with amide (**1** in Fig. 5) (De Mesmaeker *et al*., 1994), the application of this neutral linkage to ASOs had not extensively been studied. However, recent systemic studies have investigated its impact on the pharmacological properties of an siRNA (Mutisya *et al*., 2017; Kotikam and Rozners, 2020) and an ASO (Epple *et al*., 2020). It was revealed that the amide linkage has no RNase H activity, but it could replace PO linkages at each wing of a gapmer to maintain RNA binding affinity and RNase H activity. The amide linkages combined with LNA (**2** in Fig. 5) enhanced potency and cellular uptake, and reduced toxicity (Baker *et al*., 2022). The exon-skipping activity of an 18-mer ASO containing 4 LNA-amide linkages and 14 2′-OMe with 13 PS linkages (**3** in Fig. 5) was higher than that of an 18-mer ASO with 17 PS linkages with all 2′-OMe or 8 LNA+10 2′-OMe. More importantly, the activity gap substantially increased when they were transfected without lipofectamine, which implied that the 18-mer ASO containing 4 LNA-amide linkages had substantially better cellular uptake ability.

Synergy by combination

The combination of 2′-OMe at the 5′-gap 2 position and two MsPA linkages at the 5′ end of the gap or in the 3′-wing improved the RNase H1 cleavage rate, considerably reduced the binding of proteins involved in cytotoxicity, and extended the elimination half-live (Zhang *et al*., 2022).

CONCLUSIONS AND PERSPECTIVES

During the last decades, advances in medicinal chemistry have led to the development of drug-like ASO drugs that are sufficiently potent to treat various rare diseases. Among many chemical modifications, PS linkage, 2′-modification with MOE or cEt, and PMO are the key achievements that have been successfully clinically applied. As numerous molecular mechanisms underlying the pharmacology, pharmacokinetics, and toxicology of ASOs, such as ASO-protein interactions, have been revealed, it is now possible to manipulate the chemical modification and design of ASOs to broaden their therapeutic margin by fine-tuning or combining available chemistries based on structure-activity relationships. For example, site-specific modifications with 2′-OMe, MsPA, or an amide linkage are efficient in reducing toxicity while maintaining potency.

For ASOs to be more widely used for common diseases as well, two key challenges remain to be resolved. The first is tissue/cell-specific delivery to organs other than the liver. Besides GalNAc ligand conjugates targeting the asialoglycoprotein receptor on hepatocytes, which have been approved for clinical uses, another specific and efficacious ligand-receptor system should be discovered for targeting another tissues or cells. The second is chemical modification or conjugation to enhance cellular uptake and endosomal escape for improved potency. Some neutral linkers, such as amides that reduce the negative charge of PS-ASOs, are promising for this purpose. They are important because they will enhance potency and safety and lower the therapeutic dose, and as a result, reduce the cost and the risk of dose-dependent toxicity.

CONFLICT OF INTEREST

The author is the founder and CEO of Qmine Co., Ltd.

REFERENCES

Ämmälä, C., Drury, W. J., 3rd, Knerr, L., Ahlstedt, I., Stillemark-Billton, P., Wennberg-Huldt, C., Andersson, E. M., Valeur, E., Jansson-Löfmark, R., Janzén, D., Sundström, L., Meuller, J., Claesson, J., Andersson, P., Johansson, C., Lee, R. G., Prakash, T. P., Seth, P. P., Monia, B. P. and Andersson, S. (2018) Targeted delivery of antisense oligonucleotides to pancreatic β-cells. *Sci. Adv.* **4**, eaat3386.

Anderson, B. A., Freestone, G. C., Low, A., De-Hoyos, C. L., Iii, W. J. D., Ostergaard, M. E., Migawa, M. T., Fazio, M., Wan, W. B., Berdeja, A., Scandalis, E., Burel, S. A., Vickers, T. A., Crooke, S. T., Swayze, E. E., Liang, X. and Seth, P. P. (2021) Towards next generation antisense oligonucleotides: mesylphosphoramidate modification improves therapeutic index and duration of effect of gapmer antisense oligonucleotides. *Nucleic Acids Res.* **49**, 9026-9041.

Baker, B. F., Lot, S. S., Kringel, J., Cheng-Flournoy, S., Villiet, P., Sasmor, H. M., Siwkowski, A. M., Chappell, L. L. and Morrow, J. R. (1999) Oligonucleotide-europium complex conjugate designed to cleave the 5' cap structure of the ICAM-1 transcript potentiates antisense activity in cells. *Nucleic Acids Res.* **27**, 1547-1551.

Baker, Y. R., Thorpe, C., Chen, J., Poller, L. M., Cox, L., Kumar, P., Lim, W. F., Lie, L., McClorey, G., Epple, S., Singleton, D., McDonough, M. A., Hardwick, J. S., Christensen, K. E., Wood, M. J. A., Hall, J. P., El-Sagheer, A. H. and Brown, T. (2022) An LNA-amide modification that enhances the cell uptake and activity of phosphorothioate exon-skipping oligonucleotides. *Nat. Commun.* **13**, 4036.

Bhingardeve, P., Madhanagopal, B. R., Naick, H., Jain, P., Manoharan, M. and Ganesh, K. (2020) Receptor-specific delivery of peptide nucleic acids conjugated to three sequentially linked N-acetyl galactosamine moieties into hepatocytes. *J. Org. Chem.* **85**, 8812-8824.

Burdick, A. D., Sciabola, S., Mantena, S. R., Hollingshead, B. D., Stanton, R., Warneke, J. A., Zeng, M., Martsen, E., Medvedev, A., Makarov, S. S., Reed, L. A., Davis, J. W., 2nd and Whiteley, L. O. (2014) Sequence motifs associated with hepatotoxicity of locked nucleic acid--modified antisense oligonucleotides. *Nucleic Acids Res.* **42**, 4882-4891.

Burel, S. A., Hart, C. E., Cauntay, P., Hsiao, J., Machemer, T., Katz, M., Watt, A., Bui, H. H., Younis, H., Sabripour, M., Freier, S. M., Hung, G., Dan, A., Prakash, T. P., Seth, P. P., Swayze, E. E., Bennett, C. F., Crooke, S. T. and Henry, S. P. (2016) Hepatotoxicity of high affinity gapmer antisense oligonucleotides is mediated by RNase H1 dependent promiscuous reduction of very long pre-mRNA transcripts. *Nucleic Acids Res.* **44**, 2093-2109.

Burel, S. A., Machemer, T., Ragone, F. L., Kato, H., Cauntay, P., Greenlee, S., Salim, A., Gaarde, W. A., Hung, G., Peralta, R., Freier, S. M. and Henry, S. P. (2012) Unique O-methoxyethyl ribose-DNA chimeric oligonucleotide induces an atypical melanoma differentiation-associated gene 5-dependent induction of type I interferon response. *J. Pharmacol. Exp. Ther.* **342**, 150-162.

Calvo, S. E., Pagliarini, D. J. and Mootha, V. K. (2009) Upstream open reading frames cause widespread reduction of protein expression and are polymorphic among humans. *Proc. Natl. Acad. Sci. U. S. A.* **106**, 7507-7512.

Cerritelli, S. M. and Crouch, R. J. (2009) Ribonuclease H: the enzymes in eukaryotes. *FEBS J.* **276**, 1494-1505.

Chelobanov, B. P., Burakova, E. A., Prokhorova, D. V., Fokina, A. A. and Stetsenko, D. A. (2017) New oligodeoxynucleotide derivatives containing N-(methanesulfonyl)-phosphoramidate (mesyl phosphoramidate) internucleotide group. *Russ. J. Bioorg. Chem.* **43**, 664-668.

Clemens, P. R., Rao, V. K., Connolly, A. M., Harper, A. D., Mah, J. K., Smith, E. C., McDonald, C. M., Zaidman, C. M., Morgenroth, L. P., Osaki, H., Satou, Y., Yamashita, T. and Hoffman, E. P. (2020) Safety, tolerability, and efficacy of viltolarsen in boys with Duchenne muscular dystrophy amenable to exon 53 skipping: a phase 2 randomized clinical trial. *JAMA Neurol.* **77**, 982-991.

Crooke, S. T., Baker, B. F., Crooke, R. M. and Liang, X. H. (2021) Antisense technology: an overview and prospectus. *Nat. Rev. Drug Discov.* **20**, 427-453.

Crooke, S. T., Baker, B. F., Kwoh, T. J., Cheng, W., Schulz, D. J., Xia, S., Salgado, N., Bui, H. H., Hart, C. E., Burel, S. A., Younis, H. S., Geary, R. S., Henry, S. P. and Bhanot, S. (2016) Integrated safety assessment of 2'-O-methoxyethyl chimeric antisense oligonucleotides in nonhuman primates and healthy human volunteers. *Mol. Ther.* **24**, 1771-1782.

Crooke, S. T., Baker, B. F., Pham, N. C., Hughes, S. G., Kwoh, T. J., Cai, D., Tsimikas, S., Geary, R. S. and Bhanot, S. (2018) The effects of 2'-O-methoxyethyl oligonucleotides on renal function in humans. *Nucleic Acid Ther.* **28**, 10-22.

Crooke, S. T., Baker, B. F., Witztum, J. L., Kwoh, T. J., Pham, N. C., Salgado, N., McEvoy, B. W., Cheng, W., Hughes, S. G., Bhanot, S. and Geary, R. S. (2017a) The effects of 2'-O-methoxyethyl containing antisense oligonucleotides on platelets in human clinical trials. *Nucleic Acid Ther.* **27**, 121-129.

Crooke, S. T., Seth, P. P., Vickers, T. A. and Liang, X. H. (2020a) The interaction of phosphorothioate-containing RNA targeted drugs with proteins is a critical determinant of the therapeutic effects of these agents. *J. Am. Chem. Soc.* **142**, 14754-14771.

Crooke, S. T., Vickers, T. A. and Liang, X. H. (2020b) Phosphorothioate modified oligonucleotide-protein interactions. *Nucleic Acids Res.* **48**, 5235-5253.

Crooke, S. T., Wang, S., Vickers, T. A., Shen, W. and Liang, X. H. (2017b) Cellular uptake and trafficking of antisense oligonucleotides. *Nat. Biotechnol.* **35**, 230-237.

De Mesmaeker, A., Waldner, A., Lebreton, J., Hoffmann, P., Fritsch, V., Wolf, R. M. and Freier, S. M. (1994) Amides as a new type of backbone modification in oligonucleotides. *Angew. Chem. Int. Ed. Engl.* **33**, 226-229.

De Santi, C., Fernández Fernández, E., Gaul, R., Vencken, S., Glasgow, A., Oglesby, I. K., Hurley, K., Hawkins, F., Mitash, N., Mu, F., Raoof, R., Henshall, D. C., Cutrona, M. B., Simpson, J. C., Harvey, B. J., Linnane, B., McNally, P., Cryan, S. A., MacLoughlin, R., Swiatecka-Urban, A. and Greene, C. M. (2020) Precise targeting of miRNA sites restores CFTR activity in CF bronchial epithelial cells. *Mol. Ther.* **28**, 1190-1199.

Djebali, S., Davis, C. A., Merkel, A., Dobin, A., Lassmann, T., Mortazavi, A., Tanzer, A., Lagarde, J., Lin, W., Schlesinger, F., Xue, C., Marinov, G. K., Khatun, J., Williams, B. A., Zaleski, C., Rozowsky, J., Röder, M., Kokocinski, F., Abdelhamid, R. F., Alioto, T., Antoshechkin, I., Baer, M. T., Bar, N. S., Batut, P., Bell, K., Bell, I., Chakrabortty, S., Chen, X., Chrast, J., Curado, J., Derrien, T., Drenkow, J., Dumais, E., Dumais, J., Duttagupta, R., Falconnet, E., Fastuca, M., Fejes-Toth, K., Ferreira, P., Foissac, S., Fullwood, M. J., Gao, H., Gonzalez, D., Gordon, A., Gunawardena, H., Howald, C., Jha, S., Johnson, R., Kapranov, P., King, B., Kingswood, C., Luo, O. J., Park, E., Persaud, K., Preall, J. B., Ribeca, P., Risk, B., Robyr, D., Sammeth, M., Schaffer, L., See, L.-H., Shahab, A., Skancke, J., Suzuki, A. M., Takahashi, H., Tilgner, H., Trout, D., Walters, N., Wang, H., Wrobel, J., Yu, Y., Ruan, X., Hayashizaki, Y., Harrow, J., Gerstein, M., Hubbard, T., Reymond, A., Antonarakis, S. E., Hannon, G., Giddings, M. C., Ruan, Y., Wold, B., Carninci, P., Guigó, R. and Gingeras, T. R. (2012) Landscape of transcription in human cells. *Nature* **489**, 101-108.

Doherty, G. J. and McMahon, H. T. (2009) Mechanisms of endocytosis. *Annu. Rev. Biochem.* **78**, 857-902.

Dowdy, S. F. (2017) Overcoming cellular barriers for RNA therapeutics. *Nat. Biotechnol.* **35**, 222-229.

Echevarría, L., Aupy, P. and Goyenvalle, A. (2018) Exon-skipping advances for Duchenne muscular dystrophy. *Hum. Mol. Genet.* **27**, R163-R172.

Eckstein, F. (2000) Phosphorothioate oligodeoxynucleotides: what is their origin and what is unique about them? *Antisense Nucleic Acid Drug Dev.* **10**, 117-121.

Epple, S., Thorpe, C., Baker, Y. R., El-Sagheer, A. H. and Brown, T. (2020) Consecutive 5′- and 3′-amide linkages stabilise antisense oligonucleotides and elicit an efficient RNase H response. *Chem. Commun.* **56**, 5496-5499.

Fire, A., Xu, S., Montgomery, M. K., Kostas, S. A., Driver, S. E. and Mello, C. C. (1998) Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. *Nature* **391**, 806-811.

Freier, S. M. and Altmann, K. H. (1997) The ups and downs of nucleic acid duplex stability: structure-stability studies on chemically-modified DNA:RNA duplexes. *Nucleic Acids Res.* **25**, 4429-4443.

Garber, K. (2016) Big win possible for Ionis/Biogen antisense drug in muscular atrophy. *Nat. Biotechnol.* **34**, 1002-1003.

Gaus, H., Miller, C. M., Seth, P. P. and Harris, E. N. (2018) Structural determinants for the interactions of chemically modified nucleic acids with the stabilin-2 clearance receptor. *Biochemistry* **57**, 2061-2064.

Gaus, H. J., Gupta, R., Chappell, A. E., Østergaard, M. E., Swayze, E. E. and Seth, P. P. (2019) Characterization of the interactions of chemically-modified therapeutic nucleic acids with plasma proteins using a fluorescence polarization assay. *Nucleic Acids Res.* **47**, 1110-1122.

Geary, R. S., Baker, B. F. and Crooke, S. T. (2015) Clinical and preclinical pharmacokinetics and pharmacodynamics of mipomersen (kynamro(®)): a second-generation antisense oligonucleotide inhibitor of apolipoprotein B. *Clin. Pharmacokinet.* **54**, 133-146.

Geary, R. S., Leeds, J. M., Fitchett, J., Burckin, T., Truong, L., Spainhour, C., Creek, M. and Levin, A. A. (1997) Pharmacokinetics and metabolism in mice of a phosphorothioate oligonucleotide antisense inhibitor of C-raf-1 kinase expression. *Drug Metab. Dispos.* **25**, 1272-1281.

Geary, R. S., Watanabe, T. A., Truong, L., Freier, S., Lesnik, E. A., Sioufi, N. B., Sasmor, H., Manoharan, M. and Levin, A. A. (2001) Pharmacokinetic properties of 2'-O-(2-methoxyethyl)-modified oligonucleotide analogs in rats. *J. Pharmacol. Exp. Ther.* **296**, 890-897.

Gupta, A., Bahal, R., Gupta, M., Glazer, P. M. and Saltzman, W. M. (2016) Nanotechnology for delivery of peptide nucleic acids (PNAs). *J. Control. Release* **240**, 302-311.

Gupta, A., Mishra, A. and Puri, N. (2017) Peptide nucleic acids: advanced tools for biomedical applications. *J. Biotechnol.* **259**, 148-159.

Hagedorn, P. H., Yakimov, V., Ottosen, S., Kammler, S., Nielsen, N. F., Hog, A. M., Hedtjarn, M., Meldgaard, M., Moller, M. R., Orum, H., Koch, T. and Lindow, M. (2013) Hepatotoxic potential of therapeutic oligonucleotides can be predicted from their sequence and modification pattern. *Nucleic Acid Ther.* **23**, 302-310.

Hammond, S. M., Sergeeva, O. V., Melnikov, P. A., Goli, L., Stoodley, J., Zatsepin, T. S., Stetsenko, D. A. and Wood, M. J. A. (2021) Mesyl phosphoramidate oligonucleotides as potential splice-switching agents: impact of backbone structure on activity and intracellular localization. *Nucleic Acid Ther.* **31**, 190-200.

Henry, S., Stecker, K., Brooks, D., Monteith, D., Conklin, B. and Bennett, C. F. (2000) Chemically modified oligonucleotides exhibit decreased immune stimulation in mice. *J. Pharmacol. Exp. Ther.* **292**, 468-479.

Hua, Y., Vickers, T. A., Baker, B. F., Bennett, C. F. and Krainer, A. R. (2007) Enhancement of SMN2 exon 7 inclusion by antisense oligonucleotides targeting the exon. *PLoS Biol.* **5**, e73.

Huang, L., Low, A., Damle, S. S., Keenan, M. M., Kuntz, S., Murray, S. F., Monia, B. P. and Guo, S. (2018) Antisense suppression of the nonsense mediated decay factor Upf3b as a potential treatment for diseases caused by nonsense mutations. *Genome Biol.* **19**, 4.

Iversen, P. L., Arora, V., Acker, A. J., Mason, D. H. and Devi, G. R. (2003) Efficacy of antisense morpholino oligomer targeted to c-myc in prostate cancer xenograft murine model and a Phase I safety study in humans. *Clin. Cancer Res.* **9**, 2510-2519.

Juliano, R. L. (2018) Intracellular trafficking and endosomal release of oligonucleotides: what we know and what we don't. *Nucleic Acid Ther.* **28**, 166-177.

Juliano, R. L., Wang, L., Tavares, F., Brown, E. G., James, L., Ariyarathna, Y., Ming, X., Mao, C. and Suto, M. (2018) Structure-activity relationships and cellular mechanism of action of small molecules that enhance the delivery of oligonucleotides. *Nucleic Acids Res.* **46**, 1601-1613.

Kamola, P. J., Kitson, J. D., Turner, G., Maratou, K., Eriksson, S., Panjwani, A., Warnock, L. C., Douillard Guilloux, G. A., Moores, K.,

Koppe, E. L., Wixted, W. E., Wilson, P. A., Gooderham, N. J., Gant, T. W., Clark, K. L., Hughes, S. A., Edbrooke, M. R. and Parry, J. D. (2015) In silico and *in vitro* evaluation of exonic and intronic off-target effects form a critical element of therapeutic ASO gapmer optimization. *Nucleic Acids Res.* **43**, 8638-8650.

Kim, J., Hu, C., Moufawad El Achkar, C., Black, L. E., Douville, J., Larson, A., Pendergast, M. K., Goldkind, S. F., Lee, E. A., Kuniholm, A., Soucy, A., Vaze, J., Belur, N. R., Fredriksen, K., Stojkovska, I., Tsytsykova, A., Armant, M., DiDonato, R. L., Choi, J., Cornelissen, L., Pereira, L. M., Augustine, E. F., Genetti, C. A., Dies, K., Barton, B., Williams, L., Goodlett, B. D., Riley, B. L., Pasternak, A., Berry, E. R., Pflock, K. A., Chu, S., Reed, C., Tyndall, K., Agrawal, P. B., Beggs, A. H., Grant, P. E., Urion, D. K., Snyder, R. O., Waisbren, S. E., Poduri, A., Park, P. J., Patterson, A., Biffi, A., Mazzulli, J. R., Bodamer, O., Berde, C. B. and Yu, T. W. (2019) Patient-customized oligonucleotide therapy for a rare genetic disease. *N. Engl. J. Med.* **381**, 1644-1652.

Kim, Y. J., Nomakuchi, T., Papaleonidopoulou, F., Yang, L., Zhang, Q. and Krainer, A. R. (2022) Gene-specific nonsense-mediated mRNA decay targeting for cystic fibrosis therapy. *Nat. Commun.* **13**, 2978.

Knerr, L., Prakash, T. P., Lee, R., Drury Iii, W. J., Nikan, M., Fu, W., Pirie, E., Maria, L. D., Valeur, E., Hayen, A., Ölwegård-Halvarsson, M., Broddefalk, J., Ämmälä, C., Østergaard, M. E., Meuller, J., Sundström, L., Andersson, P., Janzén, D., Jansson-Löfmark, R., Seth, P. P. and Andersson, S. (2021) Glucagon Like Peptide 1 Receptor agonists for targeted delivery of antisense oligonucleotides to pancreatic beta cell. *J. Am. Chem. Soc.* **143**, 3416-3429.

Koller, E., Vincent, T. M., Chappell, A., De, S., Manoharan, M. and Bennett, C. F. (2011) Mechanisms of single-stranded phosphorothioate modified antisense oligonucleotide accumulation in hepatocytes. *Nucleic Acids Res.* **39**, 4795-4807.

Kondow-McConaghy, H. M., Muthukrishnan, N., Erazo-Oliveras, A., Najjar, K., Juliano, R. L. and Pellois, J.-P. (2020) Impact of the endosomal escape activity of cell-penetrating peptides on the endocytic pathway. *ACS Chem. Biol.* **15**, 2355-2363.

Koshkin, A. A., Singh, S. K., Nielsen, P., Rajwanshi, V. K., Kumar, R., Meldgaard, M., Olsen, C. E. and Wengel, J. (1998) LNA (Locked Nucleic Acids): synthesis of the adenine, cytosine, guanine, 5-methylcytosine, thymine and uracil bicyclonucleoside monomers, oligomerisation, and unprecedented nucleic acid recognition. *Tetrahedron* **54**, 3607-3630.

Kotikam, V. and Rozners, E. (2020) Amide-modified RNA: using protein backbone to modulate function of short interfering RNAs. *Acc. Chem. Res.* **53**, 1782-1790.

Liang, X.-h., Sun, H., Hsu, C.-W., Nichols, J. G., Vickers, T. A., De Hoyos, C. L. and Crooke, S. T. (2019a) Golgi-endosome transport mediated by M6PR facilitates release of antisense oligonucleotides from endosomes. *Nucleic Acids Res.* **48**, 1372-1391.

Liang, X.-h., Sun, H., Shen, W., Wang, S., Yao, J., Migawa, M. T., Bui, H.-H., Damle, S. S., Riney, S., Graham, M. J., Crooke, R. M. and Crooke, S. T. (2017) Antisense oligonucleotides targeting translation inhibitory elements in 5′ UTRs can selectively increase protein levels. *Nucleic Acids Res.* **45**, 9528-9546.

Liang, X. H., Nichols, J. G., Hsu, C. W., Vickers, T. A. and Crooke, S. T. (2019b) mRNA levels can be reduced by antisense oligonucleotides via no-go decay pathway. *Nucleic Acids Res.* **47**, 6900-6916.

Liang, X. H., Shen, W., Sun, H., Kinberger, G. A., Prakash, T. P., Nichols, J. G. and Crooke, S. T. (2016a) Hsp90 protein interacts with phosphorothioate oligonucleotides containing hydrophobic 2'-modifications and enhances antisense activity. *Nucleic Acids Res.* **44**, 3892-3907.

Liang, X. H., Shen, W., Sun, H., Migawa, M. T., Vickers, T. A. and Crooke, S. T. (2016b) Translation efficiency of mRNAs is increased by antisense oligonucleotides targeting upstream open reading frames. *Nat. Biotechnol.* **34**, 875-880.

Liang, X. H., Sun, H., Nichols, J. G., Allen, N., Wang, S., Vickers, T. A., Shen, W., Hsu, C. W. and Crooke, S. T. (2018) COPII vesicles can affect the activity of antisense oligonucleotides by facilitating the release of oligonucleotides from endocytic pathways. *Nucleic Acids Res.* **46**, 10225-10245.

Liang, X. H., Sun, H., Shen, W. and Crooke, S. T. (2015) Identification and characterization of intracellular proteins that bind oligonucleotides with phosphorothioate linkages. *Nucleic Acids Res.* **43**, 2927-2945.

Lim, K. H., Han, Z., Jeon, H. Y., Kach, J., Jing, E., Weyn-Vanhentenryck, S., Downs, M., Corrionero, A., Oh, R., Scharner, J., Venkatesh, A., Ji, S., Liau, G., Ticho, B., Nash, H. and Aznarez, I. (2020) Antisense oligonucleotide modulation of non-productive alternative splicing upregulates gene expression. *Nat. Commun.* **11**, 3501.

Lima, W. F., Prakash, T. P., Murray, H. M., Kinberger, G. A., Li, W., Chappell, A. E., Li, C. S., Murray, S. F., Gaus, H., Seth, P. P., Swayze, E. E. and Crooke, S. T. (2012) Single-stranded siRNAs activate RNAi in animals. *Cell* **150**, 883-894.

Lima, W. F., Vickers, T. A., Nichols, J., Li, C. and Crooke, S. T. (2014) Defining the factors that contribute to on-target specificity of antisense oligonucleotides. *PLoS One* **9**, e101752.

Lönn, P., Kacsinta, A. D., Cui, X.-S., Hamil, A. S., Kaulich, M., Gogoi, K. and Dowdy, S. F. (2016) Enhancing endosomal escape for intracellular delivery of macromolecular biologic therapeutics. *Sci. Rep.* **6**, 32301.

Makley, L. N. and Gestwicki, J. E. (2013) Expanding the number of 'druggable' targets: non-enzymes and protein–protein interactions. *Chem. Biol. Drug Des.* **81**, 22-32.

McDonald, C. M., Shieh, P. B., Abdel-Hamid, H. Z., Connolly, A. M., Ciafaloni, E., Wagner, K. R., Goemans, N., Mercuri, E., Khan, N., Koenig, E., Malhotra, J., Zhang, W., Han, B. and Mendell, J. R. (2021) Open-label evaluation of eteplirsen in patients with Duchenne muscular dystrophy amenable to exon 51 skipping: PROMO-VI trial. *J. Neuromuscul. Dis.* **8**, 989-1001.

Melton, D. A. (1985) Injected anti-sense RNAs specifically block messenger RNA translation *in vivo*. *Proc. Natl. Acad. Sci. U. S. A.* **82**, 144-148.

Migawa, M. T., Shen, W., Wan, W. B., Vasquez, G., Oestergaard, M. E., Low, A., De Hoyos, C. L., Gupta, R., Murray, S., Tanowitz, M., Bell, M., Nichols, J. G., Gaus, H., Liang, X. H., Swayze, E. E., Crooke, S. T. and Seth, P. P. (2019) Site-specific replacement of phosphorothioate with alkyl phosphonate linkages enhances the therapeutic profile of gapmer ASOs by modulating interactions with cellular proteins. *Nucleic Acids Res.* **47**, 5465-5479.

Miller, C. M., Donner, A. J., Blank, E. E., Egger, A. W., Kellar, B. M., Østergaard, M. E., Seth, P. P. and Harris, E. N. (2016) Stabilin-1 and Stabilin-2 are specific receptors for the cellular internalization of phosphorothioate-modified antisense oligonucleotides (ASOs) in the liver. *Nucleic Acids Res.* **44**, 2782-2794.

Miller, P. S., Yano, J., Yano, E., Carroll, C., Jayaraman, K. and Ts'o, P. O. (1979) Nonionic nucleic acid analogues. Synthesis and characterization of dideoxyribonucleoside methylphosphonates. *Biochemistry* **18**, 5134-5143.

Miroshnichenko, S. K., Patutina, O. A., Burakova, E. A., Chelobanov, B. P., Fokina, A. A., Vlassov, V. V., Altman, S., Zenkova, M. A. and Stetsenko, D. A. (2019) Mesyl phosphoramidate antisense oligonucleotides as an alternative to phosphorothioates with improved biochemical and biological properties. *Proc. Natl. Acad. Sci. U. S. A.* **116**, 1229-1234.

Monia, B. P., Lesnik, E. A., Gonzalez, C., Lima, W. F., McGee, D., Guinosso, C. J., Kawasaki, A. M., Cook, P. D. and Freier, S. M. (1993) Evaluation of 2'-modified oligonucleotides containing 2'-deoxy gaps as antisense inhibitors of gene expression. *J. Biol. Chem.* **268**, 14514-14522.

Mutisya, D., Hardcastle, T., Cheruiyot, S. K., Pallan, P. S., Kennedy, S. D., Egli, M., Kelley, M. L., Smith, A. V. B. and Rozners, E. (2017) Amide linkages mimic phosphates in RNA interactions with proteins and are well tolerated in the guide strand of short interfering RNAs. *Nucleic Acids Res.* **45**, 8142-8155.

Nomakuchi, T. T., Rigo, F., Aznarez, I. and Krainer, A. R. (2016) Antisense oligonucleotide-directed inhibition of nonsense-mediated mRNA decay. *Nat. Biotechnol.* **34**, 164-166.

Obika, S., Nanbu, D., Hari, Y., Morio, K.-i., In, Y., Ishida, T. and Imanishi, T. (1997) Synthesis of 2′-O,4′-C-methyleneuridine and -cytidine. Novel bicyclic nucleosides having a fixed C3, -endo sugar puckering. *Tetrahedron Lett.* **38**, 8735-8738.

Ørom, U. A., Kauppinen, S. and Lund, A. H. (2006) LNA-modified oligonucleotides mediate specific inhibition of microRNA function. *Gene* **372**, 137-141.

Patutina, O. A., Gaponova Miroshnichenko, S. K., Sen'kova, A. V., Savin, I. A., Gladkikh, D. V., Burakova, E. A., Fokina, A. A., Maslov, M. A., Shmendel, E. V., Wood, M. J. A., Vlassov, V. V., Altman, S., Stetsenko, D. A. and Zenkova, M. A. (2020) Mesyl phosphoramidate backbone modified antisense oligonucleotides targeting miR-21 with enhanced *in vivo* therapeutic potency. *Proc. Natl. Acad. Sci. U. S. A.* **117**, 32370-32379.

Plavec, J., Tong, W. and Chattopadhyaya, J. (1993) How do the gauche and anomeric effects drive the pseudorotational equilibrium of the pentofuranose moiety of nucleosides? *J. Am. Chem. Soc.* **115**, 9734-9746.

Prakash, T. P., Graham, M. J., Yu, J., Carty, R., Low, A., Chappell, A., Schmidt, K., Zhao, C., Aghajan, M., Murray, H. F., Riney, S., Booten, S. L., Murray, S. F., Gaus, H., Crosby, J., Lima, W. F., Guo, S., Monia, B. P., Swayze, E. E. and Seth, P. P. (2014) Targeted delivery of antisense oligonucleotides to hepatocytes using triantennary N-acetyl galactosamine improves potency 10-fold in mice. *Nucleic Acids Res.* **42**, 8796-8807.

Sands, H., Gorey-Feret, L. J., Cocuzza, A. J., Hobbs, F. W., Chidester, D. and Trainor, G. L. (1994) Biodistribution and metabolism of internally 3H-labeled oligonucleotides. I. Comparison of a phosphodiester and a phosphorothioate. *Mol. Pharmacol.* **45**, 932-943.

Servais, L., Mercuri, E., Straub, V., Guglieri, M., Seferian, A. M., Scoto, M., Leone, D., Koenig, E., Khan, N., Dugar, A., Wang, X., Han, B., Wang, D. and Muntoni, F. (2022) Long-term safety and efficacy data of golodirsen in ambulatory patients with Duchenne muscular dystrophy amenable to exon 53 skipping: a first-in-human, multicenter, two-part, open-label, phase 1/2 trial. *Nucleic Acid Ther.* **32**, 29-39.

Seth, P. P., Siwkowski, A., Allerson, C. R., Vasquez, G., Lee, S., Prakash, T. P., Wancewicz, E. V., Witchell, D. and Swayze, E. E. (2009a) Short antisense oligonucleotides with novel 2'-4' conformationaly restricted nucleoside analogues show improved potency without increased toxicity in animals. *J. Med. Chem.* **52**, 10-13.

Seth, P. P., Siwkowski, A., Allerson, C. R., Vasquez, G., Lee, S., Prakash, T. P., Wancewicz, E. V., Witchell, D. and Swayze, E. E. (2009b) Short antisense oligonucleotides with novel 2'-4' conformationaly restricted nucleoside analogues show improved potency without increased toxicity in animals. *J. Med. Chem.* **52**, 10-13.

Shen, W., De Hoyos, C. L., Migawa, M. T., Vickers, T. A., Sun, H., Low, A., Bell, T. A., 3rd, Rahdar, M., Mukhopadhyay, S., Hart, C. E., Bell, M., Riney, S., Murray, S. F., Greenlee, S., Crooke, R. M., Liang, X. H., Seth, P. P. and Crooke, S. T. (2019) Chemical modification of PS-ASO therapeutics reduces cellular protein-binding and improves the therapeutic index. *Nat. Biotechnol.* **37**, 640-650.

Sheng, L., Rigo, F., Bennett, C. F., Krainer, A. R. and Hua, Y. (2020) Comparison of the efficacy of MOE and PMO modifications of systemic antisense oligonucleotides in a severe SMA mouse model. *Nucleic Acids Res.* **48**, 2853-2865.

Song, J. J., Smith, S. K., Hannon, G. J. and Joshua-Tor, L. (2004) Crystal structure of Argonaute and its implications for RISC slicer activity. *Science* **305**, 1434-1437.

Stec, W. J., Zon, G. and Egan, W. (1984) Automated solid-phase synthesis, separation, and stereochemistry of phosphorothioate analogs of oligodeoxyribonucleotides. *J. Am. Chem. Soc.* **106**, 6077-6079.

Stockert, R. J. (1995) The asialoglycoprotein receptor: relationships between structure, function, and expression. *Physiol. Rev.* **75**, 591-609.

Summerton, J. and Weller, D. (1997) Morpholino antisense oligomers: design, preparation, and properties. *Antisense Nucleic Acid Drug Dev.* **7**, 187-195.

Swayze, E. E., Siwkowski, A. M., Wancewicz, E. V., Migawa, M. T., Wyrzykiewicz, T. K., Hung, G., Monia, B. P. and Bennett, C. F. (2007) Antisense oligonucleotides containing locked nucleic acid improve potency but cause significant hepatotoxicity in animals. *Nucleic Acids Res.* **35**, 687-700.

Taylor, R. E. and Zahid, M. (2020) Cell penetrating peptides, novel vectors for gene therapy. *Pharmaceutics* **12**, 225.

Teplova, M., Minasov, G., Tereshko, V., Inamati, G. B., Cook, P. D., Manoharan, M. and Egli, M. (1999) Crystal structure and improved antisense properties of 2'-O-(2-methoxyethyl)-RNA. *Nat. Struct. Biol.* **6**, 535-539.

Vickers, T. A. and Crooke, S. T. (2016) Development of a quantitative BRET affinity assay for nucleic acid-protein interactions. *PLoS One* **11**, e0161930.

Vickers, T. A., Rahdar, M., Prakash, T. P. and Crooke, S. T. (2019) Kinetic and subcellular analysis of PS-ASO/protein interactions with P54nrb and RNase H1. *Nucleic Acids Res.* **47**, 10865-10880.

Vickers, T. A., Wyatt, J. R., Burckin, T., Bennett, C. F. and Freier, S. M. (2001) Fully modified 2' MOE oligonucleotides redirect polyadenylation. *Nucleic Acids Res.* **29**, 1293-1299.

Volpi, S., Cancelli, U., Neri, M. and Corradini, R. (2020) Multifunctional delivery systems for peptide nucleic acids. *Pharmaceuticals (Basel)* **14**, 14.

Wagner, K. R., Kuntz, N. L., Koenig, E., East, L., Upadhyay, S., Han, B. and Shieh, P. B. (2021) Safety, tolerability, and pharmacokinetics of casimersen in patients with Duchenne muscular dystrophy amenable to exon 45 skipping: a randomized, double-blind, placebo-controlled, dose-titration trial. *Muscle Nerve* **64**, 285-292.

Wang, L., Ariyarathna, Y., Ming, X., Yang, B., James, L. I., Kreda, S. M., Porter, M., Janzen, W. and Juliano, R. L. (2017) A novel family of small molecules that enhance the intracellular delivery and pharmacological effectiveness of antisense and splice switching oligonucleotides. *ACS Chem. Biol.* **12**, 1999-2007.

Ward, A. J., Norrbom, M., Chun, S., Bennett, C. F. and Rigo, F. (2014) Nonsense-mediated decay as a terminating mechanism for antisense oligonucleotides. *Nucleic Acids Res.* **42**, 5871-5879.

Wu, H., Lima, W. F., Zhang, H., Fan, A., Sun, H. and Crooke, S. T. (2004) Determination of the role of the human RNase H1 in the pharmacology of DNA-like antisense drugs. *J. Biol. Chem.* **279**, 17181-17189.

Yanai, H., Chiba, S., Ban, T., Nakaima, Y., Onoe, T., Honda, K., Ohdan, H. and Taniguchi, T. (2011) Suppression of immune responses by nonimmunogenic oligodeoxynucleotides with high affinity for high-mobility group box proteins (HMGBs). *Proc. Natl. Acad. Sci. U. S. A.* **108**, 11542-11547.

Yang, B., Ming, X., Cao, C., Laing, B., Yuan, A., Porter, M. A., Hull-Ryde, E. A., Maddry, J., Suto, M., Janzen, W. P. and Juliano, R. L. (2015) High-throughput screening identifies small molecules that enhance the pharmacological effects of oligonucleotides. *Nucleic Acids Res.* **43**, 1987-1996.

Yoshida, T., Morihiro, K., Naito, Y., Mikami, A., Kasahara, Y., Inoue, T. and Obika, S. (2022) Identification of nucleobase chemical modifications that reduce the hepatotoxicity of gapmer antisense oligonucleotides. *Nucleic Acids Res.* **50**, 7224-7234.

Zamecnik, P. C. and Stephenson, M. L. (1978) Inhibition of Rous sarcoma virus replication and cell transformation by a specific oligodeoxynucleotide. *Proc. Natl. Acad. Sci. U. S. A.* **75**, 280-284.

Zhang, L., Liang, X. H., De Hoyos, C. L., Migawa, M., Nichols, J. G., Freestone, G., Tian, J., Seth, P. P. and Crooke, S. T. (2022) The combination of mesyl-phosphoramidate inter-nucleotide linkages and 2'-O-methyl in selected positions in the antisense oligonucleotide enhances the performance of RNaseH1 active PS-ASOs. *Nucleic Acid Ther.* **32**, 401-411.

Zimmermann, T. S., Karsten, V., Chan, A., Chiesa, J., Boyce, M., Bettencourt, B. R., Hutabarat, R., Nochur, S., Vaishnaw, A. and Gollob, J. (2017) Clinical proof of concept for a novel hepatocyte-targeting GalNAc-siRNA conjugate. *Mol. Ther.* **25**, 71-78.