



Splice-Modulating Antisense Oligonucleotides as Therapeutics for Inherited Metabolic Diseases

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Abstract

The last decade (2013–2023) has seen unprecedented successes in the clinical translation of therapeutic antisense oligonucleotides (ASOs). Eight such molecules have been granted marketing approval by the United States Food and Drug Administration (US FDA) during the decade, after the first ASO drug, fomivirsen, was approved much earlier, in 1998. Splice-modulating ASOs have also been developed for the therapy of inborn errors of metabolism (IEMs), due to their ability to redirect aberrant splicing caused by mutations, thus recovering the expression of normal transcripts, and correcting the deficiency of functional proteins. The feasibility of treating IEM patients with splice-switching ASOs has been supported by FDA permission (2018) of the first “N-of-1” study of milasen, an investigational ASO drug for Batten disease. Although for IEM, owing to the rarity of individual disease and/or pathogenic mutation, only a low number of patients may be treated by ASOs that specifically suppress the aberrant splicing pattern of mutant precursor mRNA (pre-mRNA), splice-switching ASOs represent superior individualized molecular therapeutics for IEM. In this work, we first summarize the ASO technology with respect to its mechanisms of action, chemical modifications of nucleotides, and rational design of modified oligonucleotides; following that, we precisely provide a review of the current understanding of developing splice-modulating ASO-based therapeutics for IEM. In the concluding section, we suggest potential ways to improve and/or optimize the development of ASOs targeting IEM.

1 Introduction

Antisense oligonucleotides (ASOs) are synthetic single-stranded oligonucleotides that can bind to precursor mRNA (pre-mRNA) and/or mRNA through Watson-Crick base pairing [1]. Upon specifically binding to their target RNAs, ASOs can modulate the function of RNA, thereby regulating gene expression [2–5]. ASO-based therapeutics

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Key Points

Despite significant efforts to date, most inborn errors of metabolism still lack an effective therapeutic approach.

Splice-modulating antisense oligonucleotides (ASOs) have offered a promising strategy to develop precision treatments for inborn defects of metabolism, such as lysosomal storage diseases, organic acidemias, and congenital disorder of glycosylation.

Chemical modifications of ASOs can further improve their therapeutic properties such as efficacy, stability, and specificity. Phosphorodiamidate morpholino oligomer (PMO), 2'-*O*-methoxyethyl (2'-MOE), 2'-*O*-methyl (2'-OMe), locked nucleic acid (LNA), 2'-*O*,4'-*C*-ethylene-bridged nucleic acid (ENA), and phosphorothioate have been utilized in constructing ASOs targeting inborn errors of metabolism.

originate from Zamecnik and Stephenson's seminal observations published in 1978, in which they discovered that a 13-deoxynucleotide ASO sequence complementary to the RNA of Rous sarcoma virus could inhibit viral RNA translation and virus production [6, 7]. One year later, a major post-RNA binding pathway, degradation of RNA by ribonuclease H (RNase H) was demonstrated [8], while modification of pre-mRNA splicing by steric blockage, another main mechanism of ASO action, was reported after 15 years, in 1993 [9]. Examples of more ASO mechanisms are provided in Sect. 2.1 of this article. Up to now, nine ASO drugs have been granted marketing approval by the United States Food and Drug Administration (US FDA) for treating several diseases, including cytomegalovirus retinitis (fomivirsen) [10–12], homozygous familial hypercholesterolemia (mipomersen) [13, 14], Duchenne muscular dystrophy (eteplirsen, golodirsen, viltolarsen, and casimersen) [15–27], spinal muscular atrophy (nusinersen) [28–30], hereditary transthyretin amyloidosis (inotersen) [31, 32], and amyotrophic lateral sclerosis (tofersen) [33] (Fig. 1). Although volanesorsen, developed for treating familial chylomicronemia syndrome, familial partial lipodystrophy, and hypertriglyceridemia, was rejected by the FDA because of safety concerns, as platelet declines were observed during clinical trials [34], the drug was approved by the European Medicines Agency (EMA) for market entry [35]. Individualized ASOs such as milasen, jacifusen, and atipeksen have been evaluated for treating Batten disease, amyotrophic lateral sclerosis, and ataxia-telangiectasia, respectively [36–38]. ASOs have also been developed as potential therapeutics for other human maladies [2, 5, 39–50], such as neurodegenerative diseases [51–53], cancers [54, 55], chronic kidney disease [56, 57], and a variety of other metabolic disorders [58–63].

Metabolism covers a range of intricate biochemical processes that are structured into various pathways to sustain everyday physiological functions. Each metabolic pathway is dependent on the catalytic activity of certain enzymes to facilitate efficient functioning [64]. Inborn errors of metabolism (IEMs), often known as inherited metabolic diseases, have been known for more than a century after the first IEM was reported by Sir Archibald Garrod in 1902 [65]. IEMs are due to a deficiency or abnormality in a protein (enzyme, receptor, or transporter) caused by genetic defects, for example, point mutations, deletions, or insertions [66–70]. The defects in genes hamper the normal functioning of the metabolic pathways in which they are involved [64, 71], leading to varying consequences: excessive accumulation of a substrate (intermediary metabolite) that cannot be processed further (which may show toxicity directly, or indirectly through the diversion of the metabolic flux to an alternative pathway) and deficiency of a specific end product [64, 67, 71]. Both of these

consequences result in significant mortality and morbidity [64, 70]. Nowadays, more than 1450 IEMs have been discovered [72]. Although each individual disorder is rare, the estimated overall prevalence of IEMs is one in 4000 newborns [73, 74]. In some regions, IEMs even represent somewhat common diseases [75]; for instance, they affect one in 784 individuals in the West Midlands of the United Kingdom (UK) [76]. Most IEMs are inherited as autosomal recessive diseases, although X-linked, autosomal dominant, and maternal inheritance (due to mutations in mitochondrial DNA) are also possible [70, 77, 78].

Significant efforts have been undertaken in managing IEMs to extend patients' life expectancy and improve their quality of life. Dietary manipulations and cofactor administrations are used for many IEMs affecting the metabolism of sugars, lipids, and amino acids [79]. This therapeutic strategy is aimed at reducing the intake of certain substrates or providing deficient products [79]. However, the specialized medical foods and supplements on which patients are relying are expensive [80], and might also affect their diet diversity and satisfaction. For lysosomal storage diseases (LSDs), a large class of IEM, enzyme replacement therapies based on intravenous or intrathecal administration of a deficient enzyme have been developed [81]. Although the clinical benefits of these therapies have been proven, there are limitations related to immune reactions and difficulties in the absorption of exogenous enzymes by refractory tissues [82]. In addition, transplantation of organs, tissues, or cells is also available for some IEMs. This approach enables the replacement of a whole organ or cells where a defective gene is expressed causing enzyme deficiency. Nevertheless, problems such as sequelae of immunosuppression, limited donor availability, high graft failure rates, and treatment-related morbidity and mortality continue to be an issue [67, 83].

Despite the abovementioned treatments, most IEMs still lack an effective therapeutic approach [60, 67]. Early diagnosis of IEMs resulting from expanded newborn screening has offered an opportunity for early therapy [84, 85], thereby increasing the urgent demand for developing more effective treatments [67]. To date, splice-modulating ASOs have been developed as, to some extent, an individualized molecular therapeutic option for IEM, due to both the ability of these ASOs to specifically suppress the aberrant splicing pattern of mutant pre-mRNA and the rarity of a certain disease and/or a certain pathogenic mutation in the disease. Very recently, Goga and Stoffel comprehensively reviewed the RNA-silencing oligonucleotides, including RNase H-dependent ASOs and small interfering RNAs (siRNAs), as therapeutic molecules for metabolic disorders [62]. Herein, we present a comprehensive analysis of advancements in the domain of splice-switching ASO-based therapeutics in inherited metabolic diseases, that is, the ASOs designed to target specific human pathogenic mutations in IEMs. Prior to this, we will

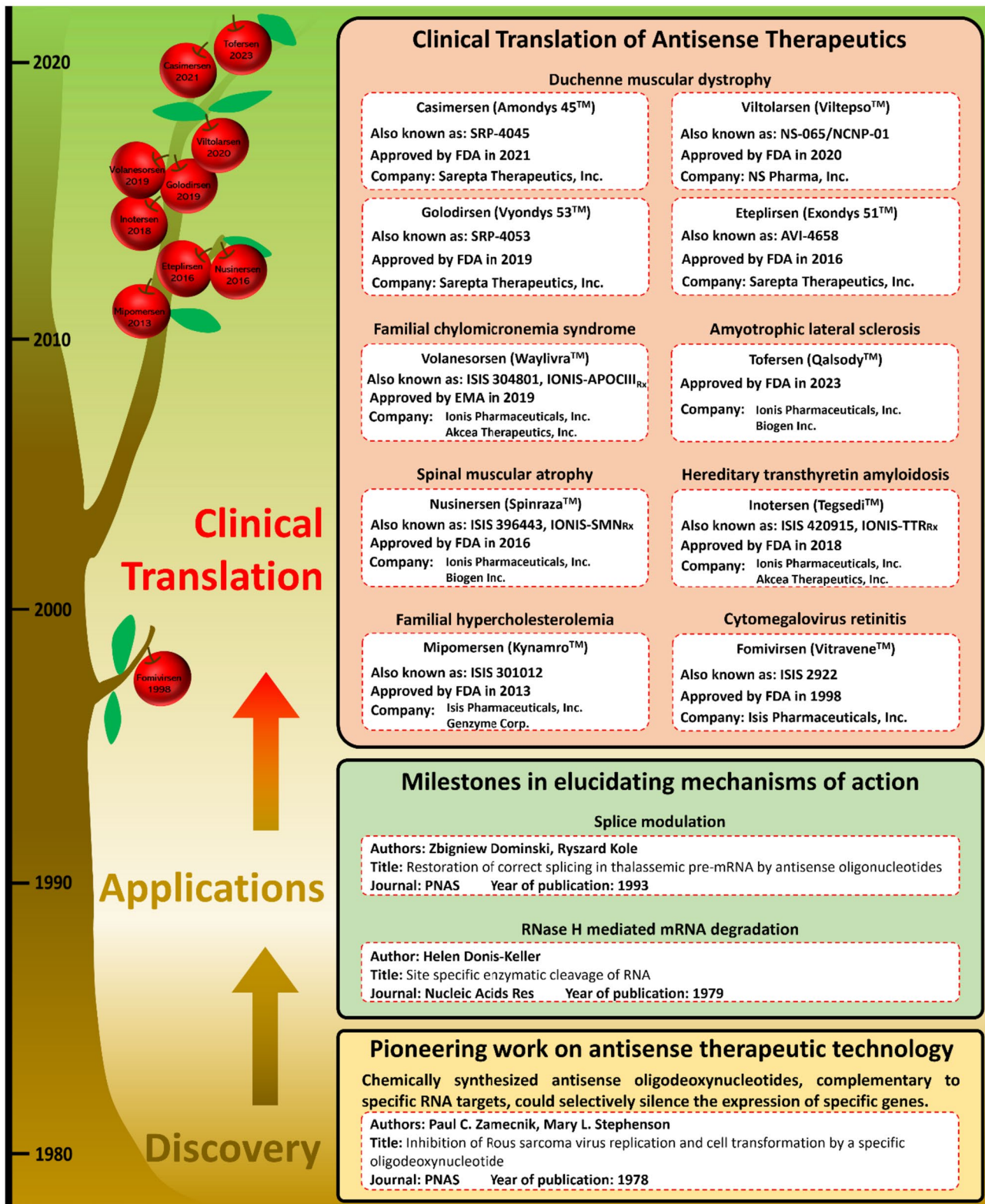
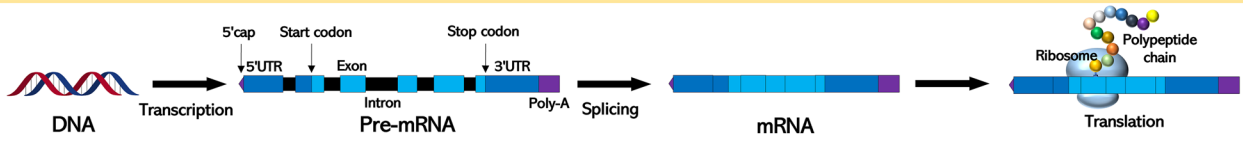


Fig. 1 Historical developments of antisense oligonucleotide therapeutics. To date, ten ASO drugs have been approved by US FDA or EMA for market entry. *EMA* European Medicines Agency, *FDA* Food and Drug Administration

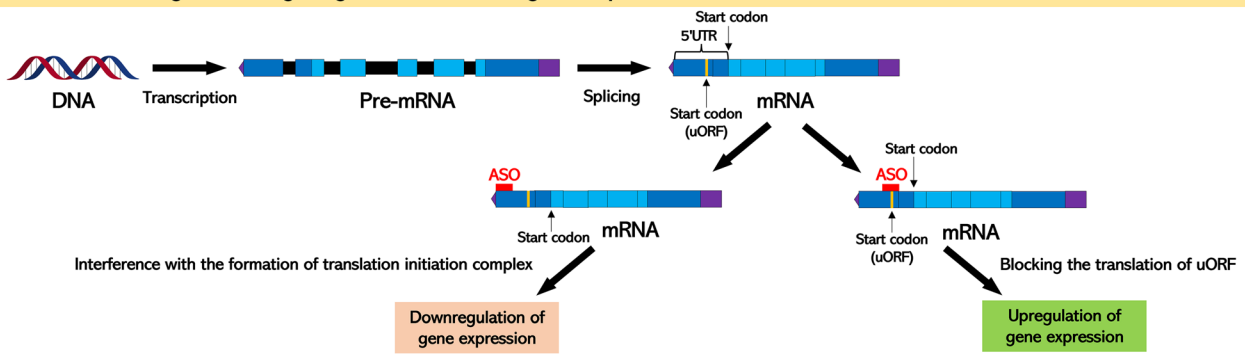
A. Gene expression without interference from ASOs



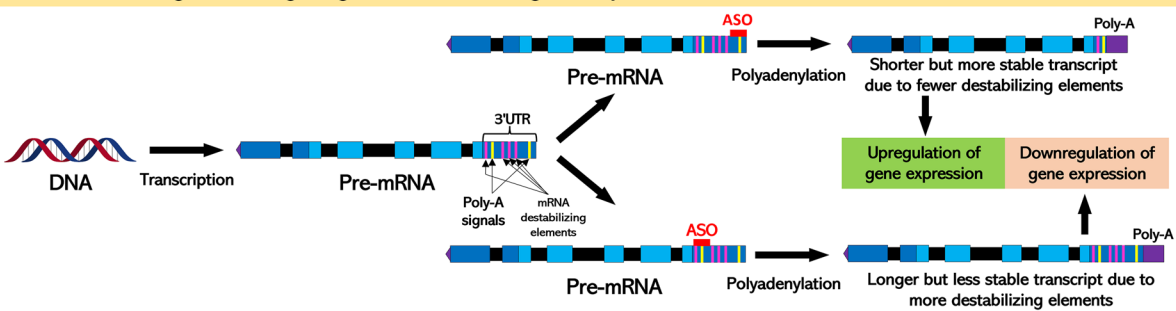
B. RNase H dependent ASOs downregulate gene expression



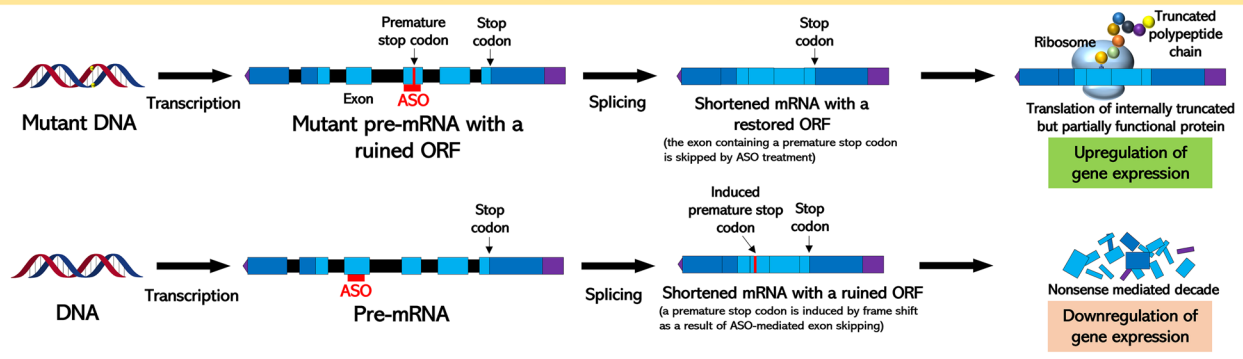
C. Steric-blocking ASOs targeting 5'UTR modulate gene expression



D. Steric-blocking ASOs targeting 3'UTR modulate gene expression



E. Steric-blocking ASOs modulate gene expression by altering splicing (using exon skipping as an example)



◀**Fig. 2** Examples of mechanisms of ASO action in modulating gene expression. **A** Normal gene expression without interference from ASOs; **B** downregulation of gene expression by RNase H-dependent ASOs; **C** up- or downregulation of gene expression by steric-blocking ASOs targeting the 5'UTR region; **D** up- or downregulation of gene expression by steric-blocking ASOs targeting the 3'UTR region; **E** up- or downregulation of gene expression by splice-switching steric-blocking ASOs. *ASO* antisense oligonucleotide, *ORF* open reading frame, *poly-A* polyadenylic acid tail, *pre-mRNA* precursor mRNA, *RNase H* ribonuclease H, *uORF* upstream open reading frame, *UTR* untranslated region

comprehensively outline the ASO approach in terms of its mechanisms of action, chemical modifications, and rational design of modified ASO.

2 Antisense Oligonucleotide (ASO)-Based Therapeutics

2.1 Mechanisms of ASO Action

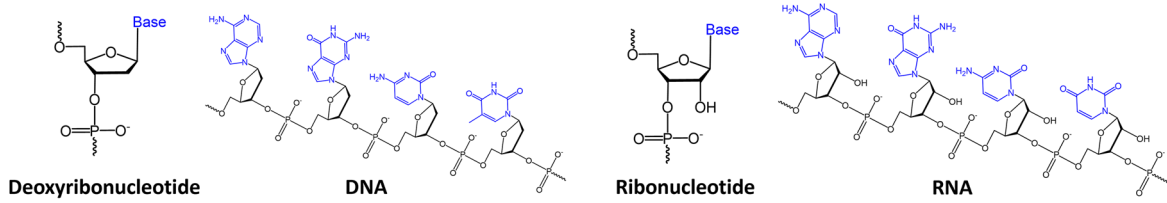
ASOs are short nucleic acid oligomers (18–30 nucleotides) usually employed to modulate gene expression through specific binding to the complementary region of their target RNA [86]. The specificity is ensured by Watson–Crick base pairing, and the entire ASO sequence is used to identify the target RNA, while, theoretically, merely 13–15 contiguous nucleotides are sufficient to achieve single-RNA specificity [41]. Several post-RNA-binding mechanisms of ASO action have been reported, and some are widely applied, and novel mechanisms are continuously being validated, enhancing the versatility of antisense technology [2]. ASO mechanisms have been categorized into two major types: RNA degradation mediated by RNase H and RNA modulation simply via steric hindrance [41, 86–89] (Fig. 2).

In mammalian cells, the RNase H family of enzymes mediate the RNA cleavage in an RNA–DNA heteroduplex [90]. Human cells contain both RNase H1 and 2, the former being responsible for the cleavage of RNA [91]. At least five, ideally eight to ten, consecutive deoxynucleotide units in an ASO are needed for an RNA–ASO duplex to become a substrate of RNase H [2, 50, 91]. Upon formation of the hybrid, RNase H is recruited and cleaves the phosphodiester (PO) bonds of the RNA strand, releasing the intact ASO strand [39, 87]. The RNA fragments that have been cleaved are then subjected to degradation by the XRN family of 5'→3' exoribonucleases and exosome complex, resulting in target RNA destruction and therefore downregulation of the target gene expression [92–94]. Since RNase H exists both in nucleus and cytoplasm, RNA cleavage by the enzyme is a robust pharmacological mechanism that enables the targeting of both nuclear pre-mRNA and cytoplasmic mRNA [92] (Fig. 2B). RNase H-dependent ASOs have been widely

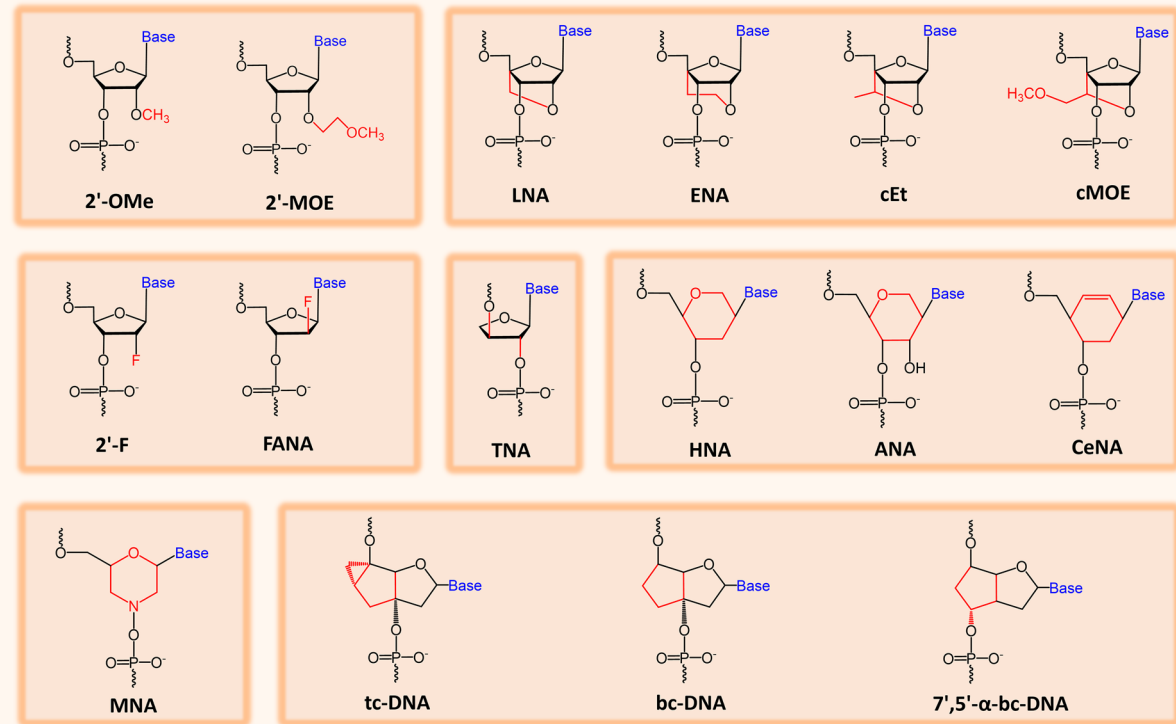
explored and developed as therapeutics for downregulating the expression of disease-causing genes. To date, four such oligonucleotides (fomivirsin, mipomersen, inotersen, and tofersen) have been FDA approved [10–13, 31–33, 95] (Fig. 1).

In contrast to the RNase H competent oligonucleotides, steric-blocking ASOs do not promote target RNA degradation [43, 50, 86]; instead, they function using different mechanisms that either downregulate or upregulate the expression of target genes [2], via masking specific sequences within a target transcript, thus interfering with interactions of the transcript with other biomolecules and/or complexes [86]. Selected examples of the steric hindrance mechanisms are shown in Fig. 2. Firstly, ASOs binding to the cap site within the 5'-untranslated region (5'UTR) of the target mRNA can repress protein synthesis by interfering with the formation of the translation initiation complex [96, 97], while ASOs targeted to the start codon of the upstream open reading frame (uORF) in the 5'UTR redirect the translational machinery to the primary open reading frame (pORF), thereby increasing protein expression, as a result of uORF blockage [98, 99] (Fig. 2C). Secondly, ASOs can alter the stability of mRNA by modulating the selection of polyadenylation sites in the 3'UTR [100]. To be more specific, ASOs targeted to the pre-mRNA 3'-most polyadenylation site can redirect polyadenylation to upstream cryptic sites, leading to the production of shorter but more stable mRNA variants, as they contain fewer destabilizing elements, thus upregulating gene expression. Conversely, ASOs that hybridize to the upstream polyadenylation sites enhance the selection of the 3'-most site, resulting in an increased level of longer (having more destabilization sequences) but less stable transcripts, thereby downregulating expression [100] (Fig. 2D). Finally, and most importantly, in addition to affecting alternative polyadenylation, steric-blocking ASOs are capable of triggering alternative splicing [9, 101–104]. ASOs that are designed to be complementary to specific pre-mRNA sites including 5' or 3' splice junctions, or exonic splicing enhancers (ESEs) or silencers (ESSs) can block the access of spliceosome or splice factors to those sequences, thereby altering the splicing decisions, leading to exclusion (skipping) or inclusion of an exon in mature mRNA [105]. Splice switching by ASOs can be used to either rescue or disrupt gene expression. Taking exon skipping as an example, an exon containing mutation-induced premature termination codon (PTC) can be excluded by ASO treatment, thereby correcting the open reading frame (ORF) and restoring production of therapeutic protein (internally truncated but partly functional protein isoforms) [106]. On the other hand, skipping of a normal exon by ASO may result in reading frame shift and induce PTC(s) in its adjacent exon, causing ORF corruption and nonsense-mediated mRNA decay (NMD) activation [88], eventually silencing protein translation

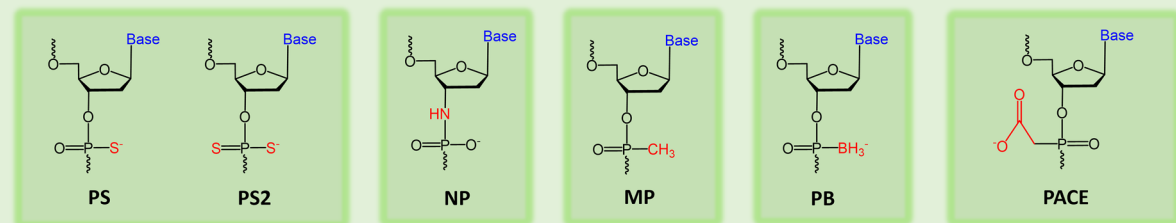
Unmodified (natural) nucleotides



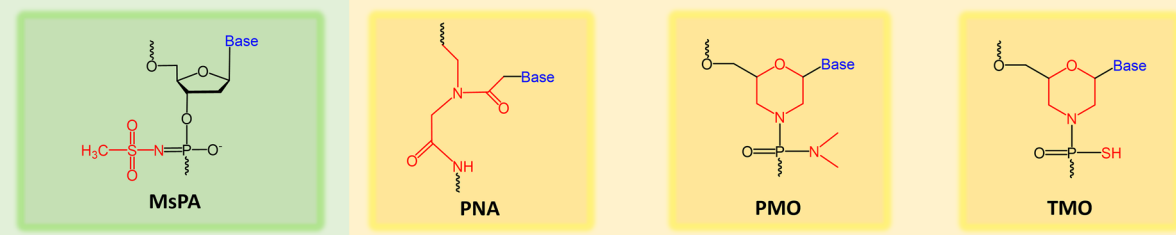
Pentose sugar modification



Phosphodiester linkage modification



Sugar-phosphate backbone modification



◀**Fig. 3** Examples of chemically modified nucleotide analogs. *2'-F* 2'-fluoro, *2'-MOE* 2'-O-methoxyethyl, *2'-OMe* 2'-O-methyl, *7',5'- α -bc-DNA* 7',5'- α -bicyclo-DNA, *ANA* altritol nucleic acid, *bc-DNA* bicyclo-DNA, *CeNA* cyclohexenyl nucleic acid, *cEt* 2',4'-constrained 2'-O-ethyl, *cMOE* 2',4'-constrained 2'-O-methoxyethyl, *ENA* 2'-O,4'-C-ethylene-bridged nucleic acid, *FANA* 2'-deoxy-2'-fluoro-arabinonucleic acid, *HNA* anhydrohexitol nucleic acid, *LNA* locked nucleic acid, *MNA* morpholino nucleic acid, *MP* methylphosphonate, *MsPA* mesyl phosphoramidate, *NP* N3'→P5' phosphoramidate, *PACE* phosphonoacetate, *PB* boranophosphate, *PMO* phosphorodiamidate morpholino oligomer, *PNA* peptide nucleic acid, *PS* phosphorothioate, *PS2* phosphorodithioate, *tc-DNA* tricyclo-DNA, *TMO* thiophosphoramidate morpholino oligomer, *TNA* threose nucleic acid

(Fig. 2E). In summary, steric-blocking ASOs are very flexible approaches and are increasingly used for therapeutic purposes. To date, five splice-switching ASOs (eteplirsen, nusinersen, golodirsen, casimersen, and viltolarsen) have been approved by the FDA for market entry [15, 17, 19, 22, 25, 28, 29].

In addition, siRNAs are usually included as antisense molecules in the literature, since their antisense (or guide) strand functions as ASO binding to target mRNA. siRNAs and ASOs share common properties regarding chemical modifications as well as delivery issues [107–111]. To date, five siRNAs (patisiran, givosiran, lumasiran, inclisiran, and vutrisiran) have been approved by the FDA for clinical use.

2.2 Chemical Modifications of ASO

DNA-based ASOs composed of natural (unmodified) deoxyribonucleotide monomers were used in the earliest studies in this field [6, 7, 112]. However, DNA exhibits a lower binding affinity for a complementary RNA target than RNA does for itself [39]. Furthermore, both unmodified single-strand DNA and RNA-based oligonucleotides have unfavorable properties that make them unacceptable systemic therapeutics [50]. The primary drawbacks include:

1. *Insufficiency in intrinsic affinity for complementary RNA:* Unmodified ASOs may not be able to disturb the secondary structure of intracellular RNA targets [39] or to compete with RNA binding proteins, leading to off-target effects and poor target specificity [48].
2. *Susceptibility to attack by endonucleases and exonucleases:* Unmodified ASOs lack stability in biological systems in which their PO linkages are cleaved by nucleases [39, 47, 113], leading to their degradation before having a chance to reach target RNAs [39].
3. *Poor protein binding capability:* Unmodified ASOs are weakly bound to proteins in plasma and thereby filtered rapidly by the kidneys and excreted in the urine [39, 114], leading to inefficient tissue uptake of the ASOs [49].

4. *Poor cellular uptake:* Due to their large size and charge, unmodified ASOs are not able to permeate plasma membranes through passive diffusion [47, 50, 115].

To overcome these problems, ASOs must be chemically modified. In fact, natural ASOs are amenable to extensive modification, and advances in ASO-based therapy have been heavily dependent on the evolution of medicinal chemistry [39, 41, 116]. To date, a large panel of modified nucleotides (analogues) have been synthesized [117]. Most of them have been explored to achieve desired pharmacokinetic and pharmacodynamic profiles, improved endocytic uptake (controlled by cell surface proteins), and reduced pro-inflammatory effects and off-target toxicity [43, 45, 114, 117–121]. Improvement of pharmacokinetics is achieved by enhancing the nuclease resistance and protein binding ability of ASOs, thus extending their half-lives and facilitating their distribution to tissues [39]. Pharmacodynamic characteristics are optimized by increasing the binding affinity of ASOs to their complementary sequences in RNA, which can be measured by the melting temperature (T_m). Based on the structure of the nucleotide, its components (phosphate group, pentose sugar, and nitrogenous base) can be modified either individually or in a combined manner.

2.2.1 Phosphate Linkage Modifications

Obviously, the phosphate group is the first target for chemical modification, owing to the inherent nuclease instability of the PO linkage [39]. Modifications to this region predominantly assist in reducing an ASO's nuclease susceptibility [49]. Some examples, such as phosphorothioate (PS) [122], phosphorodithioate (PS2) [123], N3'→P5' phosphoramidate (NP) [124], methylphosphonate (MP) [125], boranophosphate (PB) [126], phosphonoacetate (PACE) [127], and mesyl phosphoramidate (MsPA) [128] are shown in Fig. 3.

Among these analogues, PS stands out as the most effective, extensively utilized, and earliest single modification for ASO development [47, 116, 122], being present in six (fomivirsin, mipomersen, nusinersen, inotersen, tofersen, and milasen) out of the ten FDA-permitted ASO drugs (Fig. 4). PS-modified ASOs differ from their natural counterparts in that a non-bridging oxygen atom inside the phosphate group is substituted with a sulfur atom [122, 129], which alters the physicochemical properties of the phosphate group [40]. Firstly, the sulfur substitution confers ASOs sufficient metabolic stability in cells and tissues, avoiding rapid digestion by a broad spectrum of nucleases [50, 122, 129, 130]. Secondly, since the sulfur atom is twice the size of the oxygen atom, the negative charge within PS is broadly distributed compared to that in PO, which increases the lipophilicity of the modified phosphate group, thus promoting its protein binding ability [131–133]. More specifically,

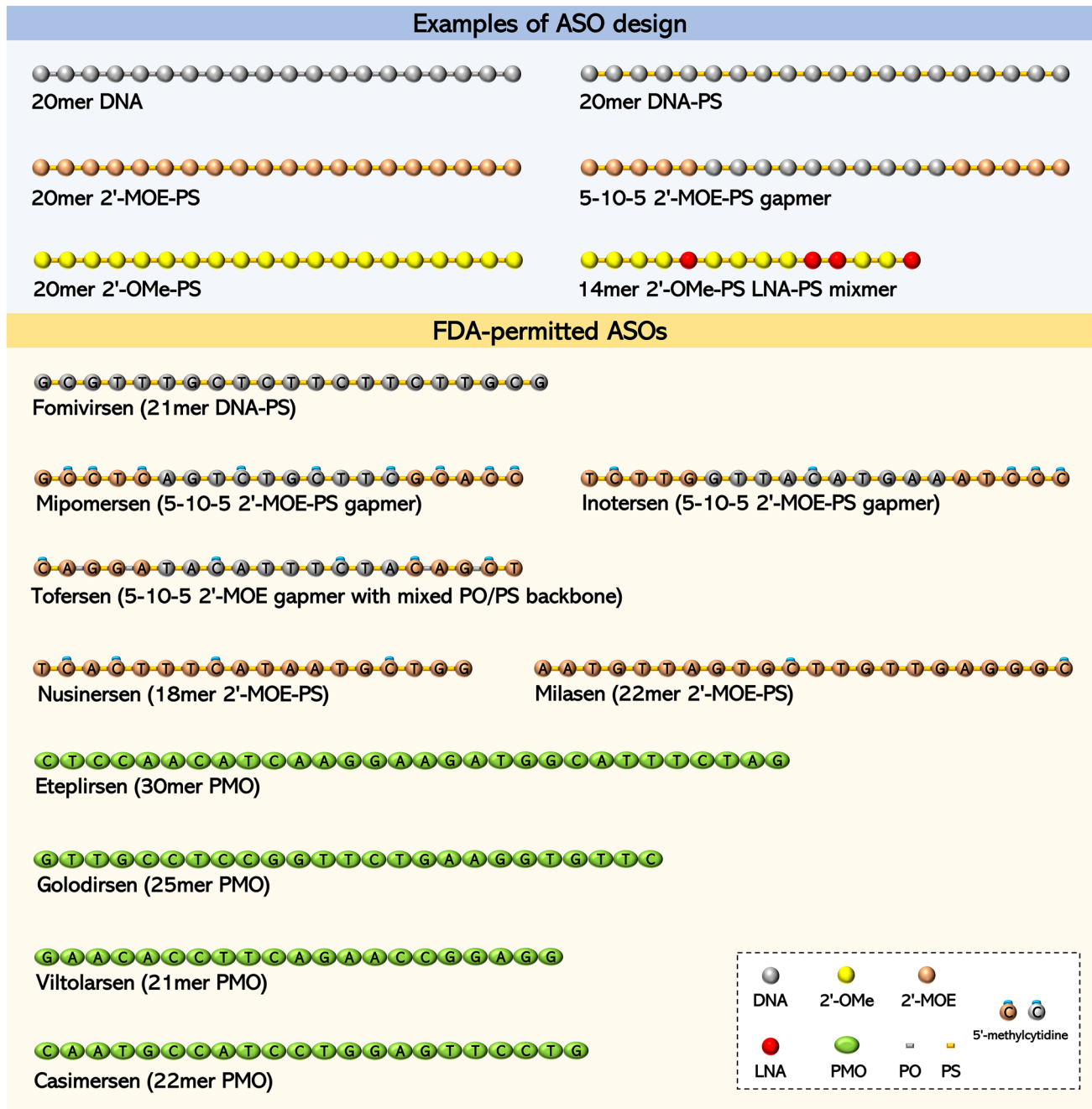


Fig. 4 Rational design of chemically modified ASOs. Among the FDA-permitted ASO drugs, fomivirsen is the only uniformly PS-modified DNA molecule, nusinersen and milasen are uniformly 2'-MOE-PS ASOs, eteplirsen, golodirsen, viltolarsen, and casimersen are PMOs, mipomersen and inotersen are 5-10-5 2'-MOE-PS gapmers, and tofersen is a 5-10-5 2'-MOE-PS gapmer with mixed PO/PS

phosphate linkages. Methylated uracil is identical to thymine. 2'-MOE 2'-O-methoxyethyl, 2'-OMe 2'-O-methyl, ASO antisense oligonucleotide, FDA Food and Drug Administration, LNA locked nucleic acid, PMO phosphorodiamidate morpholino oligomer, PO phosphodiester, PS phosphorothioate

enhanced binding to plasma proteins, e.g., albumin preserves ASOs in circulation, retards renal excretion, improves bioavailability, and finally facilitates their uptake by organs and tissues [50, 86, 134]. In addition, increased binding to cell-surface proteins (for example, scavenger receptors)

facilitates internalization of ASOs into cells, and interactions of ASOs with intracellular proteins such as nucleolin may promote their accumulation in the nucleus, which is beneficial for the action of splice-modulating ASOs, since their targets, the pre-mRNAs, are located in the nucleus

[135–143]. In an ASO sequence, at least ten PS linkages are required to support efficient protein binding [40]. Synthesis of P-stereo-defined PS oligonucleotides was developed by Professor Takeshi Wada and his team [144–146], allowing production of diastereopure PS-modified ASOs for research and therapeutic applications.

As a result of both the improved nuclease resistance and protein binding ability, PS modification extends the half-life from minutes (natural ASO) to days (PS-modified ASO) [47, 50, 147]. However, the PS moiety also has two disadvantages: reduction in binding affinity of ASO to its target (~ 0.2 – 0.5 °C per modification) [121] and pro-inflammatory toxicity resulting from nonspecific protein binding [122]. These shortcomings can be compensated for or counteracted by additional modifications on the pentose sugar moiety [2, 86]. This is supported by the fact that only fomivirsen (the earliest ASO drug) is modified by PS alone, whereas all the later approved molecules (mipomersen, nusinersen, inotersen, tofersen, and milasen) contain both PS and ribose modifications. Alternatively, researchers have been developing novel modifications to the phosphate group that are more specific and/or less toxic. For instance, the PS2 substitution increases the binding affinity of ASO to its target for 1000-fold [148], and the MsPA modification shows better target binding affinity and nuclease stability than PS [128].

2.2.2 Sugar Moiety Modifications

The sugar moiety of nucleotides is the hotspot of chemical modification. A large number of nucleotide analogs with changed sugar moiety have been used for constructing ASOs, such as 2'-*O*-methyl (2'-OMe) [149], 2'-*O*-methoxyethyl (2'-MOE) [100], 2'-fluoro (2'-F) [150, 151], 2'-deoxy-2'-fluoro-arabinonucleic acid (FANA) [152], locked nucleic acid (LNA) [153, 154], 2'-*O*,4'-*C*-ethylene-bridged nucleic acid (ENA) [155], 2',4'-constrained 2'-*O*-ethyl (cEt) [156], 2',4'-constrained 2'-*O*-methoxyethyl (cMOE) [156], threose nucleic acid (TNA) [157], anhydroxitol nucleic acid (HNA) [158], alritol nucleic acid (ANA) [158], cyclohexenyl nucleic acid (CeNA) [158], tricyclo-DNA (tc-DNA) [159], bicyclo-DNA (bc-DNA) [160], and 7',5'- α -bicyclo-DNA (7',5'- α -bc-DNA) [161] (Fig. 3).

Among these modifications, replacement of the hydrogen atom or hydroxyl group on the 2'-position of the deoxyribose/ribose moiety by other groups, most commonly 2'-OMe and 2'-MOE, provides the most value in improving an ASO's drug-like properties. Both modifications increase the nuclease resistance of ASOs by replacing the nucleophilic 2'-hydroxyl moiety [86, 116], as well as via the proximity of the 2'-substituent to the 3'-phosphate moiety [39], leading to prolonged tissue half-life and drug effects. Furthermore, 2'-OMe and 2'-MOE increase the binding affinity of ASOs to their RNA targets ($\Delta T_m \sim 2$ °C per modification,

relative to DNA) by promoting an RNA-like northern conformation (A-form 3'-endo sugar pucker) [162–164]. In addition, 2'-OMe and 2'-MOE can abrogate the immune reactions induced by oligonucleotides, reduce nonspecific protein binding, and are thus less toxic than PS modification [47, 86]. 2'-MOE is even considerably less toxic than 2'-OMe [49]. Additional modifications (such as 2'-OMe and 2'-MOE) to the sugar moiety not only increase the affinity of ASOs with PS backbone modification to target RNAs, but also decrease the toxicity induced by the PS linkages [45]. 2'-MOE is currently the most advanced and the only 2'-substituent that has been employed in the ASO drugs clinically approved by the FDA (mipomersen, nusinersen, inotersen, tofersen, and milasen).

The electronegative 2'-substituent 2'-F also increases the thermal stability of ASO–RNA heteroduplex by ~ 2 °C per modification [121]. However, this analog imparts enhanced binding affinity to drosophila behavior/human splicing (DBHS) proteins, leading to hepatotoxicity [165]. Therefore, 2'-F is not as competitive as 2'-MOE in ASO construction.

Bridged nucleic acids (BNAs) are a class of nucleotide analogs in which the 2'-oxygen atom of the furanose sugar is linked to the 4'-carbon via a bridge, which effectively locks the pucker of the parent sugar into the RNA-like 3'-endo conformation, constraining the flexibility of the sugar and preventing the DNA-like conformation [48, 50, 166–169]. Owing to the 2',4'-constraint, BNAs are especially useful for increasing the strength of hybridization with complementary RNA sequences [170]. Compared to unmodified deoxynucleotide, the presence of a single BNA substitution can increase the T_m by ~ 5 – 11 °C [48, 170]. ASOs with BNA modifications are more potent than the 2'-MOE analogs [171–173]. The most used BNAs are LNA and, to a lesser degree, ENA. LNA is characterized by a 2',4'-methylene bridge and is also considered as a constrained analog of 2'-OMe [168, 169]. ENA contains a constrained ethylene bridge and exhibits better nuclease resistance than LNA [155]. Despite their superior thermal stability, LNA-modified ASOs are associated with hepatotoxicity [173, 174], which can be dependent on either the ASO sequence or its chemistry. It has been demonstrated that such liver toxicity can be reduced by avoidance of choosing “toxic” sequences based on bioinformatic prediction [48, 175]. Alternatively, more advanced BNA analogs such as cEt and cMOE have been developed, which are less toxic and more nuclease resistant than LNA [49, 171].

DNA analogs such as tc-DNA and bc-DNA have also been devised and investigated. ASOs composed of tc-DNA monomers are distinguished from other modified oligonucleotides by their capacity to cross the blood–brain barrier and reach the central nervous system [176].

2.2.3 Sugar-Phosphate Backbone Modifications

Finally, although the majority of nucleotide analogs are derived from DNA or RNA monomer, more heterogeneous chemistries involving modification on both the phosphate and sugar moiety have been developed [47, 86]. Such analogs show little resemblance to the natural archetypes [45]. The most common representatives are phosphorodiamidate morpholino oligomers (PMOs) [177] and peptide nucleic acid (PNA) (Fig. 3) [178]. In PMOs, the five-membered furanose ring is replaced by a six-membered morpholine heterocycle, and each morpholino subunit is connected by a phosphorodiamidate linkage instead of a PO bond [179, 180]. PNA is a more radically different type of nucleotide mimic, in which the whole sugar-phosphate backbone is substituted by a pseudo peptide polymer backbone. In other words, a PNA ASO is a chain of *N*-(2-aminoethyl) glycine subunits, to which the nucleobases are connected via methyl carbonyl linker [181]. While PMOs and PNA are substantially different from natural nucleic acids, they maintain and even enhance binding ability to complementary RNA in a Watson-Crick manner, compared to unmodified ASOs [45, 182, 183]. Moreover, due to their unnatural backbone, PMOs and PNA are almost fully resistant to nucleases and proteases, conferring them tremendous stability in biological systems [2, 175, 184]. Both modifications are neutral. The fact that four PMO-based ASO therapeutics (etepirsen, casimersen, viltolarsen, and golodirsen) were approved by the FDA during 2016–2021 is testimony to their excellent drug-like properties [87]. Nevertheless, a disadvantage of PMOs and PNA is that they bind to plasma proteins with minimal affinity, meaning that they are rapidly eliminated by urinary excretion [86]. As a result, high doses or frequent administration are needed for *in vivo* activity, which may increase the risk of side effects [48].

2.2.4 Other Modifications

Modifications on the nucleobase moiety are not as common as those on the sugar and/or phosphate moiety. The main objective of base modification is to enhance binding affinity of ASOs to their complementary RNA targets [185, 186]. For instance, substitution of the 5'-hydrogen atom on cytosine with a methyl group can increase the T_m by ~ 0.5 °C per modification [121]. 5'-Methylcytidine is present in several FDA-permitted ASOs, including mipomersen, inotersen, tofersen, nusinersen, and milasen. Interesting double-headed nucleotides (nucleotides containing two nucleobases) have been reviewed elsewhere [187].

In addition to changing the structure of one or more moieties (sugar, phosphate, base) in a nucleotide, which creates nucleotide analogs, modifications can also be made

through conjugation of ASOs to different biomolecules, such as cholesterol [188], peptides [189], antibodies [190], aptamers [191], and *N*-acetylgalactosamine (GalNAc) [192], thus directing the ASO drugs to target tissues and improving cellular uptake. *In vivo* delivery and cellular uptake of therapeutic ASOs have been discussed comprehensively in useful recent or classical reviews [55, 86, 87, 114, 116, 193–202].

2.3 ASO Rational Design

As discussed in the previous section, a number of analogs (chemically modified nucleotides) have been synthesized and evaluated (Fig. 3); however, with the exceptions of only a few phosphate modifications (PS, PS2, PB, PACE, and MsPA), heteroduplexes formed between uniformly modified ASOs and their target RNA sequences are poor substrates for RNase H [50]. Therefore, uniformly modified oligomers usually do not induce the degradation of their target RNAs except, for example, the fully DNA-PS ASO fomivirsen. In order to retain the RNase H activity, “gapmer” design was devised by Inoue et al. in 1987 [203]. Since RNase H only recognizes DNA–RNA duplexes with the DNA strand containing at least five continuous deoxynucleotides [48], in a gapmer-like ASO, a stretch of unmodified DNA or DNA-PS central “gap” (at least 5mer) is flanked by two “wings,” which can be composed of non-RNase H-sensitive analogs (e.g., 2'-OMe, 2'-MOE, PNA, or LNA) [47, 204, 205] (Fig. 4). The “gap” induces RNase H-mediated cleavage of the complementary RNA target, while the “wings” enhance the ASO's nuclease stability and target binding affinity [39]. To date, mipomersen, inotersen, and tofersen are the three gapmer-like ASO drugs that have been approved by the FDA for clinical use (Fig. 4).

Although uniformly modified ASOs do not support an RNase H-based mechanism, they have been primarily used as steric blockers, and, in fact, considerably more flexibility is afforded for their action, due to the avoidance of RNase H-mediated cleavage (Fig. 2) [39, 50]. Furthermore, it is obvious that the absence of an unmodified gap region in these ASOs maximizes their thermal stability (i.e., target binding affinity) and nuclease resistance, as they are fully modified. The most employed non-terminating mechanism is splice modulation. To date, six ASO drugs that are uniformly modified (PMO: etepirsen, golodirsen, viltolarsen, and casimersen; 2'-MOE-PS: nusinersen and milasen) have been permitted by FDA for use in patients (Fig. 4).

In fact, the drug-like properties of uniformly modified ASOs can be further improved by “mixmer” design (Fig. 4), that is, two or more modifications exist in one ASO sequence. Screening of different mixmer-like ASOs (different positioning of modifications in a given sequence) can lead to the identification of the best-performing design.

Herein, we suggest three strategies to take advantage of “mixmer” design:

1. First of all, nucleotide analogs that are selected for constructing mixmer-like ASO need to be compatible with each other. Since PMO chemistry is not compatible with other modifications, morpholino nucleic acid (MNA) and thiophosphoramidate morpholino oligomer (TMO), which retain the featured morpholine ring of a PMO unit, have been envisaged to replace PMO. MNA/2'-OMe-PS, TMO/2'-OMe-PS, and TMO/DNA/2'-OMe-PS mixmers have been synthesized and investigated for their capability in inducing splice modulation (exon skipping) [206, 207].
2. Introduction of LNA modifications into a uniformly modified 2'-OMe-PS ASO, generating different patterns of LNA/2'-OMe-PS mixmers, could enhance the exon skipping efficiency of the ASO sequence, thus allowing synthesis of shorter ASO sequences to reduce cost without compromising efficacy [208].
3. As 2'-MOE is sufficiently resistant to nucleases, in a fully 2'-MOE modified ASO sequence, the internucleotide linkages do not have to be uniformly PS modified. Instead, PO/PS mixmers can be designed and screened to fine tune the pharmacokinetics of the ASO sequence [209]. For example, tofersen has mixed phosphate linkage modification (PO/PS) (Fig. 4).

3 Splice-Modulating ASOs Targeting Inborn Errors of Metabolism

In most cases, uniformly modified splice-modulating ASOs are devised to correct the aberrant splicing of mutant genes responsible for IEMs. In this section, we introduce the current developments of splice-switching ASOs towards targeting IEMs, including Batten disease, Pompe disease, Fabry disease (FD), Niemann-Pick type C disease (NPCD), inclusion-cell disease, Hunter syndrome (HS), organic acidemias (OAs), congenital disorder of glycosylation (CDG), hereditary myopathy with lactic acidosis (HML), 6-pyruvoyl-tetrahydropterin synthase (PTPS) deficiency, erythropoietic protoporphyria (EPP), complementation group cobalamin E (cblE) type of homocystinuria, and aromatic L-amino acid decarboxylase deficiency (AADCD). The feasibility of these ASOs in treating IEMs with specific mutation has been tested in patient cells or commercialized human cell lines. Further, the basic information of these works in combination with additional rodent-based research and RNase H-dependent ASO-related studies are summarized in Table 1 for the readers' reference.

3.1 Lysosomal Storage Diseases

3.1.1 Batten Disease

Batten disease is an inherited autosomal recessive neurodegenerative condition, also known as neuronal ceroid lipofuscinoses (NCLs), that clinically manifests as an LSD causing neuronal loss and lipo-pigment accumulation. The disease carries a prevalence of one to eight of 1 million live births worldwide [210]. Batten disease consists of 14 subtypes. The universal characteristic of Batten disease is a distinct mutation in ceroid lipofuscinosis neuronal (*CLN*) gene [211].

CLN3 is one of the subtypes of Batten disease that has a prevalence of one in 25,000 births in the USA, with a carrier frequency of one in 70. The most common mutation of CLN3 Batten disease is deletion of exon 7 and 8 (*CLN3*^{Δex7/8}). As a result, a shift in the reading frame causes a premature termination of CLN3 transcript, creating a truncated protein lacking 257 amino acids including the lysosomal targeting sequence (LTS). Apart from *CLN3*^{Δex7/8}, other mutations that can eliminate LTS are frame-shifting deletions and nonsense mutations [212]. According to Tang et al. [213], this is manifested as changes in lysosomal size, autophagic flux, and lysosomal dysfunction. A couple of the currently preclinical candidates are ASO-20 and ASO-28, which are highly active. ASO-20 and ASO-28 target the middle of exon 5 at the 5' splice site (ss) of the pre-mRNA and induce exon 5 skipping, creating an mRNA isoform [212].

SINE-VNTR-Alu (SVA) insertion in the *MFSD8* gene (also referred to as *CLN7*) has been identified to introduce a cryptic splice-acceptor site (i6.SA) in *MSD8* intron 6, located 119 bp upstream to the SVA site. This results in a premature translational termination, clinically manifesting as Batten disease. ASO TY777 (milasen) is a 22mer oligonucleotide with PS and 2'-MOE modifications. Milasen blocks the i6.SA cryptic splice-acceptor site and the adjoining splice enhancer, restoring *MFSD8* expression. RNA sequencing (RNA-seq) data have displayed a 34% increase in normal exon 6 splicing in patient fibroblast transfected with milasen. Clinical data have displayed that the patients treated with milasen had favorable medical outcomes where the patient's seizure count decreased by 63% and seizure duration decreased by 52% [36].

3.1.2 Glycogen Storage Disease Type II (Pompe Disease)

Glycogen storage disease type II (GSDII) also known as Pompe disease is an autosomal recessive LSD caused by suboptimal activity of acid alpha-glucosidase (GAA). GAA consists of 952 amino acids and plays a catabolic role in degrading glycogen within lysosomes. The *GAA* gene

Table 1 Developments of therapeutic ASOs for IEMs

Disease (phenotype MIM number)	Molecular target	Drug/lead candidate(s) name	ASO mechanism	ASO design	Development stage	References
Familial hypercholanemia (607748, 143890)	<i>APOB</i>	Mipomersen (Kynamro®)	mRNA degradation by RNase H	20mer 5'-10'-5 gapmer (DNA-PS gap and 2'-MOE-PS flanks)	FDA approved for marketing Phase III clinical Phase II clinical Phase I clinical Preclinical	[13, 95] [245–248] [249, 250] [251, 252] [252]
LSDs						
Batten disease (also known as NCL)	NCL7 (<i>CLN7</i>) Batten disease; 610951)	Milasen (TY777)	ORF restoration by splice modulation (prevention of pseudoexon inclusion)	22mer 2'-MOE-PS	FDA permitted for clinical investigational treatment	[36]
	CLN3 Batten disease (204200)	ASO-20 and ASO-28	ORF restoration by splice modulation (induction of exon skipping)	18mer 2'-MOE-PS	Preclinical	[212]
GSD type 1a (232200)	<i>G6PC</i>	DS-4108b	ORF restoration by splice modulation	15mer mixmer (2'-OMe-PS and ENA-PS)	Preclinical	[253]
Pompe disease (also known as GSD type II; 232300)	<i>Gys1</i> (mouse)	PMO sequence (number 10)	NMD by splice modulation (induction of exon skipping)	25mer PMO	Preclinical	[254]
	<i>GAA</i>	AON intron 15, AON intron 10, and AON exon 8	Splice modulation (prevention of pseudoexon inclusion, partial intron retention, exon skipping, and/or partial exon skipping)	25mer PMO, 21mer PMO, and 24mer PMO	Preclinical	[255]
		AON 3 and AON 4	Splice modulation (prevention of pseudoexon inclusion, exon skipping, and partial exon skipping)	21mer PMO and 25mer PMO	Preclinical	[215, 216]
		AMO 1, AMO 2, and AMO 3	Splice modulation (prevention of exon skipping)	25mer PMOs	Preclinical	[214]
		PMO 15	Splice modulation (prevention of exon skipping)	20mer PMO	Preclinical	[217]

Table 1 (continued)

Disease (phenotype MIM number)	Molecular target	Drug/lead candidate(s) name	ASO mechanism	ASO design	Development stage	References
Fabry disease (also known as α -galactosidase A deficiency; 301500)	<i>GLA</i>	3'ss-SSO	ORF restoration by splice modulation (prevention of pseudoexon inclusion)	24mer 2'-OMe-PS	Preclinical	[219]
NPCD (257220)	<i>NPCI</i>	AMO 111	ORF restoration by splice modulation (prevention of pseudoexon inclusion)	PMO (sequence was not disclosed)	Preclinical	[256]
Inclusion-cell disease (also known as mucopolidosis type II α/β ; 252500)	<i>GNPTAB</i>	H19A(+119+138)	ORF restoration by splice modulation (induction of exon skipping)	20mer 2'-OMe-PS	Preclinical	[221]
HS (also known as MPS type II; 309900)	<i>IDS</i>	All AOs (AMO1, AMO 2, AMO 3, and LNA) failed to correct aberrant splicing	Failure to prevent partial exon skipping	25mer PMOs and 20mer LNA	Preclinical	[223, 224]
Propionic acidemia (606054)	<i>PCCA</i>	N/A	Splice modulation (prevention of pseudoexon inclusion)	25mer PMO	Preclinical	[226]
		ASO-29	ORF restoration by splice modulation (prevention of pseudoexon inclusion)	18mer 2'-MOE-PS	Preclinical	[228]
	<i>PCCB</i>	N/A	Splice modulation (prevention of pseudoexon inclusion)	25mer PMO	Preclinical	[226]
Methylmalonic aciduria (251000)	<i>MUT</i>	N/A	Splice modulation (prevention of pseudoexon inclusion)	25mer PMO	Preclinical	[226]
		AMO-B	Splice modulation (prevention of pseudoexon inclusion)	25mer PMO	Preclinical	[227]
CDG	CDG-Ia (PMM2-CDG; 212065)	AMOA and AMOB	Splice modulation (prevention of pseudoexon inclusion)	25mer PMOs	Preclinical	[231]
	CDG-IIk (TMEM165-CDG; 614727)	N/A	Splice modulation (prevention of pseudoexon inclusion)	25mer PMO	Preclinical	[232]

Table 1 (continued)

Disease (phenotype MIM number)	Molecular target	Drug/lead candidate(s) name	ASO mechanism	ASO design	Development stage	References
HML (255125)	<i>ISCU</i>	N/A	ORF restoration by splice modulation (prevention of pseudoexon inclusion)	25mer PMO	Preclinical	[234]
		AO-A	ORF restoration by splice modulation (prevention of pseudoexon inclusion)	22mer 2'-OMe-PS	Preclinical	[233]
		Ionis Pharmaceuticals Inc. oligonucleotide 676672	ORF restoration by splice modulation (prevention of pseudoexon inclusion)	18mer mixmer (2'-MOE-PS and cEt-PS)	Preclinical	[235]
PTPS deficiency: hyperphenylalaninemia (261640)	<i>PTPS</i>	PTS-AMOI, PTS-AMO2, and PTS-AMO3	Splice modulation (prevention of pseudoexon inclusion)	25mer PMOs	Preclinical	[237]
EPP (177000)	<i>FECH</i>	SSO 1, SSO 5, and SSO 6	Splice modulation (prevention of pseudoexon inclusion)	25mer 2'-OMe-PS	Preclinical	[238]
Phenylketonuria (261600)	<i>PKS</i>	V1-LNA-ASO	ORF restoration by splice modulation (prevention of partial intron retention)	19mer mixmer (DNA-PS and LNA-PS)	Preclinical	[240]
cbIE type of homocystinuria (236270)	<i>MTRR</i>	19e6-ei	Splice inhibition	25mer PMO	Preclinical	[257]
		<i>MTRR</i>	Splice modulation (prevention of pseudoexon inclusion)	24mer 2'-OMe-PS	Preclinical	[242]
Canavan disease (271900)	<i>Nat8l</i> (mouse)	Nat8l gapmer	mRNA degradation by RNase H	16mer LNA-PS gapmer	Preclinical	[258]
AAADC (608643)	<i>DDC</i>	ASO-D	ORF restoration by splice modulation (prevention of partial intron retention)	25mer PMO	Preclinical	[244]

2'-MOE 2'-O-methoxyethyl, 2'-OMe 2'-O-methyl, AADC aromatic L-amino acid decarboxylase deficiency, ASO antisense oligonucleotide, *cbIE* complementation group cobalamin E, *CDG* congenital disorder of glycosylation, *cEt* 2',4'-constrained ethyl, *ENA* 2'-O,4'-C-ethylene-bridged nucleic acid, *EPP* erythropoietic protoporphyria, *FDA* Food and Drug Administration, *GSD* glycogen storage disease, *HML* hereditary myopathy with lactic acidosis, *HS* Hunter syndrome, *IEM* inborn error of metabolism, *LNA* locked nucleic acid, *LSD* lysosomal storage disease, *MIM* mendelian inheritance in man, *MPS* mucopolysaccharidosis, *N/A* not available, *NCL* neuronal ceroid lipofuscinosis, *NMD* nonsense-mediated mRNA decay, *NPCD* Niemann-Pick type C disease, *OA* organic acidemia, *ORF* open reading frame, *PMO* phosphorodiamidate morpholino oligomer, *PS* phosphorothioate, *PTPS* 6-pyruvovyl-tetrahydropterin synthase, *RNase H* ribonuclease H

contains 20 exons located in human chromosome 17q25.2-25.3. When mutations compromise the integrity of the *GAA* gene, the *GAA* protein loses its ability to degrade glycogen in lysosomes. This leads to the accumulation of glycogen in lysosomes. Lysosomal accumulation of glycogen results in lysosomal swelling, dysfunction, and defective autophagy. Scientists have discovered 497 mutations associated with the *GAA* gene; these include missense, nonsense, splice-site mutation, intragenic deletions, and insertions. Based on the clinical manifestation, GSDII is categorized into two forms, a rapidly progressing infantile-onset (IO) form and a slow progressing late-onset (LO) form [214].

The most common mutation among LO patients is c.-32-13T>G in intron 1 of the *GAA* gene, and genetic screening has shown that 40–70% of LO GSDII patients possess this mutation. This mutation in the intron region restricts spliceosomes from identifying exon 2 of *GAA*, thus inhibiting the splicing. Goina et al. [214] have developed 25mer PMOs (AMO 1, 2, and 3) for splice correction to treat LO GSDII patients. Compared to wild type, only 20% of enzyme activity was retained by myotubes of patients. Yet, 3 days after patient fibroblasts were treated with a combination of the AMOs, a 70% increase in *GAA* activity as well as a significant reduction in accumulation of glycogen has been observed [214].

PMOs also have been identified to target the putative polypyrimidine tract of a cryptic splice acceptor site created by c.-32-13T>G mutation as part of the pseudoexon created in *GAA* intron 1, blocking the cryptic splice donor, which leads to the inclusion of exon 2. Van der Wal et al. (2017) have identified AON 3 and 4 restoring exon 2 by inhibiting the usage of natural pseudoexon rather than silencing a putative intronic splice silencer [215]. Simultaneous targeting of cryptic splice acceptor and donor sites of pseudoexon has been investigated. This has displayed a 3.2-fold increase in exon 2 inclusion and sixfold reduction of natural pseudoexon inclusion [216]. Furthermore, PMOs have also been developed to improve exon 2 inclusion in patients with c.-32-13T>G. Aung-Htut et al. [217] evaluated 20 PMO candidates. The best-performing candidate (PMO 15) has displayed the most promising results, with a 1.6-fold increase in *GAA* activity when a 50- μ M concentration of this ASO was transfected using RNAiMAX.

3.1.3 Fabry Disease

FD is a recessive X-linked glycosphingolipid metabolism disorder that is caused by a mutation in the lysosomal α -galactosidase A (*GLA*) gene leading to full or partial reduction of *GLA* enzyme. Due to the impaired functionality of *GLA* enzyme, progressive accumulation of globotriaosylceramide and other related glycosphingolipids takes place within lysosomes, leading to lysosomal dysfunction.

As lysosomes are universal subcellular components, dysfunction of lysosome causes a progressive clinical manifestation of FD. FD is a multi-system disorder with characteristic clinical presentation of small vessel injury, potassium calcium channel dysfunction in endothelial cells, corneal changes, impaired autophagosome maturation, and irreversible fibrosis of cardiac and renal tissue. The prevalence of FD in the general population is estimated to range from one in 476,000 to one in 117,000, yet global epidemiological studies need to be conducted for accurate estimation [218].

There have been more than 600 pathogenic mutations identified as responsible for FD. The most prevalent mutation with a relatively higher newborn incidence rate is c.639 + 919G>A, which leads to an insertion of a 57-bp pseudoexon sequence at intron 4. The pseudoexon inclusion takes place as the mutation blocks the binding motif of the ESS at + 17 to + 22 bp and disrupts the recruitment of heterogeneous nuclear ribonucleoprotein (hnRNP) A1 and hnRNP A2/B1. The pseudoexon present in the mutated transcript creates an in-frame PTC at 27 bp in the exon, leading the misspliced mRNA to be targeted by NMD. Since the 5' and 3' canonical splice sites are still intact, splice-switching ASOs are used to block the pseudo-5' and 3' canonical splice sites. Preclinical studies have displayed multiple suitable ASO candidates to reduce pseudoexon inclusion. Among those candidates, 2'-OMe-PS 3'ss-SSO has displayed promising outcomes, becoming the leading candidate. Both 3'-targeting and 5'-targeting ASOs were investigated, and 2'-OMe-PS 3'ss-SSO has consistently been the best candidate, showing a 40–73% reduction in pseudoexon inclusion [219].

3.1.4 Niemann-Pick Type C Disease

NPCD is an autosomal recessive neurovisceral atypical lysosomal lipid storage disease that is caused by a mutation in either the *NPC1* or *NPC2* gene. NPC1 is a large protein with 1252 amino acids, while NPC2 is a smaller protein with 132 amino acids. These proteins play a key role in lipid transport in the human body, specifically egestion of cholesterol from lysosome. Mutations of *NPC1* or *NPC2* will disrupt the intracellular transport of lipids, resulting in the accumulation of cholesterol and lipids in tissues such as brain, liver, and spleen. The estimated minimal incidence rate of NPCD is one in 120,000 live births [218].

Multiple mutations, such as missense, splicing, and point mutations, could affect the function of the NPC protein. c.1554-1009G>A is a mutation affecting the *NPC1* gene at intron 9 that creates a cryptic donor splice site by adding a 194-bp intronic sequence to mature mRNA. This results in the incorporation of a pseudoexon in mRNA, leading to a PTC being included, thus leading mRNA transcript to be degraded by NMD. PMO has been used to prevent mRNA from being degraded by NMD by correcting aberrant splicing. AMO 111

is considered the leading candidate since it has been proven to restore normal splicing in preclinical studies [217].

3.1.5 Inclusion-Cell Disease (Mucopolipidosis Type II α/β)

Mucopolipidosis type II α/β (MLII) is an autosomal recessive condition caused by a deficiency of uridine diphosphate (UDP)-*N*-acetylglucosamine-1-phosphotransferase (GlcNAc-1-phosphotransferase). GlcNAc-1-phosphotransferase is responsible for producing a specific targeting signal for transporting multiple lysosomal hydrolases to lysosomes. Mutation of the *GNPTAB* and *GNPTG* genes will produce a truncated version of the protein GlcNAc-phosphotransferase. This results in abnormal glycosylation of hydrolytic enzymes, which in turn compromises the trafficking of those enzymes, leading to accumulation and secretion of those enzymes into extracellular components. These cellular pathologies clinically manifest as severe cardiac complications, respiratory complications, severe skeletal dysplasia, craniofacial abnormalities, and muscular skeletal abnormalities. The prevalence of MLII is one in 100,000–400,000 [220].

GlcNAc-1-phosphotransferase is composed of three subunits known as α , β , and γ . α and β subunits are encoded in the *GNPTAB* gene, while the γ subunit is encoded in the *GNPTG* gene. MLII is one of the most severe LSDs and is commonly caused by a TC dinucleotide deletion mutation on exon 19 of *GNPTAB* (c.3503_3504del). This deletion disrupts the ORF and produces a truncated inactive protein. 2'-OMe-modified splice-modulating ASOs have been designed and evaluated to treat this condition. These ASOs induce exon 19 skipping, producing a smaller transcript with 56 amino acid deletion, yet allowing in-frame synthesis of α and β subunits. This will restore partial activity to GlcNAc-1-phosphotransferase, reducing the severe clinical manifestation of the disease. Two ASOs, named H19D(+12–9) and H19A(+119+138), obtained the most promising results. H19A(+119+138) produced the longest *GNPTAB* transcript missing exon 19 compared to full length out-of-frame transcript [221].

3.1.6 Hunter Syndrome (Mucopolysaccharidosis II)

HS is an X-linked recessive condition caused by the deficiency of iduronate-2 sulfatase (I2S) in lysosomes. I2S is used to break down glycosaminoglycans dermatan and heparan sulfate. I2S enzyme is encoded in the *IDS* gene; mutation in the *IDS* gene causes cellular accumulation of glycosaminoglycans dermatan and heparan sulfate. Clinical presentations of HS are stiff joints, facial dysmorphism (Hunters' phenotype), and enlarged spleen and liver. The prevalence of HS is one in 170,000 male births [222].

The *IDS* gene contains nine exons and is located at Xq28. Multiple different mutations affect the expression of the *IDS*

gene. Missense and nonsense mutations like c.241.C>T and c.257C>T cause dysregulation of exon 3 splicing by activating cryptic splice sites in exon 3 and 8. These mutations also make an SRSF1 binding site while removing SRSF2 and hnRNP E2 binding sites. Synonymous mutation c.1122C>T creates a cryptic splice site at exon 8, and it has increased the splice score of 5'ss. Matos et al. [223, 224] developed 25mer PMOs and 20mer LNA modified ASOs to block the new 5'ss in exon 8 to recover the mRNA, but results still displayed spliced mRNA. The PMO and LNA ASOs that were used failed to fully block the aberrant splicing.

3.2 Organic Acidemias

OAs are a group of medical conditions that are caused by defects in biochemical pathways converting isoleucine, cholesterol, valine, threonine odd-chain fatty acids, and methionine to organic acids by degradation. This disruption results in accumulation of toxic organic acids that disrupts other metabolic pathways and increases anion gap metabolic acidosis, ketosis, hyperammonemia, hypoglycemia, and elevated lactate [225]. Propionic acidemia (PA) and methylmalonic acidemia (MMA) are the two most prominent examples of OA caused by the disruption of the propionate oxidative pathway (POP). OAs are autosomal recessive inherited conditions that affect the synthesis or functionality of two major enzymes of the POP, propionyl coenzyme A (CoA) carboxylase (PCC) and methylmalonyl CoA mutase (MCM), or their coenzymes (biotin and adenosyl-cobalamin) [226].

The PCC enzyme has two subunits encoded in the *PCCA* gene and *PCCB* gene. The MCM enzyme is encoded in the *MUT* gene. The *MMAA* and *MMAB* genes are responsible for adenosylcobalamin synthesis in mitochondria. Mutation in *MUT*, *MMAA*, or *MMAB* genes causes isolated MMA, while a mutation in the *PCCA* or *PCCB* gene causes PA [226].

Various mutations pertinent to OA have been extensively researched. Over 50 different mutations have been studied, and the majority of them are missense mutations. The molecular mechanism of pathology is mostly common among the conditions. A single point mutation in the gene causes an activation of a cryptic splice site followed by a complementary splicing site closer to the mutation, leading to an insertion of an intronic sequence known as a pseudo-exon into mature mRNA. In MMA, pathogenic mutations like c.1957-898A, c.458T>A, and c.458T>A have been identified in the *MUT* gene. One of the promising candidates selected for treatment of MMA is a 25mer PMO named AMO-B, which is complementary to the 5' region of the inserted intronic sequence in c.1957-898A and c.458T>A mutations. In preclinical studies, patient fibroblast cells were transfected with AMO-B for 24 h, and results displayed that

the treatment effectively recovered the production of normal *MUT* transcript [227].

An IVS14-1416A→G mutation was observed among patients with PCCA deficiency. This mutation has led to an insertion of an 84-bp pseudoexon between exon 14 and exon 15 of the *PCCA* gene. In PCCB-deficient patients, IVS6+462A→G was identified where a new 72-bp segment had been inserted between exon 6 and 7, which had created a 5' cryptic donor splice site. Currently, the most promising potential treatment for PCCA deficiency is ASO-29, which is an 18nt long 2'-MOE-PS AO. The modifications allow ASO-29 to bind to 3'ss and its alternating region with high affinity, restoring the normal splicing. A study displayed the dose-dependent nature of ASO-29 from 1-nM to 25-nM concentrations. At the 1-nM concentration, a onefold protein increase can be observed, while at the 25-nM concentration, close to a twofold PCCA protein increase can be observed [228]. PMO therapy targeting 3' and 5'ss of the pseudoexon is being studied as a potential treatment for *PCCB* mutation, and reverse transcriptase polymerase chain reaction (RT-PCR) results show at 10- μ M and 20- μ M concentrations the transcript is fully restored without the 72-bp intronic insertion [226].

3.3 Congenital Disorder of Glycosylation

CDG is a group of medical conditions characterized by disorder in protein and lipid glycosylation. This is caused by impairment of glycosyltransferases, glycosidases, nuclear-sugar transporters as well as proteins involved in Golgi trafficking and Golgi pH management. CDG is generally grouped into four types, i.e., O-linked glycosylation, N-linked glycosylation, O- and N-linked multiple glycosylation, and lipid and glycosylphosphatidylinositol (GPI) anchor biosynthesis defects [229]. These conditions lead to defective trafficking of proteins and abnormalities in channels implicated in pH or metal ion homeostasis. Since these biochemical processes are ubiquitous in all tissues, the clinical manifestation of CDG will carry mild to severe symptoms and multiorgan failure [230].

The N-linked glycosylation protein defect also known as PMM2-CDG (CDG type Ia) is the most common CDG. Patients suffering from PMM2-CDG harbor pathogenic missense mutations in the *PMM2* gene. These mutations create a deficiency in PMM2 enzyme, which catalyzes the conversion of mannose-6-phosphate (M-6-P) to mannose-1-phosphate (M-1-P) in the guanosine diphosphate (GDP) mannosyl transferase pathway [229]. There are multiple mutations that have been identified that affect the *PMM2* gene. c.256-1G>C substitution creates exon skipping of exon 3 and 4 by introducing a 3'ss in intron 3, while the c.640-9T>G mutation activates a cryptic splice site in intron 3. Mutation c.640-15479C>T has been identified to introduce

a 123-bp pseudoexon sequence in exon 7. 25mer PMOs AMOA and AMOB have been identified as leading candidates for treatment, where one binds to the 5'ss while the other binds to the 3'ss. A 20- μ M concentration of the two PMOs was used to transfect patient fibroblasts, and after 24 h, the PMM2 protein level had increased by 9–23%. After 48 h and 72 h, PMM activity had increased to 50% compared to the healthy fibroblast control [231].

A novel mutation causing CDG type II was discovered to be associated with the *TMEM165* gene. The *TMEM165* gene produces a transmembrane protein that is responsible for calcium and pH homeostasis in Golgi complex, plasma membranes, and late endosomes. Multiple mutations have been identified that would affect the activity of the TMEM165 protein. Sixty percent of patients reported with *TMEM* mutation-related CDG type II had intronic substitution c.792+182G>A, while the rest had missense mutations such as c.377G>A, c.376C>T, or c.910G>A. Yuste-Checa et al. (2015) identified that the c.792+182G>A mutation activated a pseudoexon where a 117-bp sequence is inserted into intron 4, creating a new 5' cryptic splice site. This resulted in either only exon 4 or both exon 3 and 4 being deleted from the transcript. A 25mer PMO has been identified by the same group, targeting the new 5' cryptic splice site. Patient fibroblast cells were transfected with the PMO (20- or 30- μ M concentrations). Twenty-four hours later, a tenfold increase in TMEM165 protein was observed among patients with the c.792+182G>A mutation, but no increase in protein production was observed in missense-mutated patient cells. This reveals that this PMO candidate specifically targets the c.792+182G>A mutation [232].

3.4 Hereditary Myopathy with Lactic Acidosis

HML is a recessively inherited condition with an IVS5+382 G>C mutation in the last intron of the *ISCU* gene. This single base transition introduces a PTC via retention of a pseudoexon between exon 5 and exon 6. This results in the loss of full-length iron-sulfur cluster assembly (ISCU) protein, creating a truncated protein due to loss of mRNA. This results in defects in iron- and sulfur-containing enzymes like succinate dehydrogenase, aconitase, and complex I–III of the mitochondrial respiratory chain. These enzymes fulfill a critical role in cellular respiration. Patients with HML have a characteristic clinical presentation of intolerance to exercises, fatigue, dyspnea, and muscle pain [233].

Exon skipping of the pseudoexon using ASOs to restore the wild type of mRNA of the allele is a therapeutic mechanism that has been investigated by scientists. Another approach is blocking the splice site to restore proper splicing of the pre-mRNA. A study conducted by transfecting three homozygous patient cell lines with 25mer PMO displayed that, 48 h after transfection, the splicing pattern was restored

to 100% like the wild type. Also, the effects of the transfection were stable for 21 days [234].

Multiple studies have been conducted to explore therapeutic options to treat HML. Patient-derived primary muscle cells were transfected with 300 nM of a 22mer 2'-OMe-PS oligomer named AO-A targeting the pseudoexon acceptor splice site; restoration was close to onefold [233]. Some therapeutic candidates have been investigated regarding inducing exon skipping. One of the promising candidates of treatment for the phenotype of ISCU myopathy is a 18mer ASO sequence modified either with 2'-MOE-PS or cEt-PS chemistry. According to Holmes-Hampton et al. (2016), ISCU protein levels were elevated to the control level after patient fibroblasts were transfected with 30 nM of the 18mer ASO. The results further display a twofold increase in succinate in myotube cultures while aconitase and complex III activity increased to the level of the control group [235].

3.5 6-Pyruvyl-Tetrahydropterin Synthase Deficiency: Hyperphenylalaninemia

Hyperphenylalaninemia (HPA) is an autosomal recessive disorder of the phenylalanine metabolism pathway that results in phenylalanine accumulating in blood while blood tyrosine concentration is reduced, causing phenylketonuria. The average prevalence of HPA worldwide is 1:10,000. HPA has three major genotypes: phenylalanine hydroxylase (PAH) deficiency, tetrahydrobiopterin (BH4) metabolism defects, and DnaJ heat-shock protein family (Hsp40) member C12 (DNAJC12) deficiency [236].

BH4 metabolism is an essential metabolic process in the human body that creates BH4, which is a natural cofactor for tyrosine, phenylalanine, and tryptophan hydrolases. The PTPS enzyme catalyzes the second step of BH4 metabolism. The PTPS enzyme is encoded in the *PTS* gene. Mutation in the *PTS* gene will create a deficiency in PTPS enzyme, which could affect BH4 metabolism and causes HPA with moderate to severe neurological conditions, including hypotonia, bradykinesia, and seizures. Even though oral BH4 administration can be conducted, BH4 transfer across the blood–brain barrier is very poor; therefore, alternative therapeutic interventions are needed [237]. Martinez-Pizarro et al. (2022) have identified multiple mutations that could result in deficiency of PTPS enzyme by affecting mRNA processing. For example, c.243+3A>G results in exon 4 skipping and c.164-672C>T creates four overlapping pseudoexons resulting in a 5' cryptic splice site. Studies have shown 20- and 25mer splice-modulating ASOs (SSO1–SSO6) with 2'-OMe-PS chemistry as good candidates for treatment. After transfection of patient fibroblasts with 25mer SSO1, 5, and 6, corrected *PTS* mRNA was produced and PTPS protein expression was increased from 5 to 50% relative to the untreated fibroblasts [238].

Apart from c.243+3A>G and c.164-672C>T, scientists have discovered more than 64 mutations that could cause PTPS deficiency. Some intronic mutations like c.84-322A>T, c.163+695_163+751del57, or c.164-712A>T create/delete splice regulatory elements and introduce new cryptic exons that are derived from short interspersed nuclear elements (SINEs) or long interspersed nuclear elements (LINEs) in the human genome. To reverse the pathogenic effect of these types of mutations in PTPS deficiency, Brasil et al. (2011) developed 24mer PTS PMOs (PTS-AMO1, PTS-AMO2, and PTS-AMO3). PTS-AMO1 targeted 3' cryptic splice sites, while PTS-AMO2 and PTS-AMO3 targeted 5' cryptic splice sites to block access of splicing agents to pseudoexons in pre-mRNA. Patient fibroblasts were transfected with 30 μM of each ASO, and 24 h after transfection, correct *PTS* mRNA was recovered and functional PTPS protein was observed in patient cells [237].

3.6 Erythropoietic Protoporphyrin

EPP is a rare genetic disorder in the heme metabolic pathway, characterized by ferrochelatase (FECH) deficiency. This clinically manifests in patients with an accumulation of protoporphyrin in blood, connective tissue, and erythrocytes. One of the unique clinical presentations of EPP is photodermatitis. The prevalence of EPP is estimated to be 1:75,000 and 1:200,000 births worldwide [239].

Biallelic polymorphism c.315-48T>C in intron 3 has been identified to increase the usage of a cryptic splice site between exon 3 and 4 by creating a 63-bp pseudoexon in intron 3. In wild-type alleles, the cryptic splice site can be seen to be only 20% active, yet with the alleles with cytosine substitution, it increases to 60%. c.315-48T>C creates PTC upstream from the mutation site – 63, causing aberrant splicing. This results in an unstable mature mRNA degraded by NMD. Deficiency of stable wild-type *FECH* mRNA in the body leads to a lack of stable *FECH* mRNA. FECH protein expression goes below 35% from healthy FECH levels, causing the depositing of free protoporphyrin IX. The 315-48C allele is present in more than 90% of EPP patients; therefore, researchers have taken interest in therapeutic approaches targeting the polymorphism. One of the approaches scientists have employed is an LNA-modified ASO method because of its nuclease resistance and higher affinity. Oustric et al. (2014) used two targets for LNA ASO development, i.e., the cryptic splice site at – 63 bp from intron 4 and the 315-48C nucleotide site. The optimum candidate that had the highest effect on reducing exon inclusion was 24mer V1-LNA-ASO, which targets the region of intron 3 from – 45 to – 63, covering both mutation point (– 48) and cryptic splice site (– 63). After 48 h of transfection with V1-LNA-ASO (125 nM), pseudoexon retention was reduced by 25% and the proportion between abnormally spliced mRNA and total mRNA was similar to the asymptomatic

subject. Overall, FECH protein production increased, resulting in reduced accumulation of protoporphyrin [240].

3.7 Complementation Group Cobalamin E Type of Homocystinuria

Homocystinurias are a group of disorders of homocysteine metabolism [241]. The cbIE type of homocystinuria is a rare autosomal recessive disorder caused by deficiency of methionine synthase reductase (*MTRR*) [242]. *MTRR* deficiency clinically manifests as brain demyelination and atrophy, megaloblastic anemia, psychomotor retardation, and homocystinuria. Fifteen exons have been identified in the *MTRR* gene, and 15 mutations in each exon have the potential to cause disease. The most prevalent mutation among these is the c.903+469T>C mutation in intron 6. c.903+469T>C mutation creates an ESE site binding to serine/arginine splicing factor 1 (SRFS1), which identifies a 5'ss leading to activation of a pseudoexon. Researchers have investigated different therapeutic options, and they have identified splice-switching ASOs as potential therapeutics that could be used to treat the cbIE type of homocystinuria. ASOs bind to pre-mRNA to restore normal splicing by providing steric hindrance to block the binding of splicing factors like U2AF and U1snRNP, thus inhibiting the pseudoexon inclusion. 2'-OMe-PS modified 24mer *MTRR* ESE-SSO, *MTRR* 5'ss-SSO, and 3'ss-SSO were used as candidates for transfection. The patient fibroblast cells were transfected with these three candidates, and *MTRR* ESE-SSO showed promising results by successfully inducing pseudoexon skipping and restoring correct splicing in *MTRR* mRNA to the control level [242].

3.8 Aromatic L-Amino Acid Decarboxylase Deficiency

AADCDC is a rare autosomal recessive neurometabolic disorder caused by a mutation in the dopa-carboxylase (*DDC*) gene affecting the biosynthesis pathway of dopamine and serotonin. The prevalence of the condition is 1:116,000 in the European Union and 1:64,000–1:90,000 births in the USA [243].

Mutation of the *DDC* gene causes AADCDC. This deficiency results in low levels of dopamine and serotonin in the body, which clinically manifest as developmental delays, athetoid movement, and oculogyric crises. Scientists have identified 40 distinct mutations among patients, and the most prevalent mutation of AADCDC is c.714+4A>T. This mutation causes a splicing error that removes a splice donor site, creating a +38 cryptic splice site that can produce a PTC in *DDC* mRNA. To correct the aberrant splicing, Tsai et al. (2018) suggested the therapeutic option of 25mer PMOs. The ASOs restore the aberrantly spliced 5-6+37-7 transcript of *DDC*.714+4A>T to the normal 5-6-7-8 isoform. The

leading candidate of this study, ASO-D, restored 41% of transcripts to the normal isoform. ASO-D restored mRNA to the normal form more effectively than any other candidate, and it has the highest target specificity among the candidates tested. Yet some unexpected novel splice variants were created by the ASOs. To increase efficacy, this group has suggested the combination of ASO-D with suppressor binding blocker ASO-9AA [244].

4 Conclusion

Although the individual prevalence of a single IEM or a single disease-causing mutation is extremely rare, collectively they affect a large population worldwide. Despite the availability of traditional treatments such as dietary manipulations, cofactor administrations, enzyme replacement therapies, and transplantation, most of the inherited metabolic diseases still lack effective therapeutic interventions. In addition, early diagnosis of the diseases resulting from technological advances further increases the demand for more effective treatments. To this end, splice-switching ASOs, owing to their capacity to suppress aberrant splicing patterns of mutant pre-mRNA in a mutation-specific manner and consequently recover normal protein expression, have been developed as an individualized molecular therapy for IEMs. The recent FDA permission given to milasen for investigating its therapeutic effect on CLN7 Batten disease can be considered a milestone in this field, proving the feasibility of splice-modulating ASO-based therapeutic strategies. In fact, as reviewed in this work, specific splice-modulating ASOs have been developed and evaluated in patients' cells for targeting several IEMs, including LSDs, OAs, CDG, HML, PTPS deficiency, EPP, the cbIE type of homocystinuria, and AADCDC.

Herein, integrating the knowledge on the diversity of ASOs' mechanisms, chemistry, and design, we provide suggestions on improving and optimizing the development of therapeutic ASOs towards targeting IEMs:

1. Splice modulation, rather than RNase H-mediated RNA degradation, is the first choice when choosing an appropriate ASO mechanism of action for IEM treatment. This is due to the fact that most IEMs are monogenic disorders [259], and an IEM is usually caused by the defective expression of an enzyme resulting from a specific ORF-destructing mutation. A splice-modulating ASO can be designed to skip the mutation-induced pseudoexon, restore the ORF, and consequently recover the synthesis of functional enzyme. Alternatively, in some cases, RNase H-dependent ASOs may also be developed to avoid or reduce the toxic accumulation of a metabolite through inhibition of its transporter. For example, in the

case of familial hypercholesterolemia, mipomersen, an FDA-approved gapmer-like ASO, is used to downregulate the expression of apolipoprotein B-100, thus lowering the plasma cholesterol level.

2. Although all the FDA-permitted splice-modulating ASO drugs are uniformly modified, for example, milasen is a fully 2'-MOE-PS molecule, a lead candidate may be further optimized to improve its drug-like properties through incorporating promising novel nucleotide chemistries, such as cMOE, TMO, 7',5'- α -bc-DNA, and MsPA (Fig. 3).
3. Similar to point 2, further optimization of a lead candidate can be done by screening different mixmer-like ASOs with combinations of diverse chemistries (Fig. 4), leading to identification of the best-performing mixmer ASO design.
4. Furthermore, administration of cocktails of two or more ASO sequences is worth investigating to further increase the potency of individual ASO candidates [260], as a single ASO sequence may not effectively disturb the secondary structure of its RNA target. However, ASO cocktail therapy may complicate or delay FDA approval.
5. In addition, although most of the reported ASOs targeting IEMs have been shown effective in the correction of phenotypes preclinically, few have entered the clinical stage. Therefore, we would also like to suggest researchers screen and identify more efficacious ASO sequences for the same target gene. For example, ISIS 113715 was identified and selected as the lead ASO sequence targeting PTP1B for the therapy of type 2 diabetes in preclinical and phase I stages, but a more efficacious sequence (ISIS 404173) was chosen as the lead compound for a phase II trial [59]. Another example is the development of ASO targeting *SOD1* for treating amyotrophic lateral sclerosis. ISIS 333611 (CCGTCGCCCTTCAGCACGCA) was initially selected as the lead candidate, but a better sequence, namely, tofersen (CAGGATACATTTCTACAGCT), was eventually chosen, clinically evaluated, and approved by the FDA [33, 261]. In addition to efficacy, pharmacokinetic properties and toxicity should also be considered when choosing a more promising ASO sequence as the new lead candidate.

Finally, given the considerable versatility, flexibility, and specificity of ASO-based therapeutics, which are offered by the diversity of their mechanisms, chemical modifications, and designs, we believe that more IEM-targeting ASOs listed in our work will enter clinical trials in the near future and splice-modulating ASOs will keep playing an important role in providing individualized treatment for IEMs with newly identified mutations.

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