Review

Evaluation of Chemically Modified Nucleic Acid Analogues for Splice Switching Application

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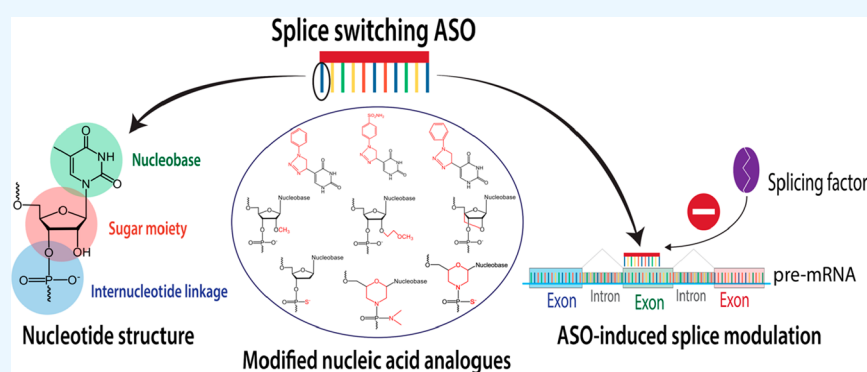Cite This: *ACS Omega* 2023, 8, 48650−48661

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ABSTRACT: In recent years, several splice switching antisense oligonucleotide (ASO)-based therapeutics have gained significant interest, and several candidates received approval for clinical use for treating rare diseases, in particular, Duchenne muscular dystrophy and spinal muscular atrophy. These ASOs are fully modified; in other words, they are composed of chemically modified nucleic acid analogues instead of natural RNA oligomers. This has significantly improved drug-like properties of these ASOs in terms of efficacy, stability, pharmacokinetics, and safety. Although chemical modifications of oligonucleotides have been discussed previously for numerous applications including nucleic acid aptamers, small interfering RNA, DNAzyme, and ASO, to the best of our knowledge, none of them have solely focused on the analogues that have been utilized for splice switching applications. To this end, we present here a comprehensive review of different modified nucleic acid analogues that have been explored for developing splice switching ASOs. In addition to the antisense chemistry, we also endeavor to provide a brief historical overview of the approved spice switching ASO drugs, including a list of drugs that have entered human clinical trials. We hope this work will inspire further investigations into expanding the potential of novel nucleic acid analogues for constructing splice switching ASOs.

1. INTRODUCTION

Alternative splicing is an important process in the regulation of gene expression. By variably removing introns and specific exons from the primary gene transcript, alternative splicing can generate a range of different mature mRNA variants from a single pre-mRNA transcript, resulting in differential gene expression.[1] However, splicing errors may arise and often result in genetic diseases. Antisense oligonucleotides (ASOs) targeting pre-mRNA to modulate splicing (splice switching ASOs) have been explored extensively in the past few years. ASO, once administered, will alter the splicing process and regulate the expression of proteins/protein isoforms by either correcting genetic mutation or inducing mRNA degradation.[1] So far, five splice switching ASO drugs have been approved by the US Food and Drug Administration (FDA) for the treatment of Duchenne muscular dystrophy (DMD) and spinal muscular atrophy (SMA).[2] Despite their success, large-scale production, poor

cellular uptake, rapid clearance, and possible toxicity issues are the major challenges.[2] To potentially address these limitations, studies have focused on evaluating various nucleic acid analogues for their splice switching efficacy. The first part of this review provides a snapshot on the history of approved splice switching drugs for DMD,[3] followed by a brief overview of the main alternative splicing mechanisms that have been utilized in therapeutic development. Then, a comprehensive review of the current nucleic acid analogues for splice switching application, as
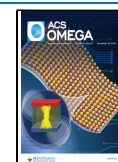
well as a list of chemically modified splice switching ASOs that have been subjected to clinical investigation, will be presented.

2. SKELETAL MUSCLE REGENERATION IN QUOKKA, AND ITS LINK TO THE FIRST APPROVED SPLICE SWITCHING DRUG

It is fascinating to note how the Rottnest quokka (*Setonix brachyurus*, Figure 1),[4] which is often called "the happiest animal

Figure 1. Quokka (*Setonix brachyurus*) on Rottnest Island, Western Australia (image credit: Dr. Yang Liu).

on earth" due to its facial muscle arrangement that resembles a human smile, is linked to the discovery of the approved splice switching drug for DMD. This only member of the genus *Setonix* is a small marsupial about the size of a domestic cat, with the main population found in Rottnest Island and Bald Island of Western Australia.[4] Prior to 1961, little was known about muscle regeneration; in fact, it was widely accepted that highly differentiated tissue such as skeletal muscle had very limited ability to regenerate.[5] However, a study on quokka led by Professor Byron A. Kakulas (then a PhD student) obtained breakthrough evidence showing that muscle lesions can be reversed. Professor Kakulas proved that the paralysis of caged quokkas was due to vitamin E deficiency. By feeding them a large daily dose of vitamin E, the quokkas that had developed paralysis showed "rapid and complete recovery".[6] Professor Kakulas's early work has inspired Professor Steve Wilton's and Professor Sue Fletcher's laboratory to further explore a treatment for neuromuscular diseases with subsequent studies, which led to a development which later became an approved drug for the treatment of DMD.[7]

On September 19, 2016, fifty-five years after the discovery of muscle regeneration in Rottnest quokka by Professor Kakulas, the first splice switching ASO drug—Exondys 51—was approved by the FDA for the treatment of DMD.[3] Although the drug is amenable to treating only around 13% of all DMD patients, the finding was significant for people who suffer from this lethal disease. DMD is an X-linked genetic disease characterized by progressive muscle weakness due to the loss of dystrophin protein, the main component that stabilizes muscle fibers during contraction. DMD patients are often bound to wheelchairs around the age of 12 and die in their early adulthood (∼25 years) mainly due to heart complications.[8] The approval of Exondys 51 was a significant milestone after a 25 year long collaborative effort between Professor Kakulas and Professor Wilton and Professor Fletcher, all of which started when the latter two scientists joined the Australian Neuromuscular Research Institute in 1991.[9] This has then paved the way for three more splice switching ASO drugs to enter the market for DMD: Vyondys 53 (2019), Viltepso (2019), and Amondys 45 (2021).[2]

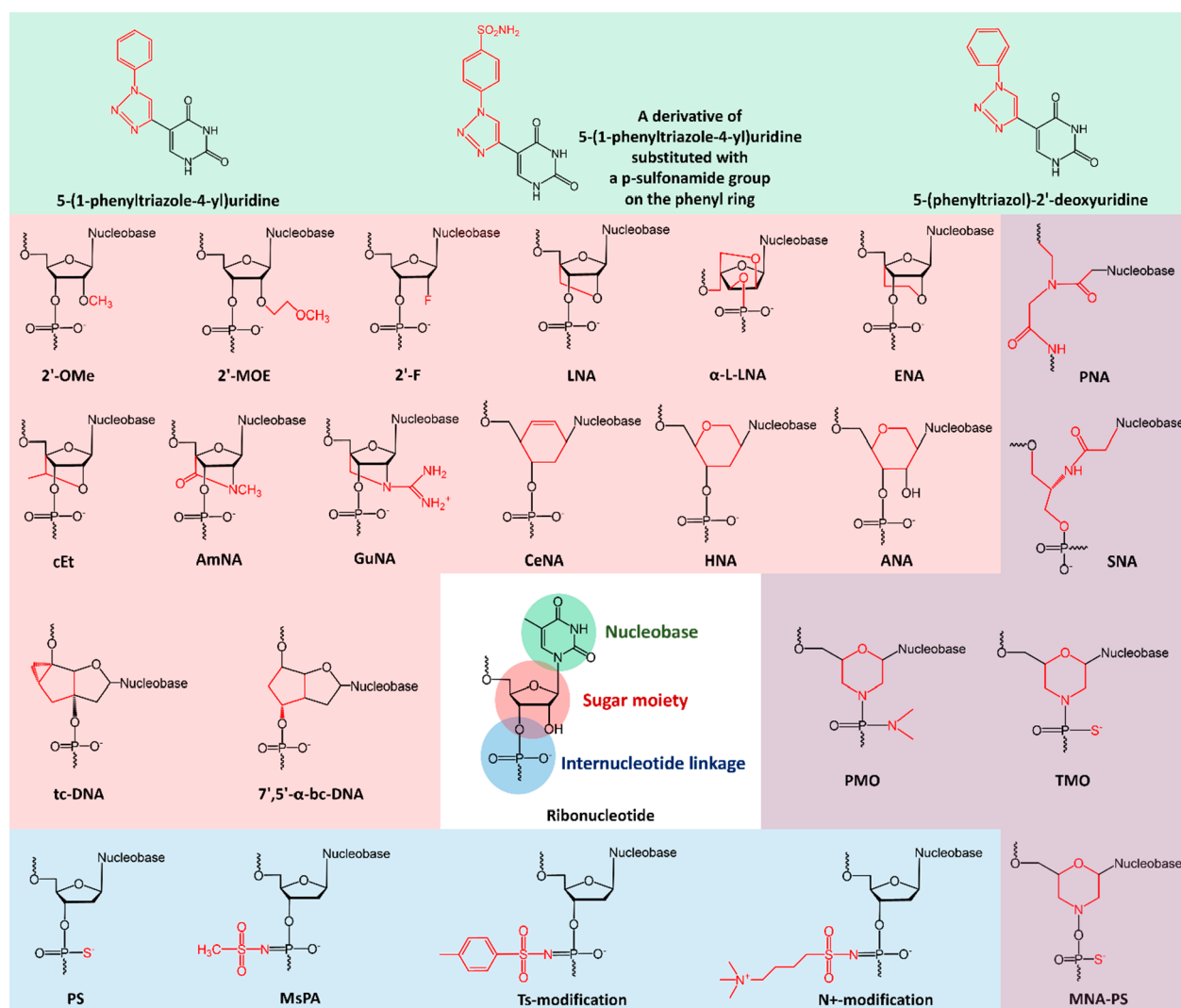## 3. SPLICE SWITCHING ANTISENSE OLIGONUCLEOTIDES: MECHANISM OF ACTIONS

Since the first report disseminated by Dominski and Kole in 1993,[10] splice switching ASO has become a truly promising tool for correcting genetic defects as well as promoting the ablation of pathogenic protein expression. Based upon the target site selection, the splice switching ASO mechanism of action generally falls into three main categories: exon skipping, exon inclusion, and intron retention (Figure 2). By specifically binding to a splice site in the pre-mRNA transcript, splice switching ASO can prevent the interaction between splicing factor and the pre-mRNA transcript, thereby manipulating the splicing event in a specific fashion.[11]

Splicing takes place inside the nucleus and is driven by a highly dynamic, multimolecular ribonucleoprotein (RNP) complex called spliceosome, which is sophisticated enough to accurately and flexibly recognize and select a splice site.[12] Splicing enhancers and silencers, proteins that bind to sequence motifs in the pre-mRNA exons and introns, play significant roles in regulating this process. Exon skipping ASOs are mainly designed to sterically block splicing enhancers to "skip" a splicing event of specific exons (Figure 2).[11] On the other hand, to "include" a desired exon or "retain" an intron, an ASO can be designed to target the splicing silencer (Figure 2). Skipping or including a partial or complete exon or intron generally causes reading frame shifting which eventually leads to two main outcomes: either restoring the reading frame to rescue a functional protein or disrupting the reading frame to create a premature stop codon in the subsequent exon or intron which results in no or nonfunctional protein expression. In addition, ASOs can also be designed to target other sites in the pre-mRNA transcript such as polyadenylation signal sequence, cleavage/polyadenylation specificity factor (CPSF), or cleavage stimulation factor (CstF).[13,14]

To further elaborate on this topic, it is worth mentioning the critical differences between splice switching and RNase H-dependent ASO therapies as the two approaches are distinctive in terms of their target sites, mechanisms of action, and ASO designs (Table 1).[15] One should consider these differences for their choice of ASO therapy toward exploring an appropriate treatment approach for a disease either via rescuing a functional protein expression or downregulating a pathogenic gene

Figure 2. Schematic illustration of the splice switching ASO mechanism of action.

Table 1. Critical Differences between Splice Switching and RNase H-Dependent ASO Therapies[a]

	Splice switching ASO	RNase H-dependent ASO
Target	Pre-mRNA	Mature mRNA
Site of action	Cell nucleus	Cytoplasm
Mechanism of action (where most active)	Steric blocking of splicing factors results in either exon skipping, or exon inclusion, or intron retention	Enzymatic degradation of the mature RNA via recruitment of RNase-H1 enzyme by RNA-DNA duplex
Application	Rescuing functional protein expression or downregulating pathogenic protein expression	Downregulating pathogenic protein expression
ASO design modality	Fully modified ASO	Gapmer ASO

[a]The ASO design illustration is based upon a 20-mer ASO synthesized on a phosphorothioate (PS) backbone. For gapmer ASO: 5−10−5 design is shown. Blue circle: chemically modified nucleic acid monomers. Red circle: DNA monomers.

expression. The former stays true for DMD and SMA where both diseases are characterized by the loss of functional protein caused by genetic mutations.[15] In these cases, employing splice switching ASOs to bypass a nonsense mutation (DMD) or including a functional coding exon (SMA) is so far the only feasible approach to rescue dystrophin or survival motor neuron (SMN) protein that would benefit patients.

4. MODIFIED NUCLEIC ACID ANALOGUES FOR SPLICE SWITCHING APPLICATIONS

Therapeutic ASOs are typically synthesized by using one or more forms of modified nucleic acid analogues. Selection and optimization of ASO chemistry plays a crucial role in the success

of ASO drug development, as it can significantly affect the drug efficacy and safety profile.[15,16] Incorporation of modified nucleic acid analogues may offer superior therapeutic properties, such as increased target binding affinity, improved resistance to nuclease degradation, and low immunogenicity. In the last two decades, progress in the synthesis and purification techniques has allowed more complex designs of ASO to be synthesized with decent yield and quality. These advances in nucleic acid technology have contributed to remarkable progress in synthetic RNA therapeutic development. Modified nucleic acid can be classified based on the site of modification: internucleotide linkage, sugar moiety, and nucleobase (Figure 3). Due to the limited scope of this review, we only discuss the chemical modifications that have

Figure 3. Modified nucleic acid analogues for splice switching applications. 2′-OMe, 2′-*O*-methyl; 2′-MOE, 2′-*O*-methoxyethyl; 2′-F, 2′-fluoro; LNA, locked nucleic acid; ENA, 2′-*O*,4′-*C*-ethylene-bridged nucleic acid; cEt, 2′,4′-constrained 2′-*O*-ethyl; AmNA, amido-bridged nucleic acid; GuNA, guanidine-bridged nucleic acid; CeNA, cyclohexenyl nucleic acid; HNA, anhydrohexitol nucleic acid; ANA, altritol nucleic acid; tc-DNA, tricyclo-DNA; 7′,5′-*α*-bc-DNA, 7′,5′-alpha-bicyclo-DNA; PNA, peptide nucleic acid; SNA, serinol nucleic acid; PMO, phosphorodiamidate morpholino oligomer; TMO, thiomorpholino oligonucleotide; MNA, morpholino nucleic acid; PS, phosphorothioate; MsPA, mesyl phosphoramidate; Ts-modification, *p*-toluenesulfonyl phosphoramidate; N+-modification, 4-(trimethylammonio)butylsulfonyl phosphoramidate.

been reported for splice switching applications along with the key examples of each modification.

4.1. Internucleotide Linkage Modifications. Toward improving the stability and bioavailability of splice switching ASOs against serum exo- and endonucleases, it is critical to modify the highly vulnerable phosphodiester backbone. The most common form of internucleotide linkage modification involves the replacement of the nonbridging oxygen atom with a sulfur (phosphorothioate, PS) (Figure 3). In 1993, Kole and colleagues demonstrated the first evidence of using a PS-modified ASO for splice switching application.[10] The study describes the use of two 18-mer 2′-*O*-methyl (2′-OMe)-PS ASOs to correct splicing defects in the human *β*-globin pre-mRNA transcript. Soon after, Wilton et al. reported the rescue of a shorter but functional dystrophin protein via deletion of exon 23 in the mouse dystrophin transcript using 2′-OMe-PS ASOs.[17] This fundamental experiment later led to the approval of three ASO drugs for DMD.

It is worth noting that among the five approved splice switching ASO drugs all DMD drugs utilize phosphorodiamidate morpholino oligomer (PMO) chemistry. This is a distinct class of nucleic acid analogues that possess a phosphorodiamidate linkage instead of a phosphodiester backbone. PMO has been widely used in splice switching applications with a proven excellent safety profile.[18] Nonetheless, tremendous effort has been focused on improving the PMO synthesis efficiency while reducing the cost of production. This is mainly due to two fundamental aspects. First, chemical synthesis of PMO cannot be done via the standard solid phase methodology. This has been a major challenge in synthesizing PMO on a large scale, which in turn contributes to the increase in cost of manufacturing. Second, PMO is considered to be a hard-to-transfect molecule due to its neutral charge nature that prevents its complexation with commercially available transfection reagents. This has become a main barrier for fast *in vitro* evaluation of PMO. To potentially overcome these limitations, various modifications of this backbone have been explored.

Chen et al. reported the synthesis of morpholino nucleic acid (MNA) uridine monomer and the evaluation of an MNA/2′-OMe-PS mixmer ASO for its exon skipping efficacy in primary *mdx* myotubes.[19] The results indicated that the mixmer ASO performed on-par with the fully 2′-OMe-PS modified ASO. In another study exploring mesyl phosphoramidate oligonucleotides as potential splice switching agents, Hammond et al. described the use of 2′-OMe and 2′-*O*-methoxyethyl (2′-MOE) oligonucleotides with either a methanesulfonyl (mesyl, *μ*) or 1-butanesulfonyl (busyl, *β*) phosphoramidate backbone for exon inclusion in SMA patient-derived fibroblasts.[20] The results revealed that there is no significant difference in terms of splice switching activity between the mesyl ASOs and their corresponding controls. In an effort to investigate novel internucleotide linkage modification, Filichev and co-workers reported the synthesis and evaluation of 2′-OMe ASO incorporated with 4-(trimethylammonio)butylsulfonyl or tosyl phosphoramidates (N+- and Ts-modifications, respectively) in a cellular model of DMD.[21] Interestingly, the exon skipping efficacy was much lower in the case of N+-modified ASO, while the Ts-modified ASO showed a similar level of exon skipping compared with the control 2′-OMe-PS ASO.

Peptide nucleic acid (PNA) represents another class of backbone-modified nucleic acid analogues, where the sugar phosphate backbone is replaced with neutral amide linkages. PNA possesses tremendously high binding affinity to a complementary target, while showing complete resistance to nuclease degradation due to its nonconventional backbone.[22,23] Similar to PMO, PNA has been explored for exon skipping in DMD. In such a study, Yin et al. examined the effects of PNA ASO and PNA conjugated with cell penetrating peptide (CPP) *in vitro* and *in vivo*.[24] The study outcome indicated that both the PNA and CPP-conjugated PNA efficiently induced exon skipping in cultured *mdx* mouse myoblasts. Furthermore, a significant number of dystrophin-positive fibers was observed after a single intramuscular injection in *mdx* mice.[24] In a comparison study using the same cellular model system, Veedu and colleagues described the synthesis of an ASO fully modified with serinol nucleic acid (SNA) and its efficacy in inducing exon skipping against PNA and 2′-OMe-PS control ASOs.[25] Notably, although structurally related to PNA, the SNA ASO could effectively induce exon skipping, while the PNA ASO failed to induce any meaningful amount of the exon skipped product. In addition, SNA ASO also showed very high stability against exonuclease degradation.[25] In another study toward inhibiting the expression of Her-2 receptor, Pankratova et al. reported the evaluation of PNA ASOs directed against intron−exon junctions of the erbB-2 transcript in cultured SK-BR-3 and HeLa cells.[26] The results suggested that exon 19 skipping could be achieved at the mRNA level, although the shorter form of protein could not be detected in Western blot due to various reasons.

More recently, stemming from the fundamental synthesis work by Caruthers and colleagues,[27,28] Le et al. demonstrated for the first time the use of a newly developed and unique class of nucleic acid analogue called thiomorpholino oligonucleotide (TMO) for splice switching application.[29] This robust class of therapeutic chemistry showed efficient internalization into the nuclei of mouse *mdx* myotubes and more importantly induced excellent exon skipping compared with a conventional PMO control and other widely used analogues such as 2′-OMe and 2′-MOE. Remarkably, TMO ASO performed exceptionally well at lower range of molar concentration (5–20 nM), making it a promising analogue for future design of splice switching ASO

therapeutics with a better safety profile and improved therapeutic efficacy.[29]

4.2. Sugar Moiety Modifications. The sugar moiety of a nucleotide is considered a "hotspot" for chemical modification. A large number of nucleotide analogues with modified sugar rings have been reported, some of which have been applied for synthesizing splice switching ASOs (Figure 3). Among these, 2′-OMe is undoubtedly the most widely used chemistry of choice. Notably, a 20-mer 2′-OMe-PS ASO named drisapersen (previously known as PRO-051) which is capable of inducing DMD exon 51 skipping was identified by Aartsma-Rus and co-workers[30] and tested in patients with DMD. However, drisapersen was rejected by the FDA due to unsatisfactory safety profile and lack of efficacy.[31,32] Despite being inappropriate for clinical usage, 2′-OMe is ideal for initial screening of ASO sequences *in vitro*, owing to its low synthesis cost. For instance, according to the guidelines of splice switching ASO development in Wilton and Fletcher's molecular therapy lab, multiple target specific 2′-OMe-PS ASO candidates are screened, leading to the identification of the best performing sequence, which will be subsequently synthesized as a PMO oligomer for further studies.[33] On the other hand, although none of the 2′-OMe modified ASOs have reached the clinic, another type of 2′-alkyl modification, 2′-MOE, has exhibited improved drug-like properties, including increased target binding affinity and nuclease resistance, and considerably less toxicity compared to the 2′-OMe chemistry.[34] As a result, FDA has approved Spinraza, a fully 2′-MOE-PS splice switching drug for the therapy of SMA in 2016.[35] 2′-MOE-PS is so far still the only 2′-alkyl modification used in FDA approved splice switching ASO on the market. Nevertheless, TMO chemistry has been considered a promising alternative to 2′-MOE-PS modification, and as mentioned earlier, TMO induces efficient splice switching at lower concentrations,[29] thus potentially reducing side effects by lowering the dosage.

In addition to the 2′-alkyl analogues, 2′-fluoro (2′-F) has emerged as a special 2′ modification due to its unique exon skipping capability. Rigo et al. discovered that, when an ASO sequence capable of inducing *SMN2* exon 7 inclusion was chemically modified by 2′-F, the ASO induced exon 7 exclusion instead of inclusion, which was opposed to its 2′-MOE counterpart.[36] The authors also demonstrated that the unexpected exon skipping capability is due to the recruitment of a protein called interleukin enhancer-binding factor 2 and 3 complexes (ILF2/3) by the 2′-F ASO/pre-mRNA duplex. This intriguing observation indicates that ASO mediated splice switching is dependent not only on its sequence but also on its chemistry. This finding suggests that 2′-F modified splice switching ASO could be utilized solely for exon skipping purpose. In other words, 2′-F modified ASO may be useful for treating DMD rather than SMA, as exon skipping is the desired therapeutic strategy for DMD. To this end, Jirka et al. examined the exon skipping effect of 2′-F-PS ASOs using DMD as a disease model.[37] This study demonstrated that 2′-F-PS ASOs possessed enhanced exon skipping *in vitro* over that of their 2′-OMe-PS counterparts. However, as *in vivo* toxicity was also identified in the study, the safety profile of 2′-F modified ASOs needs to be improved prior to clinical application. Later, Chen et al. synthesized chimeric 2′-F/2′-OMe and 2′-F/lock nucleic acid (LNA) ASOs and evaluated their ability to induce *Dmd* exon 23 skipping in *mdx* mouse myoblasts. This work not only confirms the increased exon skipping efficiency of 2′-F chemistry over 2′-OMe but also may provide a way for optimizing the

drug-like properties of 2′-F ASOs, through screening different mixmer designs.

Bridged nucleic acids are a class of nucleic acid analogues in which the 2′-oxygen of the sugar moiety is connected to the 4′-carbon, which constrains the flexibility of the sugar ring and "locks" the pucker of the sugar into RNA-like conformation.[38−43] The 2′,4′-bridge therefore confers these modified nucleic acids increased binding affinity to their target RNA sequences.[44] So far, several bridged nucleic acid analogues, including 2′-O,4′-C-ethylene-bridged nucleic acid (ENA), LNA, α-L-LNA, amido-bridged nucleic acid (AmNA), guanidine-bridged nucleic acid (GuNA), and 2′,4′-constrained 2′-O-ethyl (cEt) have been utilized as building blocks to construct splice switching ASOs (Figure 3).

In order to alleviate the toxicity associated with PS modification, Yagi et al. designed and evaluated multiple chimeric ENA/2′-OMe ASOs without a PS backbone for inducing exon 19 skipping in DMD primary muscle cells. The best performing candidate, a 5−21−5 ENA-2′-OMe-ENA gapmer-like ASO exhibited much stronger exon 19 skipping efficiency (by at least 40-fold) than the 31-mer fully DNA-PS ASO control, thus presenting a promising alternative to the conventional PS-modified candidate.[45] In a later *in vivo* study by Takaishi et al.,[46] a chimeric ENA/2′-OMe modified ASO named DS-5141b induced extremely higher *Dmd* exon 45 skipping in *mdx* mice, compared to its fully modified 2′-OMe and PMO counterparts, at a dose of 30 mg/kg, highlighting the improved target binding affinity of splice switching ASO containing ENA monomers over other modifications.

Among the bridged nucleic acids, LNA is the most commonly used analogue. LNA is considered a constrained analogue of the 2′-OMe chemistry.[41,42] Aartsma-Rus et al. reported a comparative study of uniformly modified splice switching ASOs in inducing DMD exon 46 skipping in patient myotubes.[47] Results showed that fully modified LNA ASO yielded a significantly higher level of exon 46 skipping (98%) compared to other chemistries (2′-OMe-PS: 75%, PMO: 5−6%, PNA: 0%). However, mismatch experiments showed that the LNA ASO also possessed lower sequence specificity than the fully 2′-OMe-PS control,[47] increasing the risk of off-target effects. Therefore, a fully modified LNA may not be the best choice for drug development. Later, Shimo et al. reported the evaluation of a series of chimeric LNA-modified ASOs, including various designs of LNA/DNA and LNA/DNA/2′-OMe mixmers, in inducing DMD exon 58 skipping *in vitro*.[48] According to their results, the mixmers exhibited an much stronger exon 58 skipping ability than the fully modified LNA controls, suggesting that chimeric LNA modifications may be a preferred option for optimizing the efficacy of splice switching ASO. Further, Le et al. synthesized a series of systematically truncated chimeric LNA/2′-OMe ASOs on a PS backbone and tested their ability in inducing *Dmd* exon 23 skipping in *mdx* myotubes.[49] According to the results, mixmer ASOs with reduced length (16- to 14-mer) could induce exon 23 skipping more efficiently than their corresponding truncated fully 2′-OMe-PS controls.[49] Therefore, the design of short LNA-modified chimeric ASOs could be a useful strategy to reduce the cost as well as potential toxicities of splice switching drugs while not compromising the efficacy. Recently, Raguraman et al. have reported the exploration of α-L-LNA (a stereoisomeric analogue of LNA) modified 2′-OMe-PS chimeric ASOs in causing exon 23 skipping, using the same *mdx* mouse model system. Results demonstrated that, compared to the fully 2′-OMe-PS controls, 18-mer and 16-mer α-L-LNA/2′-

OMe-PS mixmers displayed slightly better exon skipping efficiency.[50] In addition, other LNA analogues including AmNA, GuNA, and cEt also have been recently reported in constructing chimeric ASOs for inducing efficient splice switching,[51,52] thereby expanding the scope of optimizing bridged nucleic acid modified splice modulating ASOs by incorporating novel analogues.

In addition to the bridged nucleic acids, tricyclo-DNA (tc-DNA) and 7′,5′-alpha-bicyclo-DNA (7′,5′-α-bc-DNA) are also conformationally constrained nucleic acid analogues that display an increased target RNA binding affinity. Renneberg et al. investigated the antisense effect of uniformly modified tc-DNA ASOs in HeLa cells expressing a mutant β-globin gene.[53] A 17-mer fully tc-DNA modified ASO successfully corrected the aberrant splicing of the mutant β-globin pre-mRNA in the nanomolar range by skipping the retained intron sequence (pseudoexon). Notably, the pseudoexon skipping induced by the tc-DNA ASO was 100-fold higher than its fully 2′-OMe-PS control with an identical sequence and length. Later, Ittig et al. reported a comparative study of tc-DNA and LNA modified splice switching AOs in inducing exon 4 skipping of *PPIA* pre-mRNA in HeLa cells.[54] It was found that the tc-DNA modified ASOs achieved an exon skipping level higher than that of their corresponding LNA counterparts, suggesting that tc-DNA could be superior to LNA in inducing splice switching *in vitro*. Further, Goyenvalle et al. reported that systemically administered tc-DNA modified *Dmd* exon 23 skipping ASOs could rescue dystrophin production in all disease-affected tissues in *mdx* mice, including the muscles, heart, and even central nerve system.[55] The authors also revealed the possible mechanism underlying the more efficient *in vivo* delivery of tc-DNA ASOs over their 2′-OMe and PMO counterparts by demonstrating self-association of tc-DNA ASOs into nanoparticles. This discovery makes tc-DNA an attractive chemical modification for exon skipping agents for patients with a disease requiring whole-body treatment, such as DMD. Moreover, Relizani et al. also demonstrated the dystrophin recovery efficacy of tc-DNA modified ASOs in *mdx* mice.[56] Importantly, their study showed that the tc-DNA treatment had an encouraging *in vivo* safety profile, which might support a clinical application for tc-DNA based oligomers. In addition, Robin et al. systemically delivered tc-DNA modified ASOs into a SMA mouse, leading to increased exon 7 retention in *SMN2* mRNA in the central nerve system,[57] indicating that tc-DNA modified ASOs could efficiently cross the blood−brain barrier. This study further supports the potential of tc-DNA oligomers as promising systemic therapy for SMA. Most recently, Evéquoz and co-workers reported the synthesis of 7′,5′-α-bc-DNA and evaluated the activity of splice switching ASOs with this novel modification in both DMD *mdx* mice and SMA mice.[58] Their data showed that 7′,5′-α-bc-DNA ASOs exhibited good splice modulating activity (DMD: exon 23 skipping; SMA: exon 7 inclusion) in these *in vivo* models. However, an improved method to deliver 7′,5′-α-bc-DNA ASOs to muscular tissues needs to be developed.

Additionally, Le et al. have reported the synthesis of 20-mer chimeric 2′-OMe-PS ASOs with six monomers of anhydrohexitol nucleic acid (HNA), cyclohexenyl nucleic acid (CeNA), or altritol nucleic acid (ANA) incorporated in the sequences.[59] These mixmer ASOs were transfected into *mdx* mouse myotubes and exhibited an efficient *Dmd* exon 23 skipping ability. Furthermore, ASOs with these modifications were more resistant to nuclease degradation and not toxic in comparison to the uniformly 2′-OMe-PS control.

Table 2. Summary of Splice Switching ASOs That Have Been Clinically Investigated and/or Approved by the FDA for Market Entry

No.	ASO name/company or foundation name	ASO chemistry and sequence (5′-3′)	Disease	Target gene	ASO mechanism	Development stage	ref
1	Exondys 51/Sarepta Therapeutics	30mer PMO CTCCAACATCAAGGAAGATGGCATTTCTAG	Duchenne muscular dystrophy (DMD)	Dystrophin (DMD)	Exon 51 skipping	FDA approval	3–8, 64–66
2	Spinraza/Biogen-IONIS Pharmaceuticals	18mer 2′-MOE-PS TCACTTTCATAATGCTGG	Spinal muscular atrophy (SMA)	Survival of motor neuron 2 (SMN2)	Exon 7 inclusion	FDA approval	67–74
3	Vyondys 53/Sarepta Therapeutics	25mer PMO GTTGCCTCCGGTTCTGAAGGTGTTC	DMD	DMD	Exon 53 skipping	FDA approval	75–78
4	Viltepso/Nippon Shinyaku	21mer PMO CCTCCGGTTCTGAAGGTGTTC	DMD	DMD	Exon 53 skipping	FDA approval	79–86
5	Amondys 45/Sarepta Therapeutics	22mer PMO CAATGCCATCCTGGAGTTCCTG	DMD	DMD	Exon 45 skipping	FDA approval	87–89
6	Milasen/Mila's Miracle Foundation - Boston Children's Hospital	22mer 2′-MOE-PS AATGTTAGTGCTTGTTGAGGGC	Batten disease	Neuronal Ceroid Lipofuscinosis 7 (CLN7)	Pseudoexon skipping	"N-of-1" clinical trial/ investigational drug	90
7	Atipeksen (AT-008)/Boston Children's Hospital	22mer 2′-MOE-PS ATATAAGCATCACAAAGTACCT	Ataxia telangiectasia	Ataxia telangiectasia mutated kinase (ATM)	Pseudoexon skipping	"N-of-1" clinical trial/ investigational drug	63, 91
8	Kyndrisa/BioMarin Pharmaceutical	20mer 2′-OMe-PS UCAAGGAAGAUGGCAUUUCU	DMD	DMD	Exon 51 skipping	Phase III	92–96
9	Sepofarsen (QR-110)/ProQR Therapeutics	17mer 2′-OMe-PS GGUGGAUCACGAGUUCA	Leber's congenital amaurosis 10	Centrosomal protein 290 (CEP290)	Pseudoexon skipping	Phase II/III	97–100
10	Ultevursen (QR-421a)/ProQR Therapeutics	21mer 2′-MOE-PS AGCUUCGGAGAAAUUUAAAUC	Usher syndrome	Usherin (USH2A)	Exon 13 skipping	Phase II/III	99
11	Vesleteplirsen (SRP-5051)/Sarepta Therapeutics	30mer PPMO CTCCAACATCAAGGAAGATGGCATTTCTAG	DMD	DMD	Exon 51 skipping	Phase II	101
12	Zorevunersen (STK-001)/ Stoke Therapeutics	18mer 2′-MOE-PS AGUUGGAGCAAGAUUAUC	Dravet syndrome	Sodium voltage-gated channel alpha subunit 1 (SCN1A)	Exon inclusion	Phase I/IIA	102
13	SPL84/SpliSense	19mer 2′-MOE-PS CUGCAACAGAUGGAAGACU	Cystic fibrosis	Cystic fibrosis transmembrane conductance regulator (CFTR)	Pseudoexon skipping	Phase I	103
14	VP-001/PYC Therapeutics	25mer CPP-PMO ATGGTCTTGGGCTCCTCGTGCCTCT	Retinitis pigmentosa 11	CCR4-NOT transcription complex subunit 3 (CNOT3)	Exon 17 skipping	Phase I	101

4.3. Nucleobase Modifications. Despite continuous efforts in exploring novel nucleobase modifications, there has been little success in improving the ASO therapeutic properties with these modifications. This is mainly due to the vital role of the nucleobase in the interaction with the complementary target through Watson−Crick base pairing. For splice switching applications, there are two reports that have been reported in the literature. The first attempt was the synthesis of two 2′-OMe-PS ASOs incorporated with 5-(phenyltriazol)-2′-deoxy-uridine monomers by Veedu and colleagues.[60] The study suggested that the $\pi-\pi$ stacking of the aromatic substituents improves duplex stability against a complementary RNA. More importantly, both nucleobase-modified ASOs induced efficient *Dmd* exon 23 skipping in *mdx* mouse myoblasts in a dose dependent manner, while showing less *in vitro* toxicity compared with the unmodified 2′-OMe-PS control ASO. Following this initial work, the study has been expanded to accommodate the synthesis of 2′-*O*-methyl-5-(1-phenyltriazole-4-yl)uridine as well as sulfonamide substituted analogues.[61] The results indicated that all tested ASOs enhanced the binding affinity against a complementary RNA target. Furthermore, all ASOs induced equivalent exon skipping when compared with the 2′-OMe-PS control, demonstrating the potential for incorporating these nucleobase modifications in ASO design for splice switching applications.

5. CLINICALLY APPROVED SPLICE SWITCHING ASO DRUGS

To date, five splice switching ASOs drugs have been permitted by the FDA for market entry to benefit the treatment of patients with DMD and SMA. In addition, Milasen is the first drug permitted by FDA for N-of-1 (personalized) clinical study. N-of-1 trials are designed for patients with ultrarare diseases (<1 in 100,000 individuals) which in some cases might just be suitable for a single individual.[62,63] In addition, a number of splice switching ASOs have been evaluated in human clinical trials targeting various conditions. We believe that these examples not only demonstrate the feasibility of splice switching ASO as an efficacious individualized strategy for treating rare diseases and correcting rare mutations but also highlight the importance of appropriate chemical modification in conferring ASO drugs sufficient efficacy and safety. A summary of these ASO drugs with supporting references is listed in Table 2.

6. CONCLUSION

Splice switching ASOs are a distinct class of therapeutic oligonucleotides that, instead of degrading mature mRNA in the cell cytoplasm, can bind to specific motifs in the pre-mRNA and correct aberrant splicing (through exon or intron exclusion or inclusion) to restore the reading frame and functional protein expression. Although more than 20 nucleotide analogues have been utilized as building blocks of splice switching ASO (Figure 3), to improve its efficacy, safety, and stability, PMO and 2′-MOE-PS are so far the only chemistries that have been adopted in clinically approved splice switching drugs. Toward expanding the scope of splice switching ASOs, we leverage this opportunity to promote further research to explore additional nucleic acid analogues in designing ASOs for splice switching applications. We believe that these modified nucleic acid analogues could further advance the existing splice switching ASO technology to benefit the therapeutic development of ASOs toward the treatment of various genetic and acquired diseases.

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