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A Chemical View of Oligonucleotides for Exon Skipping and Related Drug Applications

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Introduction

Exon skipping is one of a number of "steric blocking" applications of oligonucleotides (ONs) and their analogues that in recent years have undergone a renaissance and which have led to new therapeutic opportunities (Kurreck, 2008; Kole et al., 2012; Lightfoot and Hall, 2012). Whereas most standard antisense applications involve cleavage of the RNA by intracellular ribonuclease H (RNase H) or by argonaute2 (Ago2) enzymes, exon skipping by steric blocking merely requires binding of the ON in an antisense orientation to an RNA target and blockage of the important biological function of splicing. In exon skipping, as well as the similar splice-switching activity of exon inclusion, the target is pre-mRNA located in the cell nucleus. Thus, the ON must enter the cell nucleus in sufficient quantity to be in excess over the target pre-mRNA, bind to it strongly, and interfere with the splicing machinery. For drug use, the ON must exhibit additionally a number of other important properties such as good biodistribution to the target organ(s), lack of immune recognition effects, and a good therapeutic window between effective and toxic doses. Further, ON synthesis must be routinely achievable on varying scales. Such simultaneous requirements have taxed the ingenuity of chemists in ON design.

It is necessary in all therapeutic applications of ONs to include modifications to the ON backbone and/or sugar component to protect against nuclease degradation. Interestingly, the very first analogue to find use therapeutically was the phosphorothioate linkage (Matsukura et al., 1987), which is still utilized in most ONs in current clinical trials. Further modifications have followed in subsequent years that have improved stability to nucleases, or even result in the ON being nuclease inert. The best ON analogues both enhance RNA binding strength, compared with an unmodified ON, and reduce nuclease resistance. Increased binding strength has allowed shorter ONs to be used in some cases, thus reducing the chance of binding to an incorrect RNA site through partial sequence complementarity. For example, in the case of ONs containing locked nucleic acids (LNAs), lengths of 12 to 15 are usually used (Lanford et al., 2010; Straarup et al., 2010) and even shorter in the case of all-LNA ONs (Obad et al., 2011). More commonly, lengths of ON analogue synthesized for exon skipping and other steric blocking applications are 14–30 residues.

ONs are usually taken up into cells *via* endocytosis and then they must be released sufficiently from endosomal compartments into the cytosol. The cell nucleus is not thought to be a barrier for ONs once released from endosomes. However, cell entry behavior in culture is not a good predictor of *in vivo* activity. Thus for a particular exon skipping application, once *in vivo* delivered, the ON must be able to reach the required cell types. Leading applications for exon skipping and exon inclusion have been neuromuscular diseases such as Duchenne muscular dystrophy (DMD) and spinal muscular atrophy (SMA). In both these cases, as well as in targeting of triplet repeats in myotonic dystrophy, the ON must be able to penetrate muscles of various types. However, it has proved very hard to date to find ONs or derivatives that are, in addition to skeletal muscle, as effective in heart and/or brain. Further, parameters such as biodistribution and organ accumulation, toxicology, and pharmacology in mice or rats must each be assessed, thus requiring at least multimilligram quantities of the ON to be readily obtainable. For such studies in animals and for clinical trials, the ON must be synthesized easily and cost effectively on gram to kilo scale under good manufacturing practice (GMP). Only a very small number of splice switching ON analogues have advanced sufficiently for use in clinical trials (Muntoni and Wood, 2011; Kole et al., 2012; van Deutekom et al., 2013) but some newer analogue types appear promising. The chemistry types that have been under study for exon skipping are depicted in Fig. 1 and the applications for which they have been used are shown in Table 1.

Negatively Charged Oligonucleotide Analogues

A 31-mer oligodeoxynucleotide phosphorothioate (DNA-PS, Fig. 1A) was the first ON analogue to make it to a clinical trial in a single patient for exon skipping in DMD (Takeshima et al., 2006). A disadvantage of DNA-PS is that when bound to RNA it can induce undesirable cleavage of the RNA by cellular RNase H. Very few other types of negatively charged ON analogue that do not induce RNase H cleavage have been reported to be effective in exon skipping or exon inclusion applications (Fig. 1A). In all cases, these ON analogues mimic RNA in their binding character when forming duplex structures with an RNA target, such that binding is tighter than for DNA analogues. The internucleotide linkage is invariably phosphorothioate (PS), which also allows high protein binding

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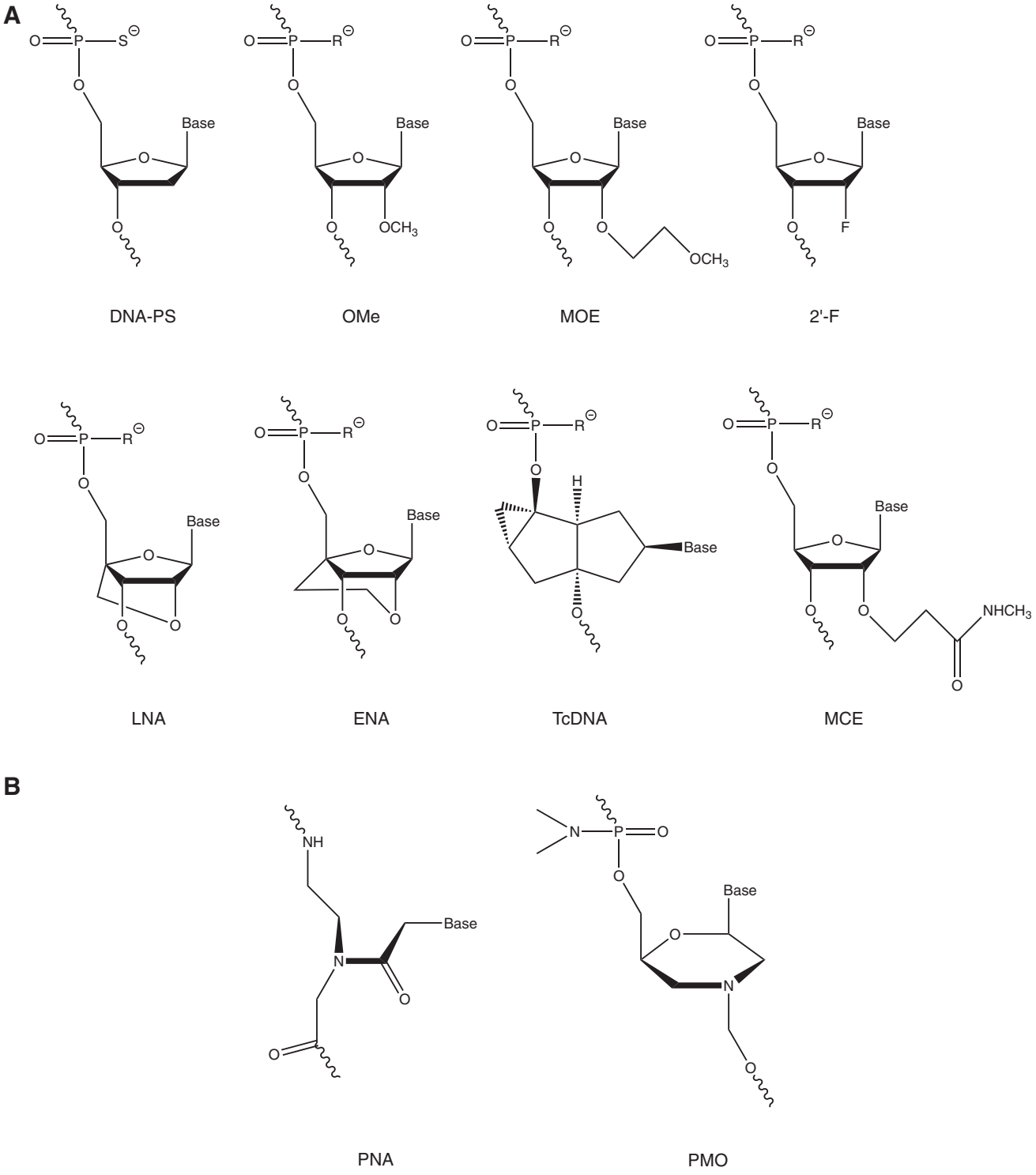

FIG. 1. Structures of repeating units of analogues of oligonucleotides (ONs) used in exon skipping and related applications. (R = O or S) **(A)** Charge-negative ONs. **(B)** Charge-neutral ONs.

in serum and increases circulation time. All of the ON analogues show high resistance to serum and cellular nucleases.

2′-O-methylphosphorothioate

Particularly ubiquitous in exon skipping studies have been 2′-ribose-modified PS ON analogues, the foremost of which is 2′-*O*-methylphosphorothioate (OMe-PS) (Fig. 1A; Table 1). Exon skipping and functional dystrophin production was demonstrated after intravenous delivery into *mdx* mice (a standard DMD mouse model) just a few years ago (Lu et al., 2005; Fletcher et al., 2006). OMe-PS quickly became the leading negatively charged ON type in exon skipping, probably because this chemistry is well established for use

Table 1. Oligonucleotide Types and Their Exon Skipping, Exon Inclusion, and Related Applications

*Oligonucleotide**	*Cellular target*	*ES*	*EI*	*Other*	*Therapeutic application*	*Reference*
Negatively charged ON Analogues						
DNA-PS	pre-mRNA	x			DMD	Takeshima et al., 2006
OMe	pre-mRNA	x			DMD	Fletcher et al., 2006; Lu et al., 2005;
		x				Goemans et al., 2011;
	pre-mRNA	x			Dystrophic epidermolysis Bullosa	Goto et al., 2006
	DMPK-mRNA			x	Myotonic dystrophy type 1	González-Barrigs et al., 2013; Mulders et al., 2009
	pre-mRNA	x			Lowering LDL cholesterol	Disterer et al., 2013
	pre-mRNA		x		SMA	Osman et al., 2012
MOE	pre-mRNA		x		SMA	Hua et al., 2010; Passini et al., 2011
MOE gapmers	DMPK-mRNA			x	Myotonic dystrophy type 1	Wheeler et al., 2012
LNA	pre-mRNA	x			DMD	Aartsma-Rus et al., 2004
	pre-mRNA	x			Rheumatoid arthritis	Graziewicz et al., 2008
	pre-mRNA	x			Chronic inflammatory disease	Yilmaz-Elis et al., 2013
TcDNA	pre mRNA	x			DMD	Goyenvalle et al., 2013
MCE	pre mRNA	x			DMD	Yamada et al., 2011
Charge-neutral ON analogues						
PNA	pre-mRNA	x			DMD	Yin et al., 2008a; Yin et al., 2010
PMO	pre-mRNA	x			DMD	For reviews see Moulton and Moulton, 2008;
		x				Moulton and Moulton, 2010
	pre-mRNA		x		SMA	Porensky et al., 2012; Zhou et al., 2013
	DMPK-mRNA			x	Myotonic dystrophy type 1	Wheeler et al., 2009; Koebis et al., 2013
Peptide or polymer ON delivery						
PEG-PEI with OMe-PS ONs	pre-mRNA	x			DMD	Sirsi et al., 2008; Ferlini et al., 2010;
		x				Bassi et al., 2012
PEG-PEI with PMO	pre-mRNA	x			DMD	Wang et al., 2013
Pip2b-PNA	pre-mRNA	x			DMD	Ivanova et al., 2008
B/R-peptide-PMO	pre-mRNA	x			DMD	Jearawiriyapaisarn et al., 2008; Wu et al., 2008;
		x				Yin et al., 2008b; Wu et al., 2012
	DMPK-mRNA			x	Myotonic dystrophy type 1	Leger et al., 2013
Pip5-PMO, Pip6-PMO	pre-mRNA	x			DMD	Yin et al., 2011; Betts et al., 2012
Vivo-morpholinos	pre-mRNA	x			DMD	Wu et al., 2009; Widrick et al., 2011; Yokota et al., 2012; Aoki et al., 2012

*For chemical structures of oligonucleotide (ON) types, see Fig. 1.

DMD, Duchenne muscular dystrophy; DMPK, dystrophia myotonica protein kinase; EI, exon inclusion; ES, exon skipping; LDL, low-density lipoprotein; LNA, locked nucleic acid; MCE, 2′-*O*-[2-(*N*-methylcarbamoyl)ethyl]uridine; MOE, 2′-O-methoxyethyl; OMe, 2′-O-methyl; PEG–PEI, polyethylene glycol–poly(ethylene imine); Pip, PMO internalization peptides; PMO, phosphorodiamidate morpholino; PNA, peptide nucleic acids; PS, phosphorothioate; SMA, spinal muscular atrophy; TcDNA, tricycloDNA.

in vivo and for scale up without onerous license requirements, and OMe-PS ONs appear also to have a relatively good safety profile. Long-term studies have shown that OMe-PS ONs can be administered safely at 200 mg/kg in various DMD mouse models of differing severity for up to 6 months (Tanganyika-de-Winter et al., 2012). An OMe-PS ON (now named Drisapersen) has been taken to clinical trials for exon 51 skipping in DMD patients (Goemans et al., 2011), showing effectiveness in extended treatment in the "6 minute walk test" for ambulatory patients, and phase 3 clinical trial data in Europe are expected imminently. ONs for skipping other exons are also now beginning clinical trials (www.prosensa.eu).

OMe-PS ON effectiveness has been reported for other genetic diseases such as exon skipping in the collagen gene in a rat model of dystrophic Epidermolysis Bullosa (Goto et al., 2006) and also for targeting a triplet repeat sequence in the mRNA of the dystrophia myotonica protein kinase (DMPK) in myotonic dystrophy type 1 (Mulders et al., 2009; González-Barriga et al., 2013). Exon skipping of *ApoB* (apolipoprotein B) pre-mRNA by OMe-PS ONs has been shown to reduce LDL cholesterol in *ApoB* transgenic mice (Disterer et al., 2013). In addition, OMe-PS ONs feature prominently in an approach for use of bifunctional RNAs to target the intronic silencer-N1 to cause exon inclusion, to increase survival motor neuron 1 (*SMN1*) pre-mRNA levels and to reduce disease severity in an animal model of SMA (Osman et al., 2012). This bifunctional steric block approach had first been proposed some years ago for this disease through cell culture studies (Skordis et al., 2003).

2′-O-methoxyethyl oligonucleotides

A 2′-*O*-methoxyethyl (MOE) ON (Fig. 1A; Table 1) is a 2′-analogue class introduced some years ago by Isis Pharmaceuticals. MOE-PS ONs were assessed as steric blocking agents first in a number of splice-switching models, such as redirection of pre-mRNA splicing of murine interleukin-5 receptor alpha chain (IL5R-α) (Karras et al., 2000), MyD88 (Vickers et al., 2006) and enhanced green fluorescent protein (EGFP) (Sazani et al., 2001). Isis Pharmaceuticals has collaborated with a number of academic groups, for example with the Krainer laboratory for exon inclusion of the SMN2 gene *in vivo* as a potential approach for treatment of SMA (Hua et al., 2010). MOE-PS appears to be better than OMe-PS (Williams et al., 2009) in this case, since it was reported that an 18-mer MOE-PS ON (ASO-10–27) was more effective after intracerebroventricular (i.c.v.) infusion into adult mice central nervous system (CNS) compared with an overlapping 20-mer OMe-PS ON, and there were fewer unwanted proinflammatory effects (Hua et al., 2010). Intrathecal infusion of ASO-10–27 ameliorated symptoms of severe SMA in cynomolgus monkeys (Passini et al., 2011) and phase 1b/2a clinical trials are now in progress (www.clinicaltrials.gov, ISIS-SMN_{Rx}).

MOE gapmers that employ an RNase H antisense mechanism are also effective *in vivo* for knockdown of toxic RNA and correction of myotonic dystrophy type 1 (Wheeler et al., 2012). Interestingly, the RNase H approach could prove more effective here than targeting of the expanded CUG repeated region in DMPK mRNA (González-Barriga et al., 2013) and further comparisons of the two approaches are to be expected. A recent report suggests that MOE-PS ONs are slightly more effective than OMe-PS ONs in an *mdx* mouse model of DMD (Yang et al., 2013).

Other negatively charged ON types

It is important to recognize in targeting pre-mRNA splicing that the type of 2′-modification matters when considering mechanism of action. This lesson has been brought home by the startling discovery that a PS ON composed entirely of supposedly steric blocking 2′-deoxy-2′-fluoronucleotides (2′-F, Fig. 1A), became recognized instead by the interleukin enhancer binding factors 2 and 3 (ILF2/3) to direct exon inclusion through activation of the splicing machinery (Rigo et al., 2012). Although in principle a fully 2′-F modified ON could be used in an exon skipping mode if the right pre-mRNA target site is found, 2′-F may perhaps find better use in modulating binding strength of ONs when used in mixmers with other analogue types (Davis et al., 2009).

A well-known analogue type, of which LNA (also known as bridged nucleic acids [BNA]) and ethylene-bridged nucleic acids (ENA) are the archetypes (Fig. 1A), is formed by cyclization of a 2′-alkyl substituent to the 4′-position resulting in "locking" the conformation of the sugar into that similar to that in RNA (Koshkin et al., 1998; Obika et al., 1999; Takagi et al., 2004). Inclusion of just a single LNA or ENA unit within an ON can result in an increase in the melting temperature (T_m) of 5°C or more. Some years ago, an all LNA ON was shown to be effective for exon skipping in cells from an exon 45-deleted DMD patient, but with reduced specificity, probably due to its high T_m with the RNA target (Aartsma-Rus et al., 2004). It is common now to mix LNA with DNA (Elayadi et al., 2002) or with an OMe backbone (Arzumanov et al., 2001) to modulate binding strength and temper possible *in vivo* toxic effects. In an EGFP splice-switching mouse model, a PS 15-mer 8LNA/7DNA approximately alternating mixmer showed much higher potency in liver, colon, and small intestine than an OMe ON (Roberts et al., 2006). A similar 15-mer LNA/DNA-PS ON was highly effective in splice-switching tumour necrosis factor-α pre-mRNA to induce the soluble form of the protein as a potential therapy for rheumatoid arthritis (Graziewicz et al., 2008). Only a modest increase over an all OMe-PS ON was seen for a 23-residue OMe/LNA-PS mixmer containing five LNA units used for exon-9 skipping of IL-1 receptor accessory protein as a potential strategy for treatment of chronic inflammatory diseases (Yilmaz-Elis et al., 2013). However, it is likely that a substantial survey of such mixmers of various lengths and compositions would be needed for optimization of activity in each indication and to minimize potential hepatotoxicity.

Quite surprisingly, the slightly larger 2′-4′ ringed ENA (Fig. 1A) has not been developed further following an initial report that a 21-mer chimeric ENA/OMe-PS ON showed 40 times the exon skipping activity compared to a 31-mer DNA/PS used originally in exon-19 skipping in myocyte culture from cells derived from a DMD patient (Yagi et al., 2004). Perhaps this is due to restricted availability of ENA. ENA would be predicted to have similar activity to LNA ONs but comparative *in vitro* and *in vivo* data are lacking. Other bicyclic derivatives such as 2′,4′-constrained-[*S*]-2′-*O*-ethyl (*S*-cEt) nucleotides have been developed by Isis Pharmaceuticals (Seth et al., 2009) as a rival to LNA, but so far do not seem to have featured in exon skipping or inclusion strategies.

The unusual tricycloDNA analogue (TcDNA, Fig. 1A) was first used in a steric block mode targeting HIV RNA a few years ago (Ivanova et al., 2007). The multistep syntheses required for monomer production hampered development until recently. In an RNase H gapmer mode with a central DNA core, TcDNA has shown better activity against the scavenger receptor B1 mRNA than MOE in the flanks of gapmer ONs in a range of tissue types in systemic treatment of mice (Murray et al., 2012). Now, fully TcDNA ONs are being evaluated for exon skipping and inclusion in models of neuromuscular diseases. For example, a recent conference abstract has suggested substantial levels of exon skipping activity have been obtained in the *mdx* mouse model of DMD and, more excitingly, restoration of dystrophin in heart muscle was achieved as well as detection of exon skipping in brain (Goyenvalle et al., 2013).

Whilst the use of chemically modified nucleotides that increase binding affinity of an ON for a target RNA (such as LNA or TcDNA) is the most often studied way of searching for steric blocking or splice switching ONs, a novel alternative approach involves attachment at or near the end of (for example) a OMe ON an *N,N*-diethyl-4-(4-nitronapthalene-1-ylazo)-phenylamine substituent (ZEN) that both increases binding affinity, presumably by an intercalative base interaction, as well as confers additional stability to exonucleases. ZEN-modified OMe ONs (without PS) have proved effective in inhibition of microRNA-122 after cellular transfection and appeared to be less toxic and equally effective as LNA/DNA-PS and LNA/OMe-PS ONs (Lennox et al., 2013). Such novel methods for boosting binding affinity for ON are now likely to be evaluated also in exon skipping and other steric blocking applications.

Although it is likely that a few other analogue types have been evaluated, the only report of a new 2′-analogue being tested in an *mdx* mouse model of DMD is that of a 30-mer OMe-PS ON in which the 6 uridine residues were substituted by 2′-*O*-[2-(*N*-methylcarbamoyl)ethyl]uridine (MCE, Fig. 1A). By intramuscular delivery, this MCE/OMe-PS ON showed a greater level of exon skipping than the parent OMe-PS ON (Yamada et al., 2011).

Charge-Neutral Peptide Nucleic Acids and Phosphorodiamidate Morpholino Oligonucleotides

Charge-neutral ON backbones feature strongly in exon skipping and exon inclusion applications. Peptide nucleic acids (PNA) developed in the laboratory of Nielsen (Egholm et al., 1992) are essentially nucleobase-functionalized derivatives of 2-aminoethylglycine (Fig. 1B). They are synthesized chemically by methods similar to peptide synthesis, and the PNA monomers are readily available commercially. Additional amino acids can be also be added (such as Lys) to maintain good water solubility. Cationic residues in particular help PNA to bind to cells and aid cell uptake. PNA exhibits strong and sequence specific binding to both RNA and DNA targets (Egholm et al., 1993).

A 15-mer PNA-Lys was as effective as a MOE-PS ON when electroporated into BCL_1 lymphoma cells in redirecting splicing of the IL5R-α pre-mRNA to give the soluble form (Karras et al., 2001). A PNA-Lys_4 18-mer was very effective in a number of organs, such as kidney, liver, heart, and lung, in a splice-switching EGFP mouse model after i.p. injection (Sazani et al., 2002). However, a PNA-Lys_4 14-mer was not effective in inducing exon skipping in exon 45-deleted patient myoblasts in the DMD model (Aartsma-Rus et al., 2004), perhaps because this PNA was too short for good binding to this target. By contrast, an electroporated 14-mer PNA-Lys_4 was very effective (and a PNA-Lys_8 even better) in redirecting splicing in a murine CD40 receptor model in BCL_1 cells as well as when incubated at high micromolar concentration in the absence of a transfection agent (Siwkowski et al., 2004). A 20-mer PNA was moderately effective in exon skipping and dystrophin production in the *mdx* mouse model of DMD following intramuscular injection into the tibialis anterior muscle (Yin et al., 2008a), but a longer 25-mer PNA was more effective under intravenous delivery, and doses up to 100 mg/kg were well tolerated (Yin et al., 2010).

In contrast to PNA, which is not yet available in GMP grade, phosphorodiamidate morpholino oligonucleotide (PMO) first described in the laboratory of Summerton (Summerton and Weller, 1997) (Fig. 1B) has become the uncharged ON analogue of choice for DMD exon skipping and inclusion in muscle and has also been used in many other therapeutic applications (Moulton and Moulton, 2008, 2010). Custom synthesis for researchers is available up to 30-mers and additional Lys residues are not needed because PMO is more water-soluble than PNA. Direct comparisons of PMO and OMe-PS chemistries in exon skipping in the *mdx* mouse (25-mers targeting exon 23 of the dystrophin pre-mRNA) showed that PMO generated higher exon skipping and dystrophin production by both intramuscular and systemic delivery than OMe-PS (Alter et al., 2006; Fletcher et al., 2006). However, for a range of human exons in humanized DMD mice, the effectiveness of exon skipping for OMe-PS and PMO ONs varied depending on the target exon sequence and on ON length (Heemskerk et al., 2009), suggesting that the *mdx* mouse exon 23 target is not an ideal comparator for human muscle effectiveness.

PMO has proven to be a remarkably safe drug as demonstrated at the maximum feasible repeated doses over 12 weeks of 320 mg/kg in cynomolgus monkeys (Sazani et al., 2011). However, establishment of good dystrophin production through exon skipping in heart in *mdx* mice required very high (up to 3000 mg/kg) dosing (Wu et al., 2010). The first systemic delivery results of human clinical trials of a 30-mer PMO (known as Eteplirsen) for exon 51 skipping in DMD patients showed some dystrophin production when delivered intravenously for 12 weeks at up to 20 mg/kg (Cirak et al., 2011). More recent results at 30 and 50 mg/kg have demonstrated higher levels of dystrophin and effectiveness in the 6-minute walk test in extension studies, but the number of patients is small (Mendell et al., 2013). A clinical trial using PMO has also begun in Japan (www.nippon-shinyaku.co.jp/). Further clinical trials are expected. Note that clinically used PMOs have tended to be longer than the corresponding OMe-PS ONs.

A single i.c.v. injection of a PMO ON has been effective in exon inclusion of the *SMN1* pre-mRNA in the brains of SMA model mice and prolonged the life of such mice dramatically (Porensky et al., 2012). Systemic delivery into neonatal mice, where the blood-brain barrier is incompletely formed, was also highly effective at extending the life of mice in a severe SMA model (Zhou et al., 2013). Both PMOs target the ISS-N1 enhancer sequence that regulates splicing of intron 7 of the *SMN1* gene. For PMO as well as for MOE (Passini et al., 2011),

a single ON could prove sufficient to treat all SMA cases, which is not the case for DMD patients.

A PMO targeted to the extended CUG triplet repeat sequence in the mRNA for DMPK has been effective in a transgenic mouse model of myotonic dystrophy type 1 (Wheeler et al., 2009), but this approach has been competitive with MOE gapmers, also targeted at this gene (Wheeler et al., 2012), and it is not clear at this stage which of these approaches will prove the most effective for clinical development. An interesting new method of *in vivo* delivery of PMO to treat myotonic dystrophy in mice has been reported that involves use of liposomal bubbles and ultrasound that increased the efficiency of exon skipping compared to intravenous injection (Koebis et al., 2013).

Conjugates of Oligonucleotides with Peptides and Other Moieties

In the absence of organ targeting, most ON types tend to accumulate in liver and kidneys. For many other organs, naked ON delivery remains much poorer. Strategies for enhancing delivery include the co-administration of cationic complexing agents (e.g., lipid or polymer) or covalent conjugation with molecules that may enhance cell penetration. Polyethylene glycol–poly(ethylene imine) (PEG–PEI) copolymer complexes with OMe-PS ONs have been shown to enhance effectiveness for exon skipping in *mdx* mice (Sirsi et al., 2008). Similarly OMe-PS ONs absorbed on ZM2 nanoparticles allowed lower dose restoration of dystrophin in *mdx* mice by intraperitoneal delivery (Ferlini et al., 2010), which was persistent even after 90 days (Bassi et al., 2012). Further, PEI-modified pluronics have also been shown to enhance delivery of PMO into *mdx* mice after systemic delivery (Wang et al., 2013).

Most studies by far have involved attachment of cell-penetrating peptides (CPPs) to ONs, however. For negatively charged ONs such as OMe-PS, covalent conjugation with peptides has met with limited success so far in enhancing *in vivo* delivery. By contrast, significant enhancement of splice switching and exon skipping for conjugates of CPPs has been seen with charge neutral ONs. Such applications of CPPs have been reviewed recently (Lebleu et al., 2008; Said Hassane et al., 2010).

PNA-Lys_8 or PNA conjugates to an amphipathic D-Lys-rich peptide or to an Arg/homoArg-rich peptide targeting a splice junction in the PTEN pre-mRNA showed strong inhibition of PTEN expression activity in adipose tissue in mice, but the amphipathic peptide conjugate also showed substantial nephrotoxicity (Wancewicz et al., 2010). In the HeLa pLuc 705 model system, early good splice switching results obtained with PNA conjugates of the CPP R_6-penetratin (Abes et al., 2007) later led to the development of the PMO internalization peptides (Pip) series of Arg-rich CPPs and Pip2b-PNA was found to be effective by intramuscular delivery for exon skipping and dystrophin production in the *mdx* mouse (Ivanova et al., 2008).

However, side-by-side comparisons of CPP-PNA and CPP-PMOs in the *mdx* mouse model showed that CPP-PMO (now usually called peptide-PMO or P-PMO) had higher activity than CPP-PNA in muscle (Yin et al., 2010). Indeed, conjugation of the AVI Biopharma (Sarepta) Arg-rich "B" or "R" peptide to PMO targeting exon 23 led to dramatic enhancement of exon skipping and dystrophin production over naked PMO in the *mdx* mouse (Jearawiriyapaisarn et al., 2008; Wu et al., 2008; Yin et al., 2008b). Long-term administration of such P-PMO in *mdx* mice showed improvements in muscle pathology and function (Wu et al., 2012). In addition, recent papers have reported further Arg-rich Pip peptides in the series, notably Pip5e (Yin et al., 2011) and Pip6a (Betts et al., 2012), which differ from each other only slightly in CPP sequence. The salient feature of Pip-PMOs is their ability to show dystrophin production in heart, albeit less than in skeletal muscle. This class of P-PMO is being developed now for clinical use in DMD.

P-PMO is also effective in the mouse model of DM1 myotonic dystrophy. Here, a 25-mer PMO targeted to the extended CUG repeat sequences was conjugated to either B or R peptide and intravenous delivery led to redistribution of the muscleblind-1 protein and corrections of abnormal downstream splicing events, as well as a reduction in myotonia (Leger et al., 2013). P-PMO application in SMA is also a natural development that is expected.

A non-peptidic octaguanidino dendrimer conjugate of PMO, marketed as "Vivo-morpholinos," has shown good exon skipping and dystrophin production in the *mdx* mouse (Wu et al., 2009) and improved muscle function (Widrick et al., 2011). Such Vivo-morpholinos have also been used in a "cocktail" for multiple exon skipping in dystrophic dogs (Yokota et al., 2012). A cocktail of ten Vivo-morpholinos has also been used recently to skip exons 45–55 of dystrophin pre-mRNA in dystrophic *mdx52* mice by systemic delivery (Aoki et al., 2012). Spontaneous deletions of this specific region are associated with exceptionally mild phenotypes. It is not clear as yet whether a subset of these PMOs is responsible for producing the large deletion in this specially constructed mouse model of DMD and as to whether this cocktail approach could be applicable to DMD patients.

In principle, ONs can also be conjugated with cell targeting peptides. For example, a conjugate of a OMe-PS ON with a bivalent Arg-Gly-Asp (RGD) peptide that is known to target integrin receptors, enhanced splicing redirection in a reporter luciferase construct in melanoma cells in the absence of a transfection agent (Alam et al., 2008) and a bombesin peptide conjugated to the same OMe-PS ON enhanced splice switching *via* delivery through G-protein coupled endocytosis in prostate cancer cells (Ming et al., 2010). Surprisingly, however, cell-targeting peptides, for example discovered by phage display, have not yet found significant utility *in vivo*, although it is known from conference reports that a peptide conjugate of an OMe-PS ON is being considered for development for use in treatment of myotonic dystrophy type 1 (www.prosensa.eu).

Prospects for Novel Chemistries in Exon-Skipping Applications

Although hundreds of promising ON analogues have been described in recent years, very few are being pursued in exon skipping and related applications (see Table 1). In the last 5 years, only TcDNA has shown sufficient promise for development to rival the more standard OMe, MOE, LNA, PNA, and PMO ON varieties (Fig. 1). Clearly further 2′-*O*-substituted and conformationally restricted 2′-4′-cyclized

RNA analogues that have followed on from LNA and ENA, such as *S*-cEt, would be worth evaluation, particularly in combinations with 2′-F, OMe, or DNA residues. Such analogue types used in steric blocking applications are usually subject to significant intellectual property (IP) barriers and therefore are only investigated for exon skipping applications if it is in the interests of the company holding that IP, but it is likely that such nucleotide derivatives will make it closer to the clinic for some types of steric blocking applications in coming years. In principle, further PNA-like or other charge-neutral ONs, several of which have been published in recent years, may also see development, but none of these appear to be on the immediate horizon.

More likely developments may stem from additional end modifications to ONs, particularly for charge neutral PMO or PNA backbones, in order to modify their pharmacological and/or cellular uptake properties. The key issue here is to ensure that any substituent does not interfere with the ability of the ON to reach its pre-mRNA target and hybridize efficiently. Chemical space is vast and the easy ability to attach moieties of varied chemistry to ONs makes it attractive to pursue such research avenues. For example, there is great scope for the exploration of further synthetic peptides and other derivatives (including amino acid analogues) to tune *in vivo* parameters of the attached ONs to improve their pharmacology. Indeed, for all ON types, there is a need to redirect ONs away from the kidney and liver (unless these organs are being targeted specifically) and to increase their serum half-life.

Better targeting to specific cell types or organs (such as brain, heart, and muscle) is another generally important goal for all ON types. For example for SMA, intrathecal delivery is currently the only possible method of ON introduction to brain and efforts to find ways to help ONs across the blood–brain barrier following intravenous delivery must remain a priority. Chemical modifications of ONs by known brain penetrating compounds, such as brain-targeting peptides, are worth exploring not only in this disease context but also for many other diseases, such as brain cancers. Improved brain penetration is a goal that is extremely challenging to achieve for macromolecules such as ONs, however. In addition, a key issue for any novel peptide or other chemical moiety as an ON conjugate is the safety margin in its use *in vivo*. For example, many peptide conjugates show toxic side effects at doses below that of their naked ON counterparts.

In our own laboratory we have developed a method using "Click" chemistry in aqueous solution for labeling P-PMO (and PMO) starting from unfunctionalized, commercially available PMO (Shabanpoor and Gait, 2013). This may aid the tracking and semi-quantification of PMO *in vivo* and intracellulary. Currently, the only analytical technique available to measure PMO levels *in vivo* is based on hybridization assays (Schnell et al., 2013). Another method we have developed involves rapid parallel synthesis of sets of CPP conjugates of biocargoes, such as PNA or PMO, for use in cell screening or similar programmes (Deuss et al., 2013). The role of a chemist in the development of therapeutic ON technology may now perhaps be better devoted to exploration of chemical space with regard to what can be attached to commercially available ONs rather than trying to "reinvent the wheel" in discovery of novel ON analogue types.

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Author Disclosure Statement

No competing financial interests exist.

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