1 **Antisense oligonucleotides: the next frontier for treatment of neurological disorders**

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8 **Abstract | Despite the discovery that antisense oligonucleotides (ASOs) could**
9 **influence RNA and modulate protein expression dating back over two decades, the**
10 **progress of these drugs into the clinic has been hampered by inadequate target**
11 **engagement, insufficient biological activity and off-target toxic effects. Over the**
12 **years, novel chemical modifications have been employed to address these issues,**
13 **which, together with elucidation of the mechanism of action of this new class of**
14 **drugs, better understanding of disease pathophysiology, and improved clinical trial**
15 **design have provided momentum for translating ASO-based strategies into therapies.**
16 **This steady progress recently reached a pinnacle with approvals of the splice-**
17 **switching ASOs Nusinersen and Eteplirsen for treatment of SMA and DMD**
18 **respectively, representing a landmark in a field where disease-modifying therapies**
19 **were virtually non-existent. With the rapid development of improved next-generation**
20 **ASOs toward clinical application, this technology now holds the potential to have a**
21 **dramatic impact on the treatment of many neurological and non-neurological**
22 **conditions in the near future.**

23

24 Antisense oligonucleotides (ASOs) are short synthetic single-stranded
25 oligodeoxynucleotides, usually 8 to 50 nucleotides in length, which, by pairing to the mRNA
26 target by complementary Watson-Crick base sequences, can alter RNA and reduce, restore,
27 or modify protein expression through several distinct mechanisms. Since the first *in vivo*
28 applications showing limited clinical potential because of the high susceptibility of naked

ASOs to rapid degradation by endo- and exonucleases[1,2], ASOs have undergone a large number of chemical modifications leading to improved pharmacological characteristics. The use of a phosphorothioate (PS) backbone, in which one of the non-bridging oxygen atoms is replaced with a sulphur, significantly improved resistance to nuclease activity and increased binding to serum proteins, resulting in longer half-lives in serum[3–5], while still being compatible with applications where target RNA downregulation is desired[6]. Further modifications at the 2′ position of the ribose sugar have led to the development of another class of antisense oligonucleotides with improved safety and efficacy profiles. In this group, the 2′-O-methyl (2OMe) and 2′-O-methoxy-ethyl (MOE) are among the most studied. These second-generation antisense agents, containing a PS backbone and 2′-O-substituted oligoribonucleotide segments, have shown increased hybridization affinity to their target RNA[7–9], increased resistance towards nuclease degradation[9,10], and reduced immunostimulatory activity[11] compared with their unmodified counterparts. Other ASOs do not possess the natural phosphate-ribose backbone: in phosphorodiamidate morpholino oligomers (PMO) for example, the deoxyribose moiety is replaced by a morpholine ring, and the charged phosphodiester inter-subunit linkage is replaced by an uncharged phosphorodiamidate linkage[12]. These oligonucleotides are very resistant to nuclease and protease degradation[13] and are mostly exploited in splicing modulation approaches or translation inhibition. Recently two of these second-generation antisense agents have gained FDA approval for spinal muscular atrophy (SMA) and Duchenne muscular dystrophy (DMD), representing a landmark for the field and fuelling unprecedented excitement in this strategy for the treatment of human diseases. In this Review, we timely discuss the properties, applications, and hurdles of antisense pharmacology and the progress made thus far towards clinical applications in neurology.

Pharmacokinetics properties

The evolution of oligonucleotides as therapeutic agents has been hindered by the fact that delivery of these large molecules to their intracellular targets is a very challenging task.

Pharmacokinetic properties of ASOs are similar across species and gender and are largely driven by the chemistry of the backbone[14–16]. Following systemic administration, plasma concentrations of phosphorothioate-modified ASOs decline in a multi-exponential fashion, where rapid tissue distribution is followed by a slower terminal elimination phase when equilibrium is reached[14]. ASOs with a phosphorothioate backbone are extensively associated to plasma proteins with low affinity[14–16]. Protein binding prevents loss of drug to renal filtration and facilitates tissue uptake, with albumin being the protein that binds the greatest amount across species[17]. In contrast, neutrally charged oligonucleotides (e.g. PNAs, morpholinos, and unmodified and unformulated siRNA) bind plasma proteins more weakly and thus are more readily filtered and excreted, resulting in lower tissue uptake[18]. It has been well established that where the ASO accumulates in highest concentrations, good antisense activity is routinely observed[14,19–22]. Systemic administration results in a broad distribution of ASOs into most tissues, particularly liver, kidney, bone marrow, adipocytes, and lymph nodes[14,15,17,20–23], and with the notable exception of the central nervous system. In humans, 2OMe ASOs accumulate in proximal tubular cells in the kidney at the highest concentration, therefore requiring careful monitoring of renal function. The blood-brain barrier is largely impervious to oligonucleotides[24]. However when ASOs are administered by intrathecal injection, they distribute broadly in the central nervous system (CNS), being taken up by both neurons and glial cells both in brain and spinal cord, and with rapid distribution kinetics[25–29]. It has been demonstrated that, compared to slow infusion, bolus injection into the CSF results in better distribution in the CNS[28]. Combined with the good clinical safety profile observed to date, intrathecal administration of ASOs holds great potential for application in neurodegenerative diseases[30–32].

Intracellular delivery

Intracellular delivery is well recognized as *the* major barrier to effective ASO activity within target cells. While cell uptake is poor, those ASOs that are internalized, are taken up by endocytosis and then traffic to the nucleus, where they encounter their pharmacological

1 targets. A number of cell-surface receptors have been suggested to bind ASOs, including
2 integrins[33], scavenger receptors[34], and Toll-like receptors[35–37]. Studies have shown that
3 nuclear entry is not the rate-limiting step for ASO activity, as oligonucleotides, particularly
4 those with phosphorothioate backbones, are able to continuously shuttle between the
5 nucleus and the cytoplasm, through both passive diffusion and active transport[38,39]. Whether
6 presented in naked form, as a chemical conjugate, or associated with a carrier, an
7 oligonucleotide entering a cell encounters an intricate maze of membrane compartments
8 which include early and recycling endosomes, late endosomes/multi-vesicular bodies, and
9 lysosomes, where the endosomal content is eventually transferred for degradation[40–42].
10 While the trafficking machinery is usually quite efficient in driving internalized material to the
11 appropriate intracellular destination, ASOs must escape the endosome compartments in
12 order to reach their target. It is becoming increasingly clear that trafficking of ASOs from
13 endosomes into the cytoplasm is a significant rate-limiting step following internalization for
14 oligonucleotides therapeutics[40–42]. Pharmacological interventions aimed at enhancing
15 endosomal escape have the potential to improve oligonucleotide activity in the clinic. Current
16 strategies rely on altering the endosomal barrier[43], modulating intra-endosomal pH using
17 titratable peptides or polymers[44], or selectively permeabilizing the endosomal compartments
18 using small molecules to improve oligonucleotide release to the cytosol[45].
19
20 **Functional mechanisms**
21 The exact mechanisms by which an oligonucleotide can induce a biological effect are yet to
22 be elucidated. Unlike siRNAs, it is likely that ASOs find their targets un-assisted as there
23 seems to be no evolved cellular mechanism for promoting antisense strand recognition.
24 Once bound to the RNA, ASOs can form an RNA-DNA hybrid that becomes a substrate for
25 RNase H, which results in target mRNA degradation[6]. RNase H is a family of ubiquitously
26 expressed enzymes that hydrolyses the RNA strand of an RNA/DNA duplex, with RNase H1
27 being the necessary mediator[46] and the rate limiting step for ASO activity[6]. The products of
28 the cleaved RNAs are then processed by the normal cellular degradation pathways in both

the nucleus and cytoplasm[47]. ASOs with RNase H competent backbones include the oligodeoxynucleotide phosphodiesters, the phosphorothioates, and the 2-fluorooligodeoxynucleotides[48]. A minimum stretch of five 2'-deoxy residues is sufficient for RNase H activation *in vitro*[49,50]. In addition to exploiting cellular nucleases, ASOs can be designed to have intrinsic enzymatic activity, including the ability to directly cleave the target RNA following hybridization (e.g. ribozymes and DNAzymes)[51,52]. Alternatively, ASOs can modulate gene expression via steric block of the ribosomal machinery[53], which can lead to reduced expression, modulation of splicing and/or restoration of a functional protein[54]. Oligonucleotide binding to the pre-mRNA can also be exploited to mask polyadenylation signals on the pre-mRNA, forcing the cell to utilize alternative polyA sites[55]. Other oligonucleotide modifications (2'-O-alkyl, PNA, and morpholinos) may use different mechanisms to inhibit protein expression, e.g. they can inhibit intron excision, a key step in the processing of mRNA. In contrast to RNase H-dependent ASOs, which can inhibit protein expression when hybridised to virtually any transcript, only certain mRNA regions are effective target sites for steric-blocker ASOs.

Proof-of-concept studies for neurological disorders

Splice-switching strategies

The biochemical mechanism of splicing is highly complicated, involving interactions between pre-mRNA, small nuclear ribonucleoproteins and splicing factor proteins and relying on multiple levels of regulation. ASOs hybridizing to splice sites, enhancer or silencer elements within the transcript, allow precise and reproducible manipulation of the splicing machinery, resulting in exon skipping, restoration of a splicing pattern, or shifting the ratio between existing splice forms depending on the designed strategy (FIG. 1). Since the first proof of principle with ASO-mediated splicing correction of the beta-globin transcript[56], ASOs have emerged as promising tools for the treatment of a number of genetic conditions. In terms of therapeutic development the most advanced use of this technology has been for DMD.

Duchenne muscular dystrophy. DMD is a severe X-linked myopathy caused by mutations in the *DMD* gene encoding the dystrophin protein. DMD affects about 1/3500-1/4000 live male births globally[57,58], thus representing one of the most common fatal genetic diseases[59,60]. The disease is caused by partial or complete absence of dystrophin, which anchors proteins from the cytoskeleton to those in the myofibre membrane[61], resulting in progressive muscle weakness and atrophy, kyphoscoliosis, cardiomyopathy, and premature death[62]. Analysis of a cohort of over 7000 DMD patients (TREAT-NMD DMD Global database) revealed that the most common mutations lead to the loss of the open reading frame (ORF), with large deletions accounting for 68% of the total mutations, followed by large duplications (11%)[63]. The first proof-of-concept studies to demonstrate that modulating pre-mRNA splicing of dystrophin using ASOs to restore the dystrophin ORF is a viable therapeutic strategy for DMD were carried out in human lymphoblastoid cells and cultured muscle cells in the mid-1990s[64–71]. The rationale for pursuing an exon-skipping ASO therapy for the treatment of DMD relies on the considerations that the partly shortened protein following internal deletion retains sufficient function to substantially modify the disease course. This is based on the evidence that most of the critical functional domains at the N- and C-terminals within the dystrophin protein are typically unaffected[72] and that in-frame DMD deletions result in the much milder phenotype of Becker muscular dystrophy. Nevertheless, given the intrinsic nature of the exon-skipping strategy, not all mutations are amenable for correction. Achieving successful restoration of dystrophin expression using ASOs has proven challenging: studies in mdx mice, a mouse model carrying a premature stop codon in exon 23 of the *dmd* gene, resulted in 5–6% restoration of dystrophin levels using 2OMe ASOs[73,74]. Administration of neutrally charged PMO oligonucleotides was able to partially restore levels of the dystrophin protein and ameliorate the disease phenotype in mdx mice[75-79]. A study comparison between 2OMe and PMO to induce exon skipping in the mdx mouse concluded that PMO resulted in higher levels of dystrophin protein, although other parameters, including the length of the ASO and the identity of the target sequence may likely contribute

1 to the efficiency of individual oligonucleotides[75]. PMOs have enhanced serum stability,
2 higher resistance to nuclease degradation, a more favourable safety profile, and a wider
3 therapeutic window. In addition, PMOs have shown efficacy in the dog model of DMD. The
4 preclinical studies have raised two main issues of the exon skipping strategy using AONs:
5 the first one being the variability in dystrophin restoration between treated animals and within
6 muscle fibres and the second one related to the restoration of dystrophin expression in non-
7 skeletal muscle and cardiac restoration in particular, which is highly refractory to ASO
8 treatment. This is clinically very relevant because failure to restore dystrophin levels in the
9 heart in mice with restored dystrophin levels in skeletal muscle could exacerbate the cardiac
10 pathology[76,77].
11
12 ***Modulation of protein expression***
13 ASOs are often used to downregulate expression of the mutant protein, an approach that
14 has been extensively used in diseases caused by a toxic gain-of-function mechanism, such
15 as Huntington's disease (HD)[26] and SOD1 amyotrophic lateral sclerosis (ALS)[25]. Next to
16 RNase H-mediated degradation, ASO-mediated target suppression can be achieved by
17 blocking translation or splicing modulation to introduce an out-of-frame deletion, which
18 results in protein knockdown by nonsense-mediated decay of the corresponding transcript[54]
19 (FIG. 1). Increasing the levels of therapeutic proteins *in vivo* is more challenging:
20 approaches such as gene therapy and antisense-mediated de-repression by targeting
21 inhibitory antisense transcripts only gained partial success, due to a number of obstacles,
22 including the limited number of applicable genes[78–80]. Recently, using a class of modified
23 ASOs that bind to mRNA sequences in upstream open reading frames (uORFs), Liang et al.,
24 were able to increase the amounts of protein translated from a downstream primary ORF by
25 30–150% in a dose dependent manner in both human and mouse cells and by ~80% in mice
26 after systemic treatment[81] (FIG. 1). These findings further broaden the potential utility of
27 ASOs as therapeutic strategies, particularly considering that approximately 50% of human
28 mRNAs have AUGs upstream of the primary start codon[82,83].

Huntington's disease. HD is an adult-onset autosomal dominant neurodegenerative condition caused by an abnormal CAG repeat expansion, encoding for a polyglutamine stretch in the HTT protein. This disorder belongs to the family of polyglutamine diseases, which further consists of the spinocerebellar ataxias (SCA 1, 2, 3, 6, 7, and 17), spinal and bulbar muscular atrophy and dentatorubro-pallidoluysian atrophy (Orr H, 2007). The pathological hallmark of these diseases is the accumulation of toxic proteins in affected tissues (Davies, SW 1997; Seidel K, 2012), therefore providing a therapeutic rationale for using an antisense strategy to lower the expression levels of the mutant transcript. Intrathecal infusion of MOE-PS antisense oligonucleotides targeting the human transgene resulted in up to 75% reduction of HTT RNA, extended survival, and improved motor performance in a HD mouse model, up to 8 months post treatment (Kordasiewicz HB, 2012). In late years, in order to limit the detrimental effects of lowering wild-type HTT levels, several allele-specific silencing approaches have been employed, including ASOs targeting the CAG expansion (Hu J, 2009; Gagnon KT, 2010; Hu J, 2010), although they may be associated with the unwanted down-regulation of other transcripts (Sun X, 2014; Hu J, 2009), or single nucleotide polymorphisms (SNPs) enriched on the HD allele (van Bilsen PHJ, 2008; Lombardi MS, 2009; Carroll JB, 2011; Ostergaard ME, 2013). Interestingly, a total of 50 SNPs have been identified on the mutant alleles (Warby SC, 2009). Population genetics studies have shown that 75%–85% of the HD population could be treated using a panel of three to five ASOs targeting these mutant HTT-selective variants (Pfister EL, 2009; Warby SC, 2009).

Tauopathies. Microtubule-associated protein (MAP) tau, MAP1 (A/B) and MAP2 perform similar functions, i.e. the promotion of assembly and stability of the microtubules network in mature neurons. The essential requirement of microtubules for axoplasmic flow, which, in turn, is critical to neuronal activity, may explain this redundancy. The biological activity of tau is regulated by its degree of phosphorylation. Hyperphosphorylation of tau depresses its microtubule assembly activity and its binding to microtubules[84,85]. In Alzheimer's disease (AD) and related disorders called tauopathies, tau is abnormally hyperphosphorylated and

accumulates into toxic intraneuronal neurofibrillary tangles[86,87], an early histopathologic marker[88] which directly correlates with dementia in these patients[89–91] and is believed to underlie the widespread neuronal loss[92]. Evidence that mice that completely lack tau only develop a mild motor phenotype later in life[93–97] and reduction of endogenous tau in adult mice results in no behavioural or neuroanatomical abnormalities have prompted researchers to investigate gene silencing approaches to treat AD and other tauopathies[98]. Morpholinos targeting the start codon, splice acceptors and donors, splicing branch points, polypyrimidine track-related sequences, and splicing enhancer and inhibitor sequences, all resulted in reduction of tau expression in human neuroblastoma cell lines, with the most potent ones inducing skipping of the targeted exons to achieve out-of-frame deletion[99]. Recently treatment of mice overexpressing human tau carrying the P301S mutation[100] with 30 mg/day of a RNse H activating-ASO targeting human tau delivered via intra-cerebroventricular infusion over 28 days was found to significantly reduce tau expression and pathology[101]. Since by the time symptoms manifest, substantial neuronal loss has already begun in patients, a critical question is whether therapies can slow or even reverse the neurodegenerative process. Importantly, ASO treatment started in aged mice was able to reverse pathological changes and prevent neuronal loss, while improving behavioural deficits, and extending survival[101], suggesting that when total human tau is reduced *in vivo*, neurons retain the ability to clear pre-existing neuronal aggregates of tau. The translational potential of this approach is further supported by evidence that in Cynomolgus monkeys delivery of tau-reducing ASO in a single bolus via lumbar puncture into the intrathecal space at doses of 30mg or 50 mg decreased total endogenous tau mRNA in the spinal cord and brain in a dose-dependent manner[101]. Altogether, *these in vivo* preclinical ASO studies strengthen the case for a tau-reducing therapeutic approach for patients with AD and other tauopathies.

RNA toxicity

Myotonic dystrophy. Myotonic dystrophy 1 and 2 (DM1 and DM2) are both autosomal dominant neuromuscular conditions caused by an abnormal trinucleotide expansion (CTG) in the 3′ UTR of the DMPK (dystrophia myotonica protein kinase) gene (DM1)[102–106] and a tetranucleotide expansion (CCTG) in the first intron of the ZNF9 (zinc finger 9) gene (DM2)[104,107–109], respectively. DM1 and DM2 affect approximately 1 in 8,500 individuals, representing the most common cause of muscular dystrophy in adults[102,107,110]. The disease mechanism is believed to mainly arise from a RNA toxic gain-of-function where aberrant RNA transcripts containing pathologically expanded (CUG)n or (CCUG)n sequences fold into a hairpin-like secondary structure[111], accumulate in the nucleus and alter the functions of RNA-binding proteins, such as muscleblind-like 1 (MBNL1), involved in regulating mRNA splicing and translation[112–115]. These alterations cause a global spliceopathy, which results in a multisystemic disorder mainly characterised by myotonia and progressive muscle weakness, cardiac arrhythmias, cataracts and nervous system dysfunction[116–119]. ASO strategies to treat diseases caused by a toxic RNA can be grouped into two main groups: a) steric hindrance to prevent binding and sequestration of critical RNA-binding proteins, and b) degradation of the mutant transcript by directly targeting the expanded CUG repeat (FIG. 1). CAG25, a PMO antisense oligonucleotide designed to complementary bind with high affinity to the expanded CUG microsatellite repeat region, prevented MBNL1 sequestration, releasing it from the RNA foci, and resulted in at least partial correction of the global misplicing with amelioration of the disease phenotype following intramuscular injection in a DM1 disease model, the HSALR mouse[120]. An alternative strategy involves degradation of the mutant DMPK mRNA using antisense oligonucleotides complementary to a region of the 3′ UTR that included a (CUG)13 sequence or directly targeting the expanded CUG repeat[121,122]. This approach using 2OMe antisense oligonucleotides resulted in 90% reduction of DMPK mRNA and improved splicing abnormalities. Importantly, DMPK transcripts containing normal (CUG)n repeats were largely unaffected.

Amyotrophic lateral sclerosis. A GGGGCC hexanucleotide repeat expansion in the noncoding region of the C9ORF72 gene accounts for approximately 40% of all inherited

1 forms of ALS and FTD[123,124]. The proposed mechanisms of pathogenesis include loss of
2 C9ORF72 protein function, supported by evidence of decreased expression in patients of the
3 repeat-containing allele[123,125–129], and RNA toxic gain-of-function arising from folding of
4 repeat-containing RNAs into stable structures, similarly to other non-coding expansion
5 disorders, including myotonic dystrophy[130,131]. Recent evidence favours the RNA toxic gain-
6 of-function as central in the disease pathogenesis as mice expressing the human C9ORF72
7 gene with different sizes of expanded repeats develop age- and repeat length-dependent
8 neurological dysfunctions, while mice expressing 50% of C9orf72 mRNA only showed
9 splenomegaly, enlarged lymph nodes, and mild social interaction deficits[132]. Another
10 proposed mechanism of RNA toxicity is the production and accumulation in affected tissues
11 of aberrant dipeptide-repeat (DPR) proteins through repeat-associated non-AUG-dependent
12 (RAN) translation[133,134], although their actual contribution to the pathogenesis remains
13 controversial. Irrespective of the relative contribution to neurodegeneration of either RNA-
14 mediated toxicity mechanism, reducing the expanded RNA transcripts without exacerbating
15 a potential loss of C9ORF72 function holds great potential as a therapeutic strategy for this
16 disease. *In vivo* administration of ASOs targeting the C9ORF72 hexanucleotide expansion
17 selectively reduced the repeat-containing RNA levels via a RNase H-dependent mechanism,
18 decreased both soluble and insoluble DPR proteins, and significantly attenuated the
19 behavioural deficits, while preserving exon 1b-containing, C9ORF72 protein-encoding
20 RNAs[132], confirming previous studies in fibroblasts[135] and iPS-derived neurons[136] using a
21 similar strategy.
22
23 **ASOs en route to the clinic: the SMA experience**
24 The use of ASOs has been known since the '70s; nevertheless, it took several decades to
25 understand the basics of their pharmacology before they could reach the stage of clinical
26 experimentation. Although significant room for improvement still exists, a number of clinical
27 trials have been already completed or are currently underway (Table 1). Among others, the
28 recently approved ASO Nusinersen to treat SMA represents an exemplary case study. SMA

is one of the most prevalent and devastating genetic disorders in childhood[137]. The disease is caused by loss-of-function mutations in a single gene, the survival of motor neuron 1 (*SMN1*) gene[138]. The *SMN2* gene is the primary genetic modifier, since it can generate overall ~10% of the functional protein compared to the SMN1 locus, the majority of the product lacking exon 7[139]. Discovery of the intronic splicing silencer N1 (ISS-N1), a 15-nucleotide sequence in intron 7 of the *SMN* gene critical for *SMN2* splicing regulation, provided a major breakthrough for the development of an antisense approach in SMA[140]. A number of *in vivo* studies, using an optimized ISS-N1-blocking ASO with different modifications, increased the survival and improved the motor phenotype in SMA mouse models[27,141–143], and demonstrated a pharmacokinetic advantage of the PMO chemistry over MOE for ICV injections[144,145]. Preclinical experiments conducted in non-human primates showed that intrathecal infusion of 3mg of ASO over 24 hours was well tolerated and resulted in widespread distribution in the spinal cord. The successful preclinical studies were quickly followed by an open-label phase 1 clinical trial, showing that intrathecal administration of four ascending single-dose levels (1, 3, 6, and 9 mg) of Nusinersen (ISIS-SMNRx) in 28 SMA patients (age 2-14 years) was well-tolerated, resulted in dose-dependent plasma and CSF drug levels, and provided some preliminary evidence of clinical efficacy at the 9 mg dose[146]. Following this, a phase 2, open-label, dose-escalation study performed in 20 infants (age 3 weeks and 7 months) where either 6 or 12 mg of Nusinersen were delivered intrathecally on day 1, 15, 85 and 253, with follow up treatments every 4 months, showed mild improvements in motor function at high dose compared to baseline. Importantly, analysis of post-mortem tissue indicated that intrathecal Nusinersen was broadly distributed throughout the spinal cord and brain and that drug concentrations in target motor neurons were above those predicted to produce SMN2 mRNA exon 7 inclusion[30]. While no control group was included, results from this trial greatly informed the design of a large phase 3 clinical trial of Nusinersen in infantile-onset SMA, whose results have been recently disclosed. Overall 173 patients were included in the study, 121 as part of a multicentre, randomized, double-blind, sham-controlled investigation (ENDEAR study),

with the remaining included in an open-label study. In an interim analysis conducted on 82 patients treated for at least 183 days, Nusinersen reduced by 47% the risk of death or permanent ventilation in infantile-onset SMA compared to control. In addition, some patients achieved milestones, such as independent sitting (in four cases) and standing (in one), that are almost never observed in the natural history of the disease. These strong results from the interim analysis supported the FDA approval of Nusinersen in the U.S. for the treatment of SMA in paediatric and adult patients, where it will be marketed by Biogen with the name of SPINRAZA™, representing the first approved treatment for individuals with SMA. In October 2016, the European Medicines Agency (EMA) granted Accelerated Assessment status; other countries will follow in 2017.

Future challenges

Recently two ASO-mediated splice switching therapies for SMA and DMD have gained FDA approval, putting neuromuscular diseases at the very forefront of ASO drug development for neurological conditions. These drugs represent the pinnacle of years of steady progress in antisense pharmacology, a field that was virtually non-existent until no more than 2 decades ago. Nevertheless, if these two successful experiences set an example to follow, they also raise a number of issues, which need to be urgently considered in future therapeutic trials using ASOs. In the case of Nusinersen, it remains to be seen whether the efficacy observed in children can be achieved also in adult SMA patients. In addition, the intrathecal delivery does not provide effective SMN correction in peripheral tissues such as muscle, which is known to play a primary role in the disease pathogenesis similarly to other lower motor neuron diseases[143,147]. Eteplirsen (Exondys 51™), the first drug approved to treat DMD patients, is specifically indicated for patients who have mutations of the dystrophin gene amenable to exon 51 skipping, which represents only about 13 percent of the population with DMD[63]. Nevertheless, the decision form FDA was based on the surrogate endpoint of a modest 0.9% increase in dystrophin protein in skeletal muscle rather than a clinical benefit in Eteplirsen-treated patients

(https://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/ucm521263.htm). Thus the future advancement of ASOs in the clinic urgently requires optimization of the following: sequence selection, biological activity, and delivery technology (FIG. 2). These issues are currently being addressed by the field and will hopefully result in better therapeutic efficacy and specificity. Several methods can be employed to improve ASO design, ranging from the more empirical testing of large numbers of mRNA complementary sequences to systematic RNase H mapping, use of combinatorial arrays and of secondary structure prediction by computational methods[148], and in silico pre-screening approach based on predictive statistical modelling[149]. In addition, ASOs can achieve allele selective suppression of gene expression by targeting SNPs associated with mutant allele, preserving the function of wild type copy, as it has been shown in models of HD[32]. Next-generation ASOs with improved pharmacological properties are being tested in animal models and some of them represent promising candidates for clinical testing: one example are tricyclo-DNAs, where an ethylene bridge is fused with a cyclopropane unit, which have been recently reported to promote a higher degree of dystrophin splicing correction in skeletal muscles, heart and brain compared to a 2OMe-PS oligonucleotide following peripheral administration in two DMD mouse models[150]. Another recent promising approach to optimise ASO chemistry is centred on the notion that nucleic acid therapeutics consists of a mixture of thousands of stereoisomers; some of them have therapeutic effects, while others are less beneficial or can even contribute to toxic effects. The pharmaceutical company WAVE Life Sciences (Cambridge, MA) has developed a novel chemistry platform to control for ASO chirality. These rationally designed stereopure nucleic acid therapeutics have demonstrated improved activity, stability, specificity and immunogenicity compared with stereoisomer mixtures (Chanda Vargeese: ‘Development of Stereopure Nucleic Acid Therapeutics’, meeting: Oligonucleotide therapeutics and Delivery. April 2016, Cambridge, MA). Beyond potency and specificity, another critical feature of a good candidate molecule is the ability to reach its intracellular target at sufficient concentration[151]. An optimal delivery system needs to be cell specific, controllable, and able to protect the nucleic acids from nuclease degradation[152].

Significant progress has been made in recent years in employing lipid- and polymer-based nanocarriers to facilitate antisense delivery[153]. Neutrally charged ASO backbones such as PMO or PNA oligonucleotides conjugated with short cationic peptides, known as cell-penetrating peptides (CPPs) have shown strong transmembrane capacity and great potential for treating neurodegenerative disorders[154]. The search for novel CPPs, with a more favourable safety profile and increased efficacy, has led to the identification of a series of peptides known as PNA or PMO internalization peptides (Pips), arisen from structural modifications of an original Drosophila melanogaster R6–Penetratin peptide[155]. Systemically delivered PPMOs effectively restored therapeutic levels of dystrophin not only in skeletal muscle but also in the heart in animal models[156–158]. Recently, the advanced peptide-oligonucleotide Pip6a-PMO has demonstrated higher efficacy in both the CNS and peripheral tissues in severe SMA mice following systemic administration than the standard naked ASO[159].

Conclusions

Advances in the understanding of ASO pharmacology, together with the optimization of their efficacy and safety profiles have certainly provided a momentum for translating ASO-based therapeutics into the clinic. The recent approvals of Nusinersen and Eteplirsen for treatment of SMA and DMD respectively will likely pave the way for the use of ASO strategies to treat a wide range of diseases in which the mechanism of disease pathogenesis has been identified. With the growing number of ASO-mediated therapeutics being tested in clinical trials, this technology holds the potential to change the therapeutic landscape for many neurological and non-neurological conditions in the near future.

Contributions

C.R. and M.J.A.W. researched data and wrote the article.

Competing interests statement

1 C.R. declares no competing interests. M.J.A.W., through the University of Oxford, has filed
2 patents on peptide-based methods for antisense oligonucleotide delivery.

3

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Figure legends

Figure 1. Functional mechanisms of antisense oligonucleotide. A│ Once bound to the RNA, ASOs can form an RNA-DNA hybrid that becomes a substrate for RNase H, which results in target mRNA degradation. **B│** ASOs targeting the AUG start site can sterically block the binding of RNA binding protein complexes, such as ribosomal subunits, suppressing translation of target mRNA. **C│** In diseases caused by a RNA toxic gain-of-function mechanism, antisense designed to complementary bind with high affinity untranslated regions can prevent binding and sequestration of RNA-binding proteins by steric hindrance. **D│** ASOs binding to splice sites or exonic/intronic inclusion signals results in skipping or inclusion of the targeted exon. **E│** Translation of the upstream open reading frames (uORFs) typically inhibits the expression of the primary ORF. ASOs binding to the uORFs are able to increase the amounts of protein translated from a downstream ORF.

Figure 2. Development of next-generation oligonucleotides. Advancement of ASOs as a viable therapeutic approach for human diseases urgently requires further optimization of sequence selection, chemistry and delivery technology. This schematic figure shows the most recent strategies adopted in the development of next-generation ASOs. **A│** ASO potency and specificity can be improved by a more rational sequence selection, based on empirical testing and use of predictive modelling, and targeting allele-specific single

1 nucleotide polymorphisms. **B│** Modification of ASO chemistry, such as the most recent
2 tricyclo-DNAs, phosphoryl guanidine oligo(2′-OMe)ribonucleotides (PGO), and use of
3 stereopure chemistry, can result in more efficient target engagement and reduced toxicity *in*
4 *vivo*. **C│** One of the major hurdles that severely limit clinical application of ASOs is low
5 intracellular delivery. Strategies to improve it include targeted ligand-oligonucleotide
6 conjugates, lipid- and polymer-based nanoparticles, antibody conjugates and use of cell-
7 penetrating peptides.
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