Antisense oligonucleotides: the next frontier for treatment of neurological disorders

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Abstract | Antisense oligonucleotides (ASOs) were first discovered to influence RNA processing and modulate protein expression over two decades ago; however, progress translating these agents into the clinic has been hampered by inadequate target engagement, insufficient biological activity, and off-target toxic effects. Over the years, novel chemical modifications of ASOs have been employed to address these issues. These modifications, in combination with elucidation of the mechanism of action of ASOs and improved clinical trial design, have provided momentum for the translation of ASO-based strategies into therapies. Many neurological conditions lack an effective treatment; however, as research progressively disentangles the pathogenic mechanisms of these diseases, they provide an ideal platform to test ASO-based strategies. This steady progress reached a pinnacle in the past few years with approvals of ASOs for the treatment of spinal muscular atrophy and Duchenne muscular dystrophy, which represent landmarks in a field in which disease-modifying therapies were virtually non-existent. With the rapid development of improved next-generation ASOs toward clinical application, this technology now holds the potential to have a dramatic effect on the treatment of many neurological conditions in the near future.

In 1978, Zamecnik and Stephenson showed that antisense oligonucleotides (ASOs) can inhibit viral replication *in vitro*[1]. Two decades later, the first oligonucleotide agent was approved by the US Food and Drug Administration (FDA) to treat cytomegalovirus (CMV)-induced chorioretinitis, sparking high hopes for the potential use of this new class of drugs for a number of human conditions. First-generation ASOs were short, synthetic, single-stranded oligodeoxynucleotides, typically 8–50 nucleotides in length, which bound by complementary base pairing to a target mRNA and led to endonuclease-mediated transcript knockdown and, consequently, to reduction of the levels of a deleterious protein[2,3]. However, early expectations of these agents remained largely unfulfilled, as first generation oligonucleotides mostly failed to meet therapeutic end points in clinical trials, mainly owing to their fast turnover and inability to achieve sufficient intracellular concentrations to suppress target genes[2,3]. Since the early 1990s, a range of second-generation and third-generation ASOs with modified backbones that confer enhanced pharmacological properties have been developed. These improved ASOs can function via alternative mechanisms — for example, they can alter pre-mRNA splicing by sterically blocking splicing factors, or they can block mRNA translation by preventing ribosome recruitment[2]. Furthermore, antisense molecules can be designed to bind non-coding RNAs and toxic RNAs associated with disease pathogenesis, which greatly expands the numbers and types of selectable targets. Several features of this new class of drugs, including high specificity, ability to address targets otherwise inaccessible with traditional therapies, and reduced toxicity owing to limited systemic exposure, make these molecules ideal candidates for therapy development for human neurological conditions. The fact that oligonucleotides do not readily cross the blood-brain-barrier (BBB), and therefore require invasive delivery methods such as intrathecal or intraventricular routes, remains one the most substantial obstacles for the clinical application of oligonucleotides in CNS disorders. A large amount of work is currently being carried out to develop chemical modifications and vehicles that will improve ASO delivery and target engagement[4]. In the past few years, two antisense agents have gained approval by the FDA for Duchenne muscular dystrophy

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Key points

- Antisense oligonucleotides (ASOs) are short, synthetic, single-stranded oligodeoxynucleotides that can alter RNA and reduce, restore, or modify protein expression through several distinct mechanisms
- By targeting the source of the pathogenesis, ASO-mediated therapies have an higher chance of success than therapies targeting downstream pathways
- Advances in the understanding of ASO pharmacology have provided momentum for translating these therapeutics into the clinic
- Two ASO-mediated therapies have received approval from the US Food and Drug Administration for the treatment of Duchenne muscular dystrophy and spinal muscular atrophy
- Further advancement of ASOs in the clinic urgently requires optimization of ASO delivery, target engagement, and safety profile
- This technology holds the potential to change the therapeutic landscape for many neurological and non-neurological conditions in the near future

(DMD) and spinal muscular atrophy (SMA), representing a landmark for the field and fuelling unprecedented excitement for the potential of this strategy in the treatment of human diseases. In this Review, we discuss the properties, applications, and hurdles of antisense pharmacology and the current progress towards clinical applications in neurology.

Chemical modifications

The first *in vivo* applications of ASOs showed limited clinical potential because of the high susceptibility of ASOs with an unmodified phosphoribose backbone to rapid degradation by endonucleases and exonucleases[5,6]. However, a large number of chemical modifications have now been applied to ASOs, resulting in improved pharmacological characteristics (FIG. 1).

The use of a phosphorothioate backbone, in which one of the non-bridging oxygen atoms of the ASO backbone is replaced with a sulfur, substantially improved resistance to nuclease activity and increased binding to serum proteins. These alterations increased the half-lives of ASOs in serum[7–9], but still enabled the molecules to be used in applications that involved downregulation of target RNA[10]. In addition, modifications at the 2′ position of the ribose sugar have yielded another class of ASOs with improved safety and efficacy profiles, including 2′-*O*-methyl (2′-OMe) and 2′-*O*-methoxy-ethyl (2′-MOE) oligonucleotides, which are among the most studied of this group.

Second-generation antisense agents that contain a phosphorothioate backbone and 2′-*O*-substituted oligoribonucleotide segments have shown increased hybridization affinity to their target RNA[11–13], increased resistance to nuclease degradation[13,14], and reduced immunostimulatory activity[15] compared with their unmodified counterparts. Other ASOs do not possess the natural phosphoribose backbone, such as phosphorodiamidate morpholino oligomers (PMO), in which the deoxyribose moiety is replaced by a morpholine ring, and the charged phosphodiester inter-subunit linkage is replaced by an uncharged phosphorodiamidate linkage[16]. These oligonucleotides are very resistant to nuclease and protease degradation[17] and are mostly used in splicing modulation or translation inhibition. The newly approved agents for SMA and DMD represent examples of these second-generation ASOs.

Pharmacokinetic 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.

The pharmacokinetic properties of ASOs are similar across species and sex and are largely driven by the chemistry of the backbone[18–20]. Following systemic administration, plasma concentrations of phosphorothioate-modified ASOs decline in a multi-exponential fashion, in which rapid tissue distribution is followed by a slower terminal-elimination phase where equilibrium is reached[18]. ASOs with a phosphorothioate backbone are highly associated with low affinity to plasma proteins[18–20], with albumin being the most common of these proteins across species[21]. Binding to plasma proteins prevents loss of the ASO drug to renal filtration and facilitates uptake by tissues. By contrast, neutrally charged oligonucleotides (such as peptide nucleic acids, morpholinos, and unmodified and unformulated siRNA) bind plasma proteins more weakly than do ASOs with the negatively changed phosphorothioate backbone and thus are more readily filtered and excreted, resulting in lower tissue uptake[22].

High concentrations of ASOs are well known to be associated with good antisense activity[18,23–26]. Systemic administration of ASOs results in a broad distribution of ASOs into most tissues, particularly the liver, kidney, bone marrow, adipocytes, and lymph nodes[18,19,21,24–27], with the notable exception of the CNS. In humans, the highest concentration of 2′-OMe ASO accumulation is in the proximal tubular cells in the kidney; consequently, careful monitoring of renal function is required in patients receiving this agent. The blood–brain barrier is largely impervious to oligonucleotides[28]; however, when ASOs are administered by intrathecal injection, they distribute broadly in the CNS, and are taken up by neurons and glial cells both in brain and spinal cord, with rapid distribution kinetics[29–33]. In addition, bolus injection into the CSF results in better distribution in the CNS than does slow infusion[32]. This finding, in combination with the good clinical safety profile observed to date, indicates that intrathecal administration of ASOs holds great potential for application in neurodegenerative diseases[34–36]. Indeed, this approach has already been implemented in human clinical trials for amyotrophic lateral sclerosis (ALS) and SMA, with encouraging results[35,37]. However, muscle poses different challenges in ASO pharmacology — despite being readily reachable after systemic delivery — particularly in relation to the large amount of tissue that must be targeted to achieve a clinically meaningful effect, and the large degree of muscle-to-muscle variability. In fact, data collected from *mdx* mice — a model for DMD carrying a premature stop codon in exon 23 of the *Dmd* gene — have shown that, compared with single injection, repeated intravenous injections of 100 mg/kg of PMO resulted in improved restoration of dystrophin, suggesting a cumulative effect, albeit variable between muscles[38].

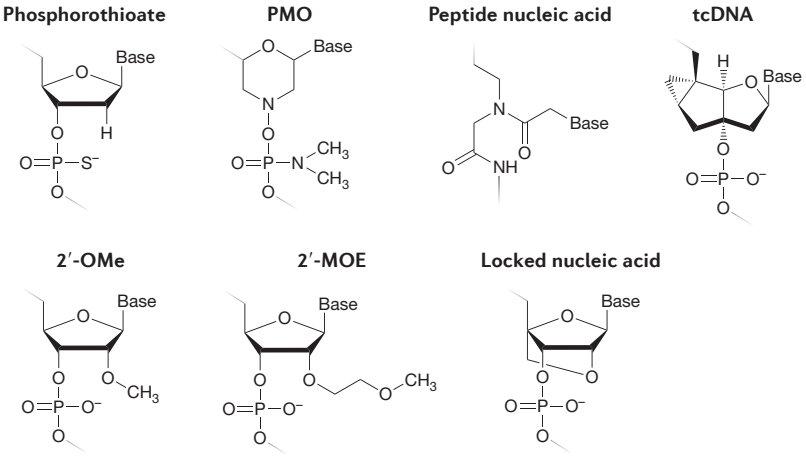

Figure 1 | **Chemical modifications of the ASO backbone.** In the past few decades a number of chemical modifications to the phosphodiester backbone have been made to improve antisense oligonucleotide (ASO) pharmacokinetic properties, tolerability profile, and target binding affinity. Phosphorothioate DNA, phosphorodiamidate morpholino (PMO), and peptide nucleic acid designs all confer resistance to nucleases and enhanced uptake in cells, resulting in increased potency of the ASO. Tricyclo-DNAs (tcDNA) are conformationally constrained DNA analogues with increased potency and enhanced uptake in tissues after systemic administration. Ribose substitutions, including 2′-*O*-methyl (2′-OMe), 2′-*O*-methoxyethyl (2′-MOE), and locked nucleic acid, are frequently used in combination to further increase stability, enhance target binding, and generally confer less toxicity than unmodified designs.

Intracellular delivery

Intracellular delivery is recognized as the major barrier to effective ASO activity within target cells. Although cell uptake is poor, those ASOs that are internalized are taken up by endocytosis and traffic to the nucleus, where they encounter their pharmacological targets. A number of cell-surface receptors have been suggested to bind ASOs, including integrins[39], scavenger receptors[40], and Toll-like receptors[41–43]. Studies have shown that nuclear entry is not the rate-limiting step for ASO activity, as oligonucleotides with phosphorothioate backbones are able to continuously shuttle between the nucleus and the cytoplasm, through passive diffusion and active transport[44,45]. Whether presented in naked form, as a chemical conjugate, or in association with a carrier, an oligonucleotide entering a cell encounters an intricate maze of membrane compartments that include early and recycling endosomes, late endosomes and multi-vesicular bodies, and lysosomes, to which the endosomal content is eventually transferred for degradation[46–48]. The trafficking machinery is usually quite efficient in driving internalized material to the appropriate intracellular destination, but ASOs must escape the endosome compartments to reach their target. After internalization, ASOs are trafficked from endosomes into the cytoplasm; this step is increasingly being recognized as an important rate-limiting step for oligonucleotide therapeutics[46–48]. Consequently, pharmacological interventions that aim to enhance the escape of ASOs from endosomes have the potential to improve oligonucleotide activity in the clinic. Current strategies rely on altering the endosomal barrier[49], modulating intra-endosomal pH using titratable peptides or polymers[50], or selectively permeabilizing the endosomal compartments using small molecules to improve oligonucleotide release to the cytosol[51].

Functional mechanisms

Depending on their chemistry, binding sequence, and target, single-stranded ASOs can modulate gene expression or modify pre-mRNA splicing through several distinct mechanisms of action (FIG. 2). Unlike siRNAs, ASOs are thought to find their targets unassisted by auxiliary proteins, as no cellular mechanism seems to have evolved to promote antisense strand recognition. Once bound to the RNA through Watson–Crick base pairing, ASOs can form an RNA–DNA hybrid that becomes a substrate for RNase H, resulting in target mRNA degradation[10]. The RNase H family consists of ubiquitously expressed enzymes that hydrolyse the RNA strand of an RNA–DNA duplex. RNase H1 is the necessary mediator[52] and the rate-limiting step for ASO activity[10]. The products of the cleaved RNAs are then processed by the normal cellular degradation pathways in the nucleus and cytoplasm[53]. RNase H-recruiting ASOs include the oligodeoxynucleotide phosphodiesters, phosphorothioates, and 2-fluorooligodeoxynucleotides[54]. A minimum of five consecutive 2′-deoxy residues is sufficient for RNase H activation *in vitro*[55,56].

In addition to this exploitation of cellular nucleases, ASOs can be designed to have intrinsic enzymatic activity, including the ability to directly cleave the target RNA after hybridization (for example, via the incorporation of ribozymes and DNAzymes)[57,58]. Alternatively, ASOs can modulate gene expression via steric block of the ribosomal machinery[59], which can lead to reduced expression, modulation of splicing and/or restoration of a functional protein[60]. In addition, binding of ASOs to intron–exon junctions can destabilize splicing sites, or can displace or recruit splicing factors (the identities of which have yet to be fully characterized), resulting in the exclusion or inclusion of particular exons[61]. This approach might be most beneficial for disorders with a known splicing defect, and can be used to restore normal gene function, either by re-establishing the normal reading frame following a pathogenic frame shift, or by excluding mutated segments of DNA. The most notable agent that uses this approach is nusinersen, an ASO designed to promote inclusion of exon 7 in the survival motor neuron (*SMN1*) gene. This therapy was approved by the FDA in December 2016 for the treatment of SMA[62].

Binding of oligonucleotides to the pre-mRNA can also be exploited to mask polyadenylation signals on the pre-mRNA, forcing the cell to use alternative polyadenylation sites[63]. Other oligonucleotide modifications (such as 2′-*O*-alkyl, peptide nucleic acids, and morpholinos) might use different mechanisms to inhibit protein expression; for example, they can inhibit intron excision, a key step in mRNA processing. 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 ASOs that act as a steric block.

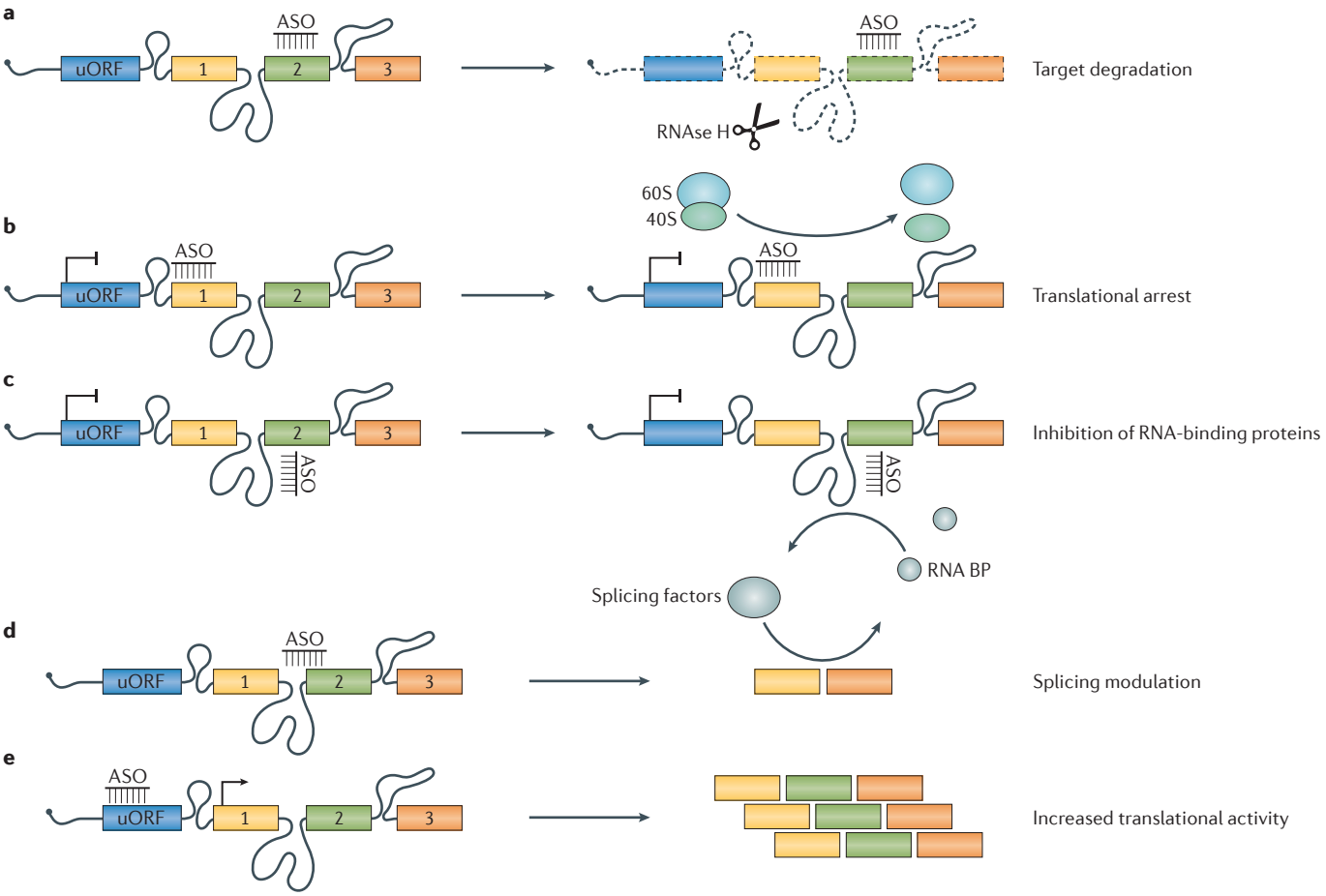

Figure 2 | **Functional mechanisms of ASOs. a** | Once bound to the RNA, antisense oligonucleotides (ASOs) can form an RNA–DNA hybrid that becomes a substrate for RNase H, which results in target mRNA degradation. **b** | ASOs targeting the AUG start site can sterically block the binding of RNA binding protein complexes, such as ribosomal subunits, suppressing translation of target mRNA. **c** | In diseases caused by a toxic RNA gain-of-function mechanism, ASOs designed to bind complementarily with high-affinity to untranslated regions can prevent binding and sequestration of RNA-binding proteins by steric hindrance. **d** | ASO binding to splice sites or to exonic or 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.

Proof-of-concept studies

Splice-switching strategies

The biochemical mechanism of splicing is highly complicated, involving interactions between pre-mRNA, small nuclear ribonucleoproteins and splicing factor proteins, and relies on multiple levels of regulation. Hybridization of ASOs to splice sites, enhancer elements or silencer elements within the transcript enables 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 intended strategy (FIG. 2).

In 1993, the first proof of principle was provided for ASO-mediated splicing correction, in which aberrant splicing of the beta-globin transcript[64] was rectified. Since this study was published, ASOs have emerged as promising tools for the treatment of a number of genetic conditions. In terms of therapeutic development for neurological conditions, one of the most advanced uses of this technology has been for DMD.

Duchenne muscular dystrophy. DMD is a severe X-linked myopathy caused by mutations in the *DMD* gene, which encodes dystrophin. DMD affects between about 1 in 3,500 and 1 in 4,000 live male births globally[65,66], and thus represents one of the most common fatal genetic diseases[67,68]. The disease is caused by partial or complete absence of dystrophin, which normally anchors proteins from the cytoskeleton to those in the myofibre membrane[69]. The loss of this function results in progressive muscle weakness and atrophy, kyphoscoliosis, cardiomyopathy, and premature death[70]. Analysis of a cohort of over 7,000 patients with DMD (from the TREAT-NMD DMD global database) revealed that the most common DMD-causative mutations lead to the loss of the *DMD* open reading frame (ORF), with large deletions accounting for 68% of the total mutations, followed by large duplications, which account for 11%[71].

In the mid-1990s, studies were carried out in human lymphoblastoid cells and cultured muscle cells that provided the first proof-of-concept that modulation of the

pre-mRNA splicing of dystrophin using ASOs could restore the dystrophin ORF, and thus represents a viable therapeutic strategy for treatment of DMD[72–79]. The rationale for pursuing this strategy for the treatment of DMD is that it results in the expression of a functional — albeit partially shortened — protein, by skipping DMD-causative mutations. This idea is based on evidence that most of the crucial functional domains of dystrophin, which lie at the *N*-terminal and *C*-terminal of the protein, are typically unaffected by internal exon skipping[80], and in-frame *DMD* deletions result in the much milder phenotype of Becker muscular dystrophy. Overall, a single-exon or multi-exon skipping strategy to restore dystrophin expression would be applicable for 90% of DMD mutations[81]. Successful restoration of dystrophin expression using ASOs has proven challenging: studies in the *mdx* mouse model of DMD achieved a 5–6% restoration of dystrophin levels after treatment with 2′-OMe ASOs[82,83]. Neutrally charged PMOs were also able to partially restore levels of the dystrophin protein and ameliorate the disease phenotype in *mdx* mice[75–79]. A study comparing the ability of 2′-OMe oligonucleotides and PMOs to induce exon skipping in the *mdx* mouse concluded that PMO treatment resulted in higher levels of dystrophin protein; however, other parameters, including the length of the ASO and the identity of the target sequence, could contribute to the efficiency of individual oligonucleotides[84]. PMOs have higher serum stability, higher resistance to nuclease degradation, a more favourable safety profile, and a wider therapeutic window than 2′-OMe ASOs[4]. In addition, PMOs have shown efficacy in the dog model of DMD[85].

Preclinical studies have raised two main issues regarding the exon skipping strategy using ASOs: first, restoration of dystrophin levels is highly variable between treated animals and even within different muscles from the same animal and, second, non-skeletal and cardiac muscle in particular are highly refractory to ASO treatment[38,83,84,86]. These issues are very clinically relevant, as studies in *mdx* mice have suggested that failure to restore dystrophin levels in the heart in the presence of restored dystrophin levels in skeletal muscle exacerbates cardiac pathology owing to increases in voluntary activity[86,87].

Following these preclinical studies, a 30-nucleotide PMO that triggers excision of exon 51 during pre-mRNA splicing of the dystrophin RNA transcript has been tested via intravenous infusion in phase II and III clinical trials (dose range 0.5–50 mg/kg per week, up to 96 weeks), in cohorts of patients with genetically confirmed DMD[88,89]. This agent elicited variable increases in the overall dystrophin expression in muscles of different patients after treatment, only yielding up to a modest 0.9% increase in total protein levels, reflecting an increased dystrophin intensity in positive fibres from 9.4% to 22.6% in confirmatory studies[90]. These findings provided the basis for the controversial accelerated approval of eteplirsen (trade name Exondys 51) from the FDA in late 2016 (REFS 91,92). The data from this trial were hampered by issues regarding the reproducibility and reliability of the methods used to assess the increase in dystrophin expression as a surrogate end point, as well as by shortfalls in clinical trial design, including the small sample size of 12 patients, all of which poses serious questions regarding the actual efficacy of the ASO[92]. Additionally, whether the increased dystrophin levels observed as a result of eteplirsen treatment are likely to translate into a clinical benefit for patients has been the subject of dispute[92]. Nevertheless, eteplirsen is the first drug approved to treat patients with DMD, and is specifically indicated for patients who have *DMD* mutations that are amenable to exon 51 skipping, who represent ~13% of the total DMD population[71].

Modulation of protein expression

ASOs are often used to downregulate expression of a mutant protein, an approach that has been extensively used in diseases caused by a toxic gain-of-function mechanism, such as Huntington disease (HD)[30] and ALS resulting from an *SOD1* mutation[29]. In addition to RNase H-mediated degradation, ASO-mediated target suppression can be achieved by blocking translation or by modulating splicing to introduce an out-of-frame deletion, which results in protein knockdown by nonsense-mediated decay of the corresponding transcript[60] (FIG. 2). Compared with the reduction of protein levels, increasing the levels of proteins *in vivo* is more challenging: approaches such as gene therapy and antisense-mediated derepression via the targeting of inhibitory antisense transcripts have gained only partial success, owing to a number of obstacles, including the limited number of genes to which these strategies are applicable[93–95]. In one study, a class of modified ASOs that bind to mRNA sequences in upstream ORFs were used to increase the amounts of protein translated from a downstream primary ORF. This strategy increased protein levels by 30–150% in a dose dependent manner in human and mouse cells, and systemic treatment in mice increased protein levels by ~80%[96] (FIG. 2). These findings further broaden the potential utility of ASOs as therapeutic strategies, as ~50% of human mRNAs have AUGs upstream of the primary start codon[97,98].

Huntington disease. HD is an adult-onset autosomal dominant neurodegenerative condition caused by an abnormal CAG repeat expansion in *HTT*, encoding a polyglutamine stretch in the huntingtin protein. This disorder belongs to the polyglutamine disease family, which also contains the spinocerebellar ataxias (SCA 1, 2, 3, 6, 7, and 17), spinal and bulbar muscular atrophy and dentatorubropallidoluysian atrophy[99]. The pathological hallmark of these diseases is the accumulation of toxic proteins in affected tissues[100,101], which provides a therapeutic rationale for the use of an antisense strategy to lower the expression levels of the mutant transcript. Intrathecal infusion of 2′-MOE-PS ASOs targeting the human *HTT* transgene in a mouse model of HD yielded an up to 75% reduction of *HTT* RNA, extended survival, and improved motor performance in the animals up to 8 months after treatment[30]. In the past few years, several allele-specific silencing approaches have been employed to limit the detrimental effects of lowering

the levels of wild-type huntingtin, including ASOs that specifically target the CAG expansion[102–104]; however, these ASOs might be associated with unwanted down-regulation of other CAG-containing transcripts[102,105]. In addition, ASOs can also be targeted to specific single nucleotide polymorphisms (SNPs) enriched on the HD allele[36,106–108]. Interestingly, a total of 50 SNPs have been identified on the mutant alleles[109]. Population genetics studies have shown that 75–85% of patients with HD could be treated using a panel of three to five ASOs targeting these mutant HTT-selective variants[109,110].

SOD1. Mutations in *SOD1*, the gene encoding superoxide dismutase 1 (SOD1), account for ~20% of familial ALS cases[111]. Toxicity resulting from these mutations is mainly mediated by a toxic gain-of-function mechanism associated with the aggregation of the misfolded SOD1 protein; therefore, ASO-mediated degradation of SOD1 transcript holds great potential for therapy development. In one study carried out in rats and monkeys, intraventricularly delivered ASOs targeting SOD1 distributed widely throughout the CNS, including the regions affected in the major neurodegenerative diseases, and effectively reduced SOD1 protein and mRNA levels throughout the brain and spinal cord[29]. Importantly, the ASO treatment significantly slowed disease progression and extended survival of rats carrying a mutation in the human *SOD1* transgene[29]. Following these promising preclinical results, the first-in-man study for intrathecal administration of an ASO targeting *SOD1* transcript was performed from 2010–2012 (TABLE 1). Owing to the low concentrations of ASO used, the study did not show a reduction in SOD1 protein in participants; however, the agent demonstrated an excellent safety profile, and the trial enabled crucial pharmacokinetic measurements to be established, including measurement of plasma and CSF ASO concentration[35]. In late 2015, a second-generation *SOD1*-targeted ASO compound, BIIB067 (IONIS-SOD1Rx), entered phase I–II trial (TABLE 1) with a single dose cohort, which is due to be followed by a multiple-dose cohort in ALS patients with a *SOD1* mutation. To determine efficacy of *SOD1*-targeted ASO in this ongoing study, SOD1 protein measured in the CSF will be employed as a pharmacodynamic marker[112,113].

Tauopathies. Human tau is encoded by *MAPT*, which comprises 16 exons on chromosome 17q21 (REF. 114). The adult human brain contains six main tau isoforms, which are generated by alternative splicing of exons 2, 3, and 10. These isoforms can be classified as 0N, 1N, and 2N, depending on the number of near-amino-terminal inserts present. In addition, isoforms can be categorized according to whether they contain three (3R) or four (4R) carboxy-terminal repeat domains[115]. In the brain, tau is found mainly in neurons, but is also present at low levels in glia. In mature cells, tau promotes assembly and stability of the microtubule network. The biological activity of tau is regulated by its degree of phosphorylation, where hyperphosphorylation depresses its microtubule assembly activity[116,117]. In Alzheimer disease (AD) and other tauopathies, tau is abnormally hyperphosphorylated and accumulates into toxic intraneuronal neurofibrillary tangles[118,119], an early histopathologic marker[120] that directly correlates with dementia in these patients[121–123] and is believed to underlie the widespread neuronal loss[124].

Evidence that mice completely lacking tau only develop a mild motor phenotype later in life[125–129] and that reduction of endogenous tau in adult mice results in no behavioural or neuroanatomical abnormalities, has prompted researchers to investigate gene silencing approaches to treat AD and other tauopathies[130]. Experiments in human neuroblastoma cell lines have shown that morpholinos targeting the start codon, splice acceptors and donors, splicing branch points, polypyrimidine track-related sequences, and splicing enhancer and inhibitor sequences can all elicit a reduction of tau expression, with the most potent reduction yielded by agents that induce skipping of the targeted exons to achieve an out-of-frame deletion[131]. Furthermore, tau expression and pathology was significantly reduced in mice overexpressing human Pro301Ser mutant tau[132] after treatment with 30 mg per day of an RNase H-activating ASO targeted to human tau delivered via intracerebroventricular infusion over 28 days[133]. Owing to the fact that substantial neuronal loss has already begun in patients with AD by the time symptoms manifest, a crucial question is whether therapies can slow or even reverse the neurodegenerative process. Importantly, ASO treatment started in aged mice reversed pathological changes, prevented neuronal loss, improved behavioural deficits, and extended survival[133], suggesting that neurons retain the ability to clear pre-existing neuronal aggregates of tau when total human tau is reduced *in vivo*. The translational potential of this approach is further supported by a study in cynomolgus monkeys, which showed that delivery of tau-reducing ASO in a single bolus via lumbar puncture into the intrathecal space at doses of 30 mg or 50 mg decreased total endogenous tau mRNA in the spinal cord and brain in a dose-dependent manner[133]. Altogether, these *in vivo* preclinical ASO studies strengthen the case for the use of tau-reducing therapeutic strategies for patients with AD or other tauopathies.

Alternative splicing of exon 10, which encodes the second carboxy-terminal repeat domain and is only included in 4R tau, is also of particular interest, as inclusion of this domain is associated with tauopathies that are distinct from those caused by 3R tau. Consequently, a treatment approach aimed at selectively modulating tau splicing to lower the levels of the 4R isoform alone has also been proposed. One study showed that treatment with an ASO triggering exon 10 inclusion, resulting in a shift towards 4R tau expression, increased tau phosphorylation and worsened the disease phenotype in two mouse models that expressed human tau[134]. The converse approach was shown to effectively lower 4R tau expression, but left total protein levels unaltered, highlighting the potential for ASO corrections of tau splicing for therapeutic development.

RNA toxicity

Myotonic dystrophy. Myotonic dystrophy type 1 and type 2 (DM1 and DM2) are both autosomal dominant neuromuscular conditions. DM1 is caused by an abnormal trinucleotide expansion (CTG) in the 3′ untranslated region (UTR) of the *DMPK* gene[135–139], whereas DM2 is caused by a tetranucleotide expansion (CCTG) in the first intron of *CNBP* (also known as *ZNF9*)[137,140–142]. DM1 and DM2 affect approximately 1 in 8,500 individuals, representing the most common cause of muscular dystrophy in adults[135,140,143]. The disease mechanism is believed to arise mainly from a toxic RNA gain of function, whereby aberrant RNA transcripts containing pathologically expanded $(CUG)_n$ or $(CCUG)_n$ sequences fold into a hairpin-like secondary structure[144], accumulate in the nucleus, and alter the functions of RNA-binding proteins, such as muscleblind-like 1 (MBNL1), which are involved in the regulation of mRNA splicing and translation[145–148]. 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[149–152].

Strategies that use ASOs to treat diseases caused by a toxic RNA can be grouped into two main groups: steric hindrance of the toxic RNA to prevent binding and sequestration of critical RNA-binding proteins, or degradation of the mutant RNA transcript via direct targeting of the expanded CUG repeat (FIG. 2). CAG25, a PMO designed to bind with high affinity to the expanded CUG microsatellite repeat region, prevented sequestration of MBNL1 by the repeats, releasing it from the RNA foci, and resulted in at least partial correction of global missplicing. Furthermore, intramuscular injection of this ASO in a mouse model carrying a long CTG repeat in a human skeletal actin gene, which replicates signs of the disease including the prominent myotonia, strongly ameliorated the disease phenotype[153]. An alternative strategy involves degradation of the mutant *DMPK* mRNA either by using ASOs complementary to a region of the 3′ UTR that included a $(CUG)_{13}$ sequence, or by directly targeting the expanded CUG repeat[154,155]. Application of this approach using 2′-OMe ASOs resulted in 90% reduction of *DMPK* mRNA and improved splicing abnormalities in DM1 mice. Importantly, *DMPK* transcripts containing normal $(CUG)_n$ repeats were largely unaffected.

C9orf72. A GGGGCC hexanucleotide repeat expansion in the non-coding region of the *C9orf72* gene accounts for ~40% of all inherited forms of ALS and FTD in studies of pan-European, North American, and Australian patient populations[156–158]. The proposed mechanisms of pathogenesis include loss of C9orf72 protein function, supported by evidence of decreased expression of the gene in patients with the repeat-containing allele[157,159–163], and toxic RNA gain-of-function arising from folding of repeat-containing RNAs into stable structures, similarly to other non-coding expansion disorders, including myotonic dystrophy[164,165]. Evidence from the past few years suggests that the toxic RNA gain-of-function is central in the disease pathogenesis, as mice expressing the human *C9orf72* gene with different sizes of expanded repeats develop age-dependent and repeat-length-dependent neurological dysfunctions, whereas mice with a 50% reduction in *C9orf72* mRNA only showed splenomegaly, enlarged lymph nodes, and mild social interaction deficits[166]. Another proposed mechanism of toxicity is the production and accumulation of aberrant dipeptide-repeat (DPR) proteins translated from the hexanucleotide repeat RNA in all reading frames through a process called repeat-associated non-AUG-dependent (RAN) translation[167,168], although the mechanisms of their relative contribution to disease pathogenesis remains unclear[169]. Irrespective of the relative contribution to neurodegeneration of either RNA-mediated or protein-mediated mechanisms of toxicity, reduction of the expanded RNA transcripts without exacerbating any loss of C9orf72 function holds great potential as a therapeutic strategy for this disease. *In vivo* administration of ASOs targeting the *C9orf72* hexanucleotide expansion selectively reduced the repeat-containing RNA levels via a RNase H-dependent mechanism, decreased both soluble and insoluble DPR proteins, and significantly attenuated the behavioural deficits in transgenic mice, but preserved levels of alternatively spliced C9orf72 protein-encoding isoforms that do not include the repeats[166]. These findings confirm previous studies in fibroblasts[170] and iPS-derived neurons derived from patients with the *C9orf72* repeat expansion[171], which showed that administration of ASOs could rescue disease phenotypes in a similar manner.

ASOs en route to the clinic

The potential for the therapeutic use of ASOs has been recognized since the 1970s, but several decades of research was required to understand the basics of the pharmacology of these agents before clinical experimentation could commence. Although substantial room for improvement of ASO design still exists, a number of clinical trials using these molecules have been completed or are currently underway (TABLE 1). Among these, the ASO nusinersen, which was approved by the FDA in December 2016 to treat SMA, represents an exemplary case study.

SMA is one of the most prevalent and devastating genetic disorders in childhood[172]. The disease is caused by loss-of-function mutations in a single gene: *SMN1* (REF. 173). The *SMN2* gene is the primary genetic modifier of the disease, as this locus generates ~10% of the normal physiological levels of functional SMN protein; however, the majority of SMN produced by *SMN2* lacks exon 7, which results in a protein that is more prone to degradation[174]. A major breakthrough in the development of an antisense approach in SMA was the discovery of a 15-nucleotide sequence in intron 7 of *SMN2*, termed the intronic splicing silencer N1 (ISS-N1), which is crucial for splicing regulation; this finding led to the development of the first medical therapy for SMA[175,176]. A number of *in vivo* studies used an optimized ISS-N1-blocking ASOs with different modifications to increase the survival and improve the motor phenotype

Table 1 | **Clinical trials using antisense oligonucleotides for neurological diseases**

Drug	Target gene	Chemistry	Mechanism	Treatment route	Design (phase)	Clinical trials identifier
Duchenne muscular dystrophy						
Drisapersen	DMD (exon 51)	2′-OMe	Splicing modulation	Subcutaneous or intravenous	Open label (I–II)	NCT01910649 (REF. 200)
				Subcutaneous	Placebo-controlled (I)	NCT01128855 (REF. 201)
					Placebo-controlled (II)	NCT01153932 (REF. 202)
					Placebo-controlled (III)	NCT01254019 (REF. 203)
					Open label (III)	NCT01480245 (REF. 204)
					Placebo-controlled (II)	NCT01462292 (REF. 205)
					Open label (III)	NCT01803412 (REF. 206)
					Open label (III)	NCT02636686 (REF. 207)
Eteplirsen	DMD (exon 51)	PMO	Splicing modulation	Intravenous	Single blind (I–II)	NCT00159250 (REF. 208)
					Open label (I–II)	NCT00844597 (REF. 88)
					Placebo-controlled (II)	NCT01396239 (REF. 89)
					Open label (II)	NCT01540409 (REF. 209)
					Open label (III)	NCT02255552 (REF. 210)
					Open label (II)	NCT02286947 (REF. 211)
					Open label (II)	NCT02420379 (REF. 212)
PRO044	DMD (exon 45)	2′-OMe	Splicing modulation	Subcutaneous or intravenous	Open label (II)	NCT01037309 (REF. 213)
PRO045	DMD (exon 45)	2′-OMe	Splicing modulation	Subcutaneous	Open label (II)	NCT01826474 (REF. 214)
SRP-4045	DMD (exon 45)	PMO	Splicing modulation	Intravenous	Placebo-controlled (III)	NCT02500381 (REF. 215)
SRP-4053	DMD (exon 53)	PMO	Splicing modulation	Intravenous	Placebo-controlled (III)	NCT02500381 (REF. 215)
Spinal muscular atrophy						
Nusinersen	SMN2	2′MOE-PS	Splicing modulation	Intrathecal	Open label (I)	NCT01494701 (REF. 216)
					Open label (I–II)	NCT01703988 (REF. 217)
					Open label (I)	NCT01780246 (REF. 218)
					Open label (II)	NCT01839656 (REF. 34)
					Open label (I)	NCT02052791 (REF. 219)
					Sham-controlled (III)	NCT02193074 (REF. 220)
					Sham-controlled (III)	NCT02292537 (REF. 221)
					Open label (II)	NCT02386553 (REF. 222)
					Sham-controlled (II)	NCT02462759 (REF. 223)
					Open label (III)	NCT02594124 (REF. 224)
					Open label (III)	NCT02865109 (REF. 225)
Familial amyloid polyneuropathy						
IONIS-TTR_{Rx}	TTR	2′-MOE-PS	RNase H-mediated degradation	Subcutaneous	Placebo-controlled (III)	NCT01737398 (REF. 226)
Huntington disease						
IONIS-HTT_{Rx}	HTT	2′-MOE-PS	RNase H-mediated degradation	Intrathecal	Placebo-controlled (I–II)	NCT02519036 (REF. 227)
Amyotrophic lateral sclerosis						
IONIS-$SOD1_{Rx}$	SOD1	2′-MOE-PS	RNase H-mediated degradation	Intrathecal	Placebo-controlled (I)	NCT01041222 (REF. 35)
					Placebo-controlled (I–II)	NCT02623699 (REF. 228)
Multiple sclerosis						
ATL1102	CD49d	2′-MOE-PS	RNase H-mediated degradation	Subcutaneous	Placebo-controlled (II)	ACTRN12608000226303 (REF. 229)

Table 1 (cont.) | **Clinical trials using antisense oligonucleotides for neurological diseases**

Drug	Target gene	Chemistry	Mechanism	Treatment route	Design (phase)	Clinical trials identifier
Myotonic dystrophy 1						
ISIS-DMPK$_{Rx}$	DMPK	2′-MOE-PS	RNase H-mediated degradation	Subcutaneous	Placebo-controlled (I–II)	NCT02312011 (REF. 230)

2′-MOE PS, 2′-O-methoxy-ethyl phosphorothioate; 2′-OMe, 2′-O-methyl; DMPK, myotonic dystrophy protein kinase; HTT, huntingtin; PMO, phosphorodiamidate morpholino oligomer; SMN2, survival of motor neuron 2; SOD1, superoxide dismutase 1; TTR, transthyretin.

in mouse models of SMA[31,177–179], and demonstrated a pharmacokinetic advantage of the PMO chemistry over 2′-MOE for intracerebroventricular injections[180,181]. Preclinical experiments conducted in nonhuman primates showed that intrathecal infusion of 3 mg of ASO over 24 h was well tolerated and resulted in widespread distribution in the spinal cord.

The successful preclinical studies were quickly followed by an open-label phase I clinical trial, which showed that intrathecal administration of four ascending single-dose levels (1, 3, 6, and 9 mg) of nusinersen (ISIS-SMNRx) in 28 patients with SMA (age 2–14 years) was well-tolerated, resulted in dose-dependent levels of the drug in the plasma and CSF, and provided some preliminary evidence of clinical efficacy at the 9 mg dose[37]. Following this trial, a phase II, open-label, dose-escalation study was performed in 20 infants (aged between 3 weeks and 7 months). Participants received either 6 mg or 12 mg of nusinersen, delivered intrathecally, on day 1, 15, 85 and 253, with follow-up treatments every 4 months, and mild improvements in motor function were observed at high dose compared with baseline function. 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 inclusion of exon 7 in *SMN2* mRNA[34]. Although no control group was included, results from this trial greatly informed the design of a large phase III clinical trial of nusinersen in infantile-onset SMA. 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 participants included in an open-label study. In an interim analysis conducted on 82 patients treated for at least 183 days, nusinersen reduced the risk of death or permanent ventilation by 47% in infantile-onset SMA compared with control. In addition, some patients achieved milestones such as independent sitting (in four cases) and standing (in one case) that are almost never observed in the natural history of the disease.

These strong results from the interim analysis led to the approval of nusinersen by the FDA for the treatment of SMA in paediatric and adult patients in the US, where it will be marketed by Biogen under the name SPINRAZA. Consequently, this agent represents the first approved treatment for individuals with SMA. In October 2016, the European Medicines Agency granted Accelerated Assessment status for the agent; other countries will follow in 2017.

Future challenges

Two ASO-mediated splice-switching therapies for SMA and DMD have gained FDA approval within the past few years, 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 two decades ago. Nevertheless, if these successful experiences set an example to follow, they also raise a number of issues that require urgent consideration in future therapeutic trials using ASOs. In the case of nusinersen, whether the efficacy observed in children can also be achieved in adults with SMA remains to be seen. In addition, the intrathecal delivery of this agent does not provide effective correction of the loss of SMN in peripheral tissues such as muscle, which is known to have a primary role in the disease pathogenesis similarly to other lower motor neuron diseases[179,182]. Thus, the future advancement of ASOs in the clinic urgently requires optimization of sequence selection, biological activity, and delivery technology, while also keeping an optimal safety profile. These issues are currently being addressed by the field, hopefully resulting in improved therapeutic efficacy and specificity.

Several methods can be employed to improve ASO design, including empirical testing of large numbers of mRNA complementary sequences, systematic RNase H mapping, use of combinatorial arrays, use of secondary structure prediction by computational methods[183], and *in silico* pre-screening approaches based on predictive statistical modelling[184]. In addition, ASOs can achieve allele selective suppression of gene expression by targeting SNPs associated with a mutant allele, thereby preserving function of the wild-type copy, as has been demonstrated in experimental models of HD[36]. Next-generation ASOs with improved pharmacological properties are being tested in animal models, some of which represent promising candidates for clinical testing. One example is tricyclo-DNAs, in which an ethylene bridge is fused with a cyclopropane unit (FIG. 1). These agents have been reported to promote a higher degree of dystrophin splicing correction in skeletal muscles, heart and brain than do 2′-OMe-PS oligonucleotides, following peripheral administration in two mouse models of DMD[185].

Another promising new approach to optimize ASO chemistry is centred on the notion that nucleic acid therapeutics consist of a mixture of thousands of stereoisomers, some of which have therapeutic effects,

whereas others are less beneficial or can even contribute to toxic effects. The pharmaceutical company WAVE Life Sciences (Cambridge, Massachusetts) 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[186]. Beyond potency and specificity, another crucial feature of a good candidate molecule is the ability to reach its intracellular target at sufficient concentration[187]. An optimal delivery system needs to be cell specific, controllable, and able to protect the nucleic acids from nuclease degradation[188]. Substantial progress has been made in the past few years in the employment of lipid-based and polymer-based nanocarriers to facilitate ASO delivery[189]. Neutrally charged ASO backbones such as PMO or peptide nucleic acid oligonucleotides conjugated with short cationic peptides, known as cell-penetrating peptides, have shown strong transmembrane capacity and great potential for treating neurodegenerative disorders[190]. The search for novel cell-penetrating peptides with a more favourable safety profile and increased efficacy has led to the identification of a series of peptides known as peptide nucleic acid or PMO internalization peptides (Pips). These molecules arose from structural modifications of an original peptide derived from *Drosophila melanogaster* named R6-Penetratin, containing six positively charged amino acids (arginines) and a bulky side-chain composed of hydrophobic amino acids, which enable efficient cellular uptake[191]. Systemically delivered peptide-conjugated PMOs effectively restored therapeutic levels of dystrophin not only in skeletal muscle but also in the heart in animal models[192–194]. In addition, systemic administration of the advanced peptide–oligonucleotide Pip6a-PMO in mice with severe SMA demonstrated a higher efficacy than did administration of the standard naked ASO, in both the CNS and peripheral tissues[195].

The development of progressively more-effective ASOs naturally poses concerns about the safety of these new molecules, which should be addressed before these strategies are translated into the clinic. Adverse effects related to antisense therapeutics can be divided into whether they are mediated by hybridization-dependent effects, such as off-target effects, or hybridization-independent mechanisms, such as accumulation-driven degenerative changes in the kidney proximal tubules, antagonism of receptors of the innate immune system or complement activation. For first-generation ASOs, hybridization-mediated off-target effects have never been viewed as a major issue, given the poor cellular penetration and target engagement of these agents[196]. The development of new chemistries, which greatly improved potency, also enabled the design of shorter sequences[197] that further reduced the potential for complement activation [14,198]. Conversely, with decreasing sequence length, off-target effects could become a more realistic concern. The Oligonucleotide Safety Working Group (OSWG) now recommends experimental assessment of off-target effects for ASOs during drug discovery and development[199].

Conclusions

Advances in the understanding of ASO pharmacology, together with the optimization of their efficacy and safety profiles have certainly provided momentum for the translation of ASO-based therapeutics into the clinic. The approval of eteplirsen and nusinersen for treatment of DMD and SMA, respectively, is likely to 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 a growing number of ASO-mediated therapeutics now 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.

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Author contributions
The authors contributed equally to the preparation of the article.

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