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Antisense Drugs Make Sense for Neurological Diseases

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Abstract

The genetic basis for most inherited neurodegenerative diseases has been identified, yet there are limited disease-modifying therapies for these patients. A new class of drugs—antisense oligonucleotides (ASOs)—show promise as a therapeutic platform for treating neurological diseases. ASOs are designed to bind to the RNAs either by promoting degradation of the targeted RNA or by elevating expression by RNA splicing. Intrathecal injection into the cerebral spinal fluid results in broad distribution of antisense drugs and long-term effects. Approval of nusinersen in 2016 demonstrated that effective treatments for neurodegenerative diseases can be identified and that treatments not only slow disease progression but improve some symptoms. Antisense drugs are currently in development for amyotrophic lateral sclerosis, Huntington's disease, Alzheimer's disease, Parkinson's disease, and Angelman syndrome, and several drugs are in late-stage research for additional neurological diseases. This review highlights the advances in antisense technology as potential treatments for neurological diseases.

INTRODUCTION

Advances in molecular genetics in the 1980s and further refinement in the 1990s allowed researchers to identify causative genes for most of the inherited neurological disorders. These were heady times for the field, as a steady flow of news on the identification of genes that cause devasting neurological diseases occurred throughout the 1990s. One of the more notable discoveries was the causative gene for Huntington's disease, caused by a triplet repeat (CAG) expansion in exon 1 of the huntingtin gene (1). The list of diseases due to repeat expansion has expanded to include several spinocerebellar ataxias, spinal and bulbar muscle atrophy, type 1 and 2 myotonic dystrophy, fragile X syndrome, and an inherited form of amyotrophic lateral sclerosis (2–4). Completion of the sequencing of the human genome, which provides a genetic reference, and advances in DNA sequencing have dramatically accelerated the pace of identification of genetic changes that cause disease, including the private mutations harbored by a single family or in small populations. Today, it is common to identify a putative disease-causing gene within a few weeks of patient presentation.

If clinical geneticists can quickly identify the genetic cause of a disease, why do we not yet have effective therapies for these inherited neurological diseases? The answers to this question are complex and multifactorial. Perhaps a key historical limitation was the absence of interest by large pharmaceutical companies to apply their resources toward the discovery and development of drugs for what were considered rare patient populations. Fortunately, this is changing with the commercial success of several rare-disease drugs. Additional hurdles include the lack of knowledge of the normal function of the gene and how the genetic change affects the gene's function, the lack of development of model systems to evaluate potential therapies, the lack of adequate knowledge of the natural history of the disease, the lack of development of both disease-relevant and target engagement biomarkers that can be used to reduce project risk, and in many cases, the lack of targetability of the affected gene by traditional drug discovery strategies. Importantly, the expansion of drug discovery platforms beyond traditional small-molecule drugs has led to the development of designer DNA drugs, frequently termed antisense oligonucleotides (ASOs), to directly target the expression of the genes that cause neurological diseases (5). Targeting RNA rather than protein simplifies the drug discovery process and dramatically expands the types of therapeutic targets for drug discovery.

Antisense drugs have been used as research tools by neuroscientists for almost 30 years. Some of the early applications were to help determine functions of various proteins in the central nervous system (CNS) (6–10). These early studies were hindered by the adverse effects and the short duration of effect of first-generation ASOs (1 to 2 days). Advances in antisense chemistry and a better understanding of antisense mechanisms and biodistribution helped the platform progress not only as a research tool but importantly as therapeutic agents for severe neurological diseases. Today there are five licensed antisense drugs for neurological diseases (**Table 1**) and nine antisense drugs in clinical trials; several more targets are being pursued with antisense technology for neurological diseases. This review summarizes the pharmacological properties of antisense drugs being used to treat neurological diseases.

ANTISENSE MECHANISMS OF ACTION

Antisense drugs bind to RNAs through normal Watson-Crick base-pairing, although noncanonical base-pairing (e.g., G to U) is also a possibility. Following binding to a target RNA, an antisense drug can modulate the RNA through a variety of different mechanisms, including degradation of the targeted RNA through endogenous nucleases, alteration of RNA splicing, displacement of proteins bound to the RNA, and disruption of regulatory structures in the target RNA (**Figures 1**

Table 1 Examples of antisense drugs at market or in clinical development for neurological diseases

Drug	Target	Indication	Mechanism	Chemistry	Delivery route	Status	Reference(s)
Eteplirsen	Dystrophin	Duchene muscular dystrophy-exon 51	Splicing modulation	PMO	Intravenous	Marketed	155, 156
Golodirsen	Dystrophin	Duchene muscular dystrophy-exon 53	Splicing modulations	PMO	Intravenous	Marketed	157
Inotersen	Transthyretin	Familial amyloid polyneuropathy and cardiomyopathy	RNase H	2′-MOE/DNA chimera	Subcutaneous	Marketed	15
Patisiran	Transthyretin	Familial amyloid polyneuropathy	siRNA	Liposomal formulation	Intravenous	Marketed	17
Nusinersen	SMN2	Spinal muscular atrophy	Splicing modulation	Uniform 2′-MOE	Intrathecal	Marketed	22, 87, 88
Tominersen	HTT	Huntington's disease	RNase H	2′-MOE/DNA chimera	Intrathecal	Phase III	149
Tofersen	SOD1	Familial ALS due to mutations in *SOD1*	RNase H	2′-MOE/DNA chimera	Intrathecal	Phase III	46, 103
WVE-120101[a]	HTT	Huntington's disease	RNase H	Unknown	Intrathecal	Phase I/II	None
WVE-120102[a]	HTT	Huntington's disease	RNase H	Unknown	Intrathecal	Phase I/II	None
IONIS-MAPT$_{Rx}$/BIIB080[b]	MAPT (Tau)	Alzheimer's disease/primary tauopathies	RNase H	2′-MOE/DNA chimera	Intrathecal	Phase I/II	None
IONIS-C9$_{Rx}$/BIIB078[b]	C9orf72	Familial ALS due to mutations in *C9orf72*	RNase H	2′-MOE/DNA chimera	Intrathecal	Phase I/II	None
ION859/BIIB094[b]	LRRK2	Parkinson's disease	RNase H	2′-MOE/DNA chimera	Intrathecal	Phase I/II	None
GTX-101[c]	UBE3A-ATS	Angelman syndrome	RNase H	Unknown	Intrathecal	Phase I/II	None
IONIS-DMN2–2.5$_{Rx}$[b]	Dynamin 2	Centronuclear myopathy	RNase H	cET/DNA chimera	Intravenous	Phase I/II	None

[a]Unable to find published literature on the drug. Information obtained from Wave Life Sciences (**https://wavelifesciences.com/**).
[b]Unable to find published literature on the drugs. Information obtained from Ionis Pharmaceuticals, Inc. (**https://www.ionispharma.com/**) or Biogen (**https://www.biogen.com/en_us/home.html**).
[c]Unable to find published literature on the drug. Information obtained from GeneTx Biotherapeutics (**https://genetxbio.com/**).
Abbreviations: 2′-MOE, 2′-*O*-methoxyethyl; ALS, amyotrophic lateral sclerosis; cEt, constrained ethyl; HTT, huntingtin; LRRK2, leucine-rich repeat kinase 2; PMO, phosphorodiamidate morpholino oligonucleotide; RNase, ribonuclease; siRNA, small interfering RNA; SMN2, survival motor neuron 2; SOD1, superoxide dismutase 1; UBE3A-ATS, ubiquitin protein ligase E3A–antisense transcript.

and **2**). It is expected that additional mechanisms for ASOs will be identified. Both the chemistry of the oligonucleotide and the positioning on the target RNA to which the ASO binds are major design elements that dictate which antisense mechanism is utilized (5, 11). No single antisense mechanism meets all therapeutic needs and no mechanism is vastly superior to other mechanisms of action.

Recruiting ribonuclease (RNase) H1 or binding to and activating argonaute 2 (Ago2) (**Figure 1**) is commonly used to promote degradation of the targeted RNA (5, 11). RNase H1 is a ubiquitously expressed enzyme involved in DNA replication and DNA transcription that cleaves the RNA strand of a DNA-RNA heteroduplex (12). Antisense drugs that have at least 5 to 7 consecutive DNA nucleotides are capable of supporting the RNase H1 mechanism (13). There

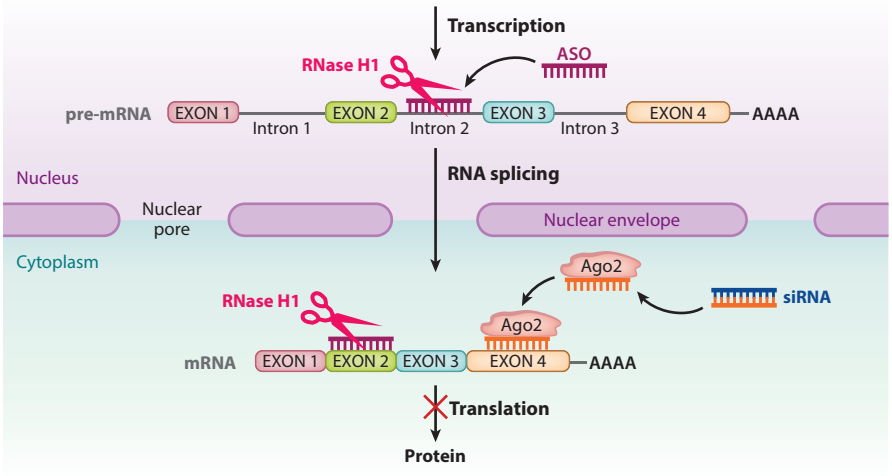

Figure 1

RNA degradation antisense mechanisms. RNA is transcribed from DNA into a pre-messenger RNA (pre-mRNA) form that undergoes several posttranscriptional processing events, such as splicing, to remove intronic sequences to form the mature mRNA. The mRNA is exported out of the nucleus to the cytoplasm, where it is translated to its protein product. Two broadly used antisense mechanisms result in selective degradation of the targeted RNA. Single-stranded antisense oligonucleotides (ASOs) that work through the ribonuclease (RNase) H1 mechanism bind to the targeted RNA and recruit RNase H1 to the ASO-RNA heteroduplex in either the nucleus or the cytoplasm. RNase H1 catalyzes the degradation of the RNA strand, releasing the ASO to bind to another target RNA. A second common antisense mechanism, small interfering RNA (siRNA), utilizes double-stranded RNA or RNA analogs, which are dissociated within the cell, and the antisense strand (also termed the guide RNA) binds to argonaute 2 (Ago2) protein in a facilitated manner. The antisense strand (guide RNA) bound to Ago2 directs the complex to the targeted RNA through Watson-Crick base-pairing to a complementary sequence in the targeted RNA. Ago2 cleaves the targeted RNA, and after cleavage the Ago2/RNA complex is released, allowing it to bind and cleave additional target RNAs.

are several approved antisense drugs that work through the RNase H1 mechanism, and multiple drugs are in clinical development (5, 14). One RNase H1 drug, inotersen, is approved to treat the neurological disease hereditary transthyretin polyneuropathy (15) and nine RNase H ASOs are in clinical development (**Table 1**). ASOs that work through the RNA interference pathway [e.g., small interfering RNAs (siRNAs)] are generally delivered to cells as an RNA duplex, or modified RNA, where the two strands dissociate within the cell and the antisense strand (also termed the guide RNA) binds to Ago2. Ago2 is a member of the argonaute family of proteins, which contains an RNase H–like domain. The bound antisense strand directs the RNA-Ago2 complex to the targeted RNA, where it cleaves the RNA, releasing the complex to bind to another RNA. ASOs that utilize the RNA interference mechanisms are also commonly used as research tools and are gaining momentum as therapeutic agents, with the approval of siRNA drugs in 2019 to treat acute hepatic porphyria (5, 16) and a second drug, patisiran, in 2018 to treat hereditary transthyretin polyneuropathy (17). To date, we know of no siRNA drugs in clinical development for CNS diseases, although optimization of the design and delivery of siRNAs for CNS diseases is continuing (18).

ASO drugs can also bind to a target pre-messenger RNA (pre-mRNA) and interfere with its maturation or interfere with the function of the mature messenger RNA (mRNA) through non-degradative mechanisms (5, 11) (**Figure 2**). Blocking protein translation by ASOs is one example

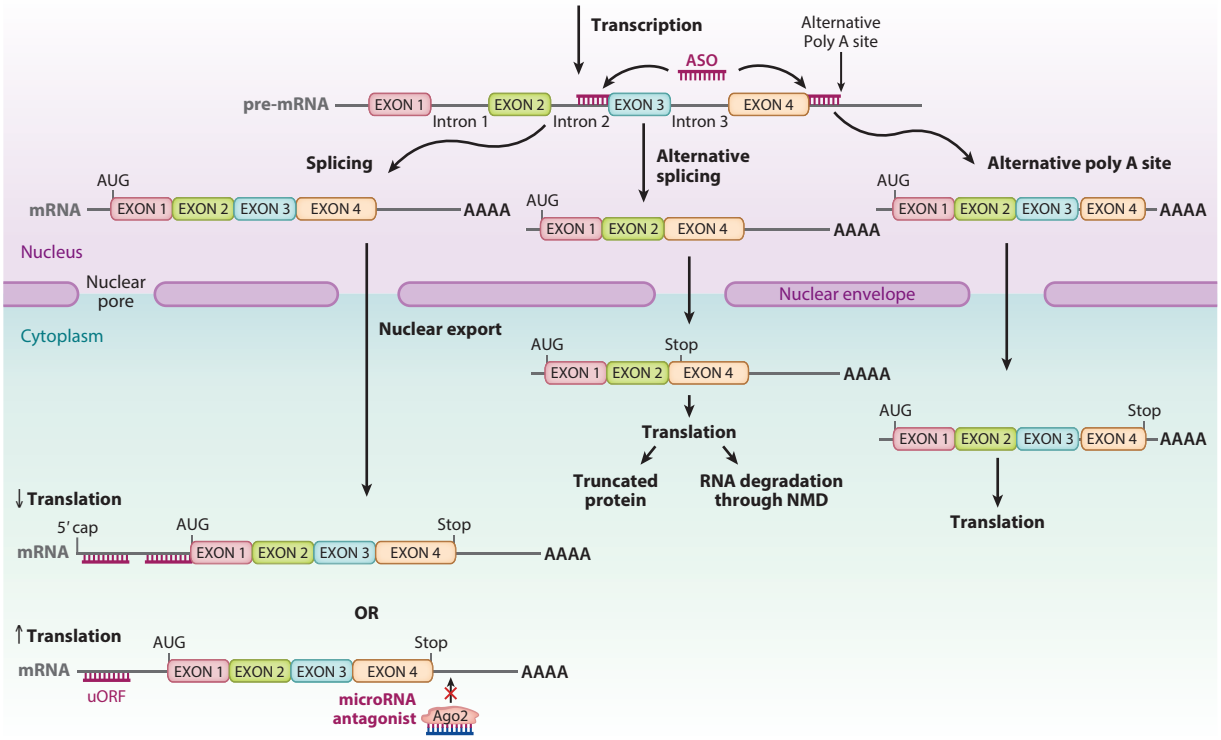

Figure 2

Non-RNA degradation antisense mechanisms (occupancy only). RNA is transcribed from DNA into a pre-messenger RNA (pre-mRNA) form that undergoes several posttranscriptional processing events, such as splicing, to remove intronic sequences and polyadenylation to form the mature mRNA. The mRNA is exported out of the nucleus to the cytoplasm, where it is translated to its protein product. Antisense oligonucleotides (ASOs) can be designed to modify RNA-processing events in the nucleus, such as modulate RNA splicing, to exclude or include a protein-coding exon. In some cases, excluding specific exons could result in a truncated protein product, or alternatively, the RNA missing a specific exon is recognized by the cell as misspliced and is degraded by the nonsense mediated decay (NMD) pathway. ASOs can also be designed to alter polyadenylation (poly A) site selection, resulting in loss of RNA regulatory sequences in 3′-untranslated regions (UTRs) of the RNA. In the cytoplasm, ASOs can be designed to decrease translation of the RNA to proteins by binding to sequences in the 5′-UTR of the mRNA or by blocking microRNA binding to the RNA. ASOs can also be designed to block translation starting at an upstream open reading frame (uORF) or disrupt regulatory RNA structures, resulting in an increase in protein translation.

of a mechanism that does not degrade the RNA transcript but decreases protein production (19). The best-characterized examples of nondegradative mechanisms are ASOs that modulate pre-mRNA splicing to promote exclusion or inclusion of exons (20). Two antisense drugs have been approved for the treatment of distinct populations of patients with Duchenne muscular dystrophy, a disease caused by point mutations or deletions of one or more exons in the gene encoding the protein dystrophin (**Table 1**). Eteplirsen and golodirsen are approved for patients who can benefit from dystrophin exon 51 or exon 53 skipping to restore the correct reading frame and synthesis of a nearly full-length dystrophin that retains partial dystrophin function (21). A second splicing strategy is to promote exon inclusion, which was the strategy used to treat spinal muscular atrophy with nusinersen (22, 23). A third splicing strategy is to promote degradation of the RNA transcript by exon skipping to produce an RNA species recognized by the nonsense mediated decay machinery, which eliminates mRNAs that harbor a premature termination codon (24). ASOs

can also be used to block or displace access to the target RNA by proteins or other RNAs. An example is blocking exon junction complex proteins from binding to the RNA downstream of a premature termination codon, thereby blocking degradation of the RNA by nonsense mediated decay (25). Another example is the use of antisense drugs to modulate polyadenylation site selection (26). Finally, ASOs can be used to increase protein translation by blocking translation of upstream open reading frames, disrupting regulatory RNA structures, and blocking microRNA access to the 3′-untranslated region of the transcript (27–30).

OLIGONUCLEOTIDE CHEMICAL MODIFICATIONS

Unmodified DNA and RNA are unstable in biological systems and are not suitable as drugs. In developing nucleic acid therapeutics, two different strategies—formulations and chemical modifications—have been used to protect the nucleic acids from degradation. Chemical modification of ASOs has been used to enhance stability against endogenous nucleases, enhance binding affinity to RNA, and decrease unwanted toxicities. Modifications to the phosphate backbone, ribose sugar, and bases have been examined for utility as potential antisense drugs (11, 31).

Nucleic acids are degraded by both endo- and exonucleases, which cleave the phosphodiester bonds that link the nucleotides together. First-generation antisense chemistry focused on stabilizing the phosphodiester bond through chemical modifications including phosphorothioate, methyl phosphonate, and phosphorodiamidate morpholinos. Of these, the phosphorothioate modification, in which one of the nonbridging oxygen atoms is replaced with sulfur, is the most widely utilized (32). Most of the approved antisense drugs as well as drugs currently in development have some phosphorothioate modifications. In addition to stabilizing the ASO against degradation by nucleases, the phosphorothioate modification enhances protein binding, which can result in better tissue distribution and cell uptake, but protein binding also contributes to some unwanted side effects (11). Phosphorodiamidate morpholino oligonucleotides (PMOs) are sugar-phosphate substitutions, in which the sugar is replaced with a morpholine ring and the phosphate with a phosphorodiamidate linkage. Because of the neutral phosphorodiamidate linkage, morpholino oligonucleotides exhibit minimal protein binding and are rapidly excreted (33). The PMO modification does not support the RNase H1 or RNA interference mechanisms of action. PMOs are used primarily for modulating splicing, in which high doses are administered to compensate for the poor tissue distribution. Two PMO drugs are approved to treat Duchenne muscular dystrophy (**Table 1**), albeit neither has been documented to provide more than minimal restoration of dystrophin expression.

The ribose sugar has proven to be an important nucleic acid constituent by which chemical modifications have enhanced pharmacological effects. Sugar modifications are used to support splicing modulation, translation suppression, enhancement of translation, RNA-induced silencing complex (RISC)-based mechanisms, and RNase H1 mechanisms (**Figures 1** and **2**). Most sugar modifications do not support RNase H1 activity; therefore, they are generally incorporated at the ends of the oligonucleotide, leaving a DNA gap in the middle, and are often referred to as gapmers or second-generation ASOs. The 2′-*O*-methyl modification is a naturally occurring ribose modification. The other commonly used sugar modifications are not naturally found in RNAs. Examples include 2′-*O*-methoxyethyl (2′-MOE), 2′-fluoro, and bridged 2′-4′ modifications such as locked nucleic acid (LNA) and constrained ethyl (cEt). Most sugar modifications show enhanced nuclease stability (with 2′-fluoro being the exception) and increased binding affinity to RNA. LNA and cEt modifications, which have a bridged 2′-4′ linkage, demonstrate the greatest binding affinity for their target RNAs (34–36). Although their general trends are similar, each sugar modification has unique attributes. The ranking of the different sugar modifications in terms of RNA affinity is

roughly DNA < RNA < 2′-*O*-methyl ≤ MOE < 2′-fluoro ≪ cEt ∼ LNA (36, 37). For resistance to nuclease degradation, the general ranking is RNA < DNA < 2′-fluoro < 2′-*O*-methyl < LNA < MOE < cEt. In addition, each modification has unique protein-binding properties (38–40). Currently, the most effective drugs use a mixture of different chemical modifications that have been optimized for the specific indications.

DISTRIBUTION OF ANTISENSE DRUGS IN THE CENTRAL NERVOUS SYSTEM

Today, most centrally delivered ASOs for neurological indications are 18–20 bases in length and thus 6–8 kDa. These negatively charged macromolecules do not cross the blood-brain barrier. In preclinical studies, ASOs are delivered directly into the cerebrospinal fluid (CSF) by injection into the lateral ventricles or the lumbar space to access the CNS. Clinically, ASOs are delivered into CSF by intrathecal injection into the lumbar space. Once introduced into CSF, small water-soluble molecules like ASOs distribute broadly throughout the CNS (22, 41–44). An ASO delivered into the nonhuman primate CSF via intrathecal bolus injection can be found throughout the spinal cord and cortex, often with higher ASO concentrations in the cortex than in the spinal cord (45). ASOs are delivered into and are effective in deeper brain regions, including the hippocampus, pons, and amygdala, although to a lesser extent than in the cortex or spinal cord (41, 45, 46).

The broad distribution of ASOs in the CNS is likely facilitated by intrinsic CSF dynamics. CSF surrounds the brain and spinal cord, occupying the open spaces of the ventricles, subarachnoid space, cisterns, sulci of the brain, and central canal of the spinal cord. In humans and nonhuman primates, CSF turns over approximately three to four times per day and accounts for approximately 10% of the total fluid volume in the intracranial space (47). CSF is in constant movement, driven largely by CSF production, cardiac cycle, and respiration (reviewed in 48, 49). In addition to bulk movement, CSF and interstitial fluid are continuously exchanged, with CSF moving through the ventricular ependymal layer, interstitial and perivascular spaces, and perineural lymphatic channels (reviewed in 50, 51). The exchange of CSF and interstitial fluid is facilitated by convective influx of CSF along the periarterial space into the brain parenchyma through the glymphatic system, with these convective forces driving movement of macromolecules through the parenchyma (52). This constant mixing and movement of CSF is likely a key factor in the broad distribution of ASOs within the CNS.

A combination of immunohistochemistry and live imaging of labeled ASOs has allowed for detailed kinetics of early ASO distribution (53). These data support a model for ASO distribution in which ASOs first associate with the pial membrane and the major cerebral surface arteries, suggesting that like other macromolecules ASOs may access deeper levels of the parenchyma by traveling through intramural perivascular spaces. ASOs then progress into the parenchyma likely by direct migration through the glia limitans via gap junctions or transcellular exchange and are detectable in the extracellular space. It is still not clear whether ASO distribution into the parenchyma occurs primarily via passive diffusion or convective forces or a combination of both. By 24 h after dosing, an ASO is present in the intracellular space and pharmacologically active. Maximal onset of pharmacological action is typically between 1 and 3 days after injection and is then maintained for weeks after dosing.

Once in the parenchyma, ASOs target the major cell types, including neurons, astrocytes, microglia, and oligodendrocytes (43, 54, 55). Cerebellar granule cells, despite robust activity in neighboring Purkinje cells (56), appear partially resistant to the actions of ASOs. The mechanism for lower distribution and activity for granule cells is not yet known.

Distribution into the parenchyma is dose dependent, with an increase in neuronal ASO levels with increasing doses (53). Conversely, ASO levels in pia and perivascular spaces tend to be more constant across pharmacologically active dose levels. This constancy is likely due to rapid absorption and saturation by vascular intramural basement membranes of ASOs, allowing for increased penetration across these membranes with increasing doses.

Insight into the pharmacology of ASOs can be gleaned by quantifying ASO activity in individual neurons in the dorsal root ganglia. Due to the unique architecture and stereotypical neuronal sizes of the dorsal root ganglia, it is possible to quantify pharmacology in three neuronal subtypes exposed to similar amounts of ASOs. Here, all three neuronal populations exhibited equivalent dose-dependent suppression in target RNA (57). This favors a model in which low doses of ASOs target most cells modestly and evenly rather than a subset of cells robustly. However, more work is needed to replicate this across cellular subtypes and in human samples, as a limited data set in human patients suggested potentially more heterogeneity (58).

Given these principles and observations, it is not surprising that modulation of the ASO dosing paradigm can alter distribution. Enhancing convective forces either by increasing dose volume or by applying percussive force improves distribution of ASOs up the neuraxis. Altering dose volume from 7% to 17% of total CSF changes the cord-to-cortex ratios, with higher cortical levels reached with higher dose volumes (59). Similarly, ASO bolus injection leads to wider and more efficient distribution than slow infusion does (42). This is likely due to higher maximal drug concentrations (C_{max}) achieved in CSF driving ASO into productive compartments, as has previously been demonstrated for the liver (60). In the liver, ASOs can be taken up into a productive compartment in the nucleoplasm or cytoplasm, where ASO can access the target RNA, and a nonproductive compartment, where ASO is unable to access the target RNA (60, 61). One can imagine a paradigm in which simple changes in delivery technique can be used to achieve a desired distribution.

PRECLINICAL AND CLINICAL EXPERIENCE OF CENTRALLY ADMINISTERED ANTISENSE DRUGS

Currently, five antisense drugs are approved to treat neurological diseases (**Table 1**). Four of the five drugs are delivered systemically, targeting either skeletal muscle or liver, and have been previously reviewed (5, 14, 62, 63). The only approved antisense drug that is administered by intrathecal injection is nusinersen, which is used to treat spinal muscular atrophy. Nine additional drugs, all delivered intrathecally, are in clinical trials for the treatment of amyotrophic lateral sclerosis, Huntington's disease, Alzheimer's disease, Parkinson's disease, and Angelman syndrome. Further, there are multiple drug discovery programs to identify intrathecally administered antisense drugs for the treatment of common, rare, and ultrarare neurological diseases (56, 57, 64–71). Highlighting the efficiency and acceptance of intrathecally administered ASOs, a personalized antisense drug was identified in 2019 and tested in a single patient with a unique form of Batten's disease (72) and in a patient with amyotrophic lateral sclerosis caused by a mutation in the fused in sarcoma (*FUS*) gene (73). A comprehensive review of antisense drugs used to treat neurological diseases is beyond the scope of this article, and the reader is referred to several reviews on the topic (5, 14, 74). Here, we focus on intrathecal antisense drugs, examples that highlight lessons that translate across the platform.

Spinal Muscular Atrophy: Nusinersen

Spinal muscular atrophy (SMA) is a severe pediatric neuromuscular disease characterized by muscle weakness and atrophy secondary to degeneration and death of neurons. Prior to therapeutic

intervention, SMA was the most common genetic cause of infant mortality, with a life expectancy of less than two years for the most severe form of the disease (75, 76). The disease is caused by mutations in or deletions of the survival motor neuron 1 (*SMN1*) gene (77). Humans have a *SMN1* paralog, *SMN2*, which was generated by an inverted duplication of the 5Q13 chromosomal region. *SMN2* differs from *SMN1* by approximately five nucleotides, one of which is a C-to-T transition in exon 7 that disrupts an exon splice enhancer and creates an exon splice suppressor. As a result, exon 7 was skipped in approximately 80% of *SMN2* transcripts. The transcripts missing exon 7 produce a truncated protein that is rapidly degraded (78), whereas the 20% of the transcripts containing exon 7 make full-length SMN protein identical to the protein derived from the *SMN1* gene. Thus, SMA can be thought of as an SMN protein deficiency disease. This is further supported by the observation that patients with more copies of the *SMN2* gene tend to have a milder form of the disease (79). SMA presents as a phenotypic spectrum on which it can roughly be classified as type 1, type 2, or type 3. Type 1 SMA is infantile onset, in which infants never gain the ability to sit and have a short life expectancy (less than 2 years). Types 2 and 3 SMA have later ages of onset, usually in early childhood. Children with type 2 SMA achieve the ability to sit but cannot walk independently, and children with type 3 SMA gain the ability to walk but often lose this ability as they develop (80).

Nusinersen is an antisense drug that binds to a site in intron 7 of the *SMN2* pre-mRNA, displacing hnRNP A1 and A2 proteins, which negatively regulate splicing of exon 7, resulting in full-length *SMN2* transcripts (23, 39). In multiple preclinical models, nusinersen enhances *SMN2* exon 7 inclusion, improves muscle function, and enhances survival (23, 81–85). These findings, plus those from safety and tolerability studies of rodents and nonhuman primates, supported advancing the drug into development. The drug was broadly studied in all SMA types, demonstrating beneficial effects across the different patient populations (22, 86–91). Nusinersen has been approved in over 40 different countries to treat SMA.

Several important lessons from nusinersen could have broader implications for antisense drugs being used to treat other neurological diseases. First, the discovery and development of nusinersen was a collaborative project by the many stakeholders that effectively capitalized on shared interests in finding a therapy. Second, the studies demonstrated for the first time that SMA is a treatable disease. Third, studies of genetically diagnosed, presymptomatic patients demonstrated remarkable benefit, with many children achieving motor milestones that included sitting, standing, and walking within the normal age-appropriate windows (91). These findings demonstrated that administering treatment before symptoms develop may prevent or markedly minimize disease symptoms for a neurodegenerative disease, which was intuitive but not previously proven. Finally, nusinersen was important for the field of ASO because it demonstrated that the technology can create commercially successful drugs that have a major impact on patients' lives.

Amyotrophic Lateral Sclerosis

Amyotrophic lateral sclerosis (ALS), also known as Lou Gehrig's disease, is a severe neurodegenerative disease with progressive muscle weakness and paralysis leading to respiratory failure and death (92). The primary pathology observed in ALS is degeneration and death of upper and lower motor neurons and denervation-induced muscle atrophy. Although the cause of disease for most cases is unknown, approximately 10–20% of cases are inherited. More than 50 genes that cause or contribute to ALS have been identified to date (93). Superoxide dismutase 1 (*SOD1*) was the first example of a gene whose mutations cause familial forms of ALS and they account for approximately 20% of familial ALS cases (94). More than 170 different ALS-causing mutations have been identified. Multiple molecular mechanisms by which mutations in SOD1 protein cause ALS

have been proposed, but to date there is no agreement on a unifying mechanism (95). Most data implicate a toxic gain of function rather than a loss of function in the SOD1 protein (95, 96).

Examining the functions of more recently identified genes whose mutation is causative or contributory to ALS has not identified a common disease mechanism. Putative ALS-causing genes appear to impact several important areas of cell biology, including RNA metabolism (e.g., *TARDBP*, *FUS*, *hnRNPA1*, *MATR3*, *C9orf72*, *ANG*), autophagy (*OPTN*, *SQSTM1*, *TBK1*), intracellular transport (*DCTN1*, *TUB4A*, *PFN1*), and proteostasis (*SOD1*, *VCP*), suggesting that perturbation of several biological pathways can lead to dysfunction and death of motor neurons.

The first use of ASOs as a treatment for a neurodegenerative disease targeted SOD1 for inherited ALS (44). A key factor in deciding to use SOD1 as a model system to learn about the behavior of ASOs delivered into the CNS was the overwhelming data supporting that the mutant SOD1 protein caused ALS through a toxic gain of function, providing a well-validated target (95–99). In addition, the initial study population was well defined and had a consistent natural history based on specific mutation (e.g., a good genotype-to-phenotype correlation) (100, 101). Finally, several systemically dosed, second-generation, 2′-MOE-modified ASOs had advanced in the clinic, providing comfort regarding safety and tolerability when applied systemically.

Preclinical studies documented dose-dependent reduction in SOD1 mRNA and protein in spinal cord and different brain regions following injection into the lateral ventricle of wild-type and transgenic rats (44). On the basis of the efficacy and safety of the ASO drug ISIS 333611, it was advanced to a phase I clinical study of ALS patients with mutations in *SOD1* (102). Because this was the first antisense drug to be delivered via the intrathecal route, the first study was focused primarily on safety. A 12-h infusion of the drug into the intrathecal space was well tolerated. While the study was conducted significant advances in screening ASOs for CNS applications had been made, resulting in more potent and well-tolerated drugs. In addition, preclinical data demonstrated that broader delivery within the CNS and better efficacy were achieved by bolus injection than by constant infusion (42); therefore, it was decided to terminate development of ISIS 333611 and advance a drug into development that took advantage of the improved screening.

Tofersen was identified following extensive screening in cell culture and transgenic rodents against the human SOD1 pre-mRNA. Tofersen was more potent than ISIS 333611 (46). It exhibited enhanced activity in mouse and rat SOD1 models of ALS (46). Tofersen was advanced to a single ascending dose and multiple ascending dose (MAD) phase I/IIa study of ALS patients with pathogenic mutations in *SOD1*. Forty-eight subjects participated in the study and received all planned doses, ranging from 20 to 100 mg in the MAD portion of the study (103). Patients in the MAD portion of the study received 5 doses on days 1, 15, 29, 57, and 85 by bolus intrathecal injection. Dose-proportional plasma exposure was observed, but trough CSF concentrations (e.g., CSF concentration of the drug prior to administration of the next monthly dose) were less than dose proportional. Tofersen was generally well tolerated, with most adverse events ascribed to the underlying disease or the intrathecal procedure. Baseline values in CSF SOD1 protein were reduced in the 40-, 60-, and 100-mg-dose groups, consistent with tofersen's mechanism of action. In this short-duration study, there was a lessening of decline in clinical measures of the disease in the 100-mg-dose group, which was significant in patients with fast-progressing mutations. Consistent with a decrease in the rate of decline in clinical measures, baseline values of plasma and CSF phosphorylated neurofilament heavy chain and neurofilament light chain declined. These encouraging data supported advancement of tofersen to a pivotal phase III study, which is currently ongoing (**https://clinicaltrials.gov/ct2/show/NCT02623699**).

Hexanucleotide expansions in the first intron of the gene *C9orf72* were initially described in 2011as a pathogenic genetic change causative of ALS and frontotemporal dementia (3, 4). Mutations in *C9orf72* account for approximately 8–10% of all ALS cases and 40% of familial cases.

The size of the hexanucleotide repeat can range from fewer than 20 repeats in healthy controls to several thousand repeats in the nervous systems of affected individuals (3, 4). The mechanism(s) by which the hexanucleotide expansion causes neurodegenerative diseases is not well understood. The protein product from *C9orf72* has a conserved DENN domain and can function as a guanine nucleotide exchange factor for Rab proteins (104). Most data support three potential nonexclusive mechanisms: RNA toxicity, in which the expanded repeat sequesters RNA-binding proteins; translation of the repeat RNA to polydipeptides (RAN translation); and a decrease in C9orf72 expression (3, 105–107). Toxicity from repeat-containing RNAs or their RAN translation products appears to synergize with reduced C9orf72 protein produced from the affected *C9orf72* allele (108).

ASOs designed to bind to the C9orf72 transcript containing the hexanucleotide expansion decreased the number of repeat-associated RNA foci, improved electrophysiological changes, and decreased sensitivity to neurotoxins and repeat-associated dipeptides in human fibroblasts and induced pluripotent stem cells (iPSCs) (106, 109–111). In mice expressing human *C9orf72* gene with a 500-nucleotide hexanucleotide expansion, one-time treatment with an ASO targeting *C9orf72* repeat-containing RNAs reduced dipeptides derived from the expansion and attenuated the behavioral effects (112).

The collective data support pathogenesis arising from an acquired toxicity combined with the reduction in C9orf72 expression observed in the clinical samples. Correspondingly, an "on disease mechanism" therapy would be to suppress the repeat-containing RNAs without exacerbating reduction in protein-coding C9orf72 mRNAs. Consistent with this, an antisense drug, BIIB078, has been identified that selectively targets C9orf72 transcripts with repeat expansions but does not target most C9orf72 protein-encoding RNAs, thereby preserving expression of C9orf72 protein from RNAs produced by the unaffected *C9orf72* allele (109). This drug is currently in phase I/II MAD clinical study (**https://clinicaltrials.gov/ct2/show/NCT03626012**).

Given the early success targeting familial forms of ALS, there is increased interest in identifying targets for sporadic forms of the disease. A frequent histological observation in motor neurons in the cortex and spinal cord from autopsies of patients with ALS is the presence of cytoplasmic inclusions that contain TAR DNA-binding protein 43 (TDP-43) (113). TDP-43 is an RNA-binding protein involved in transcription, RNA processing, and nuclear cytoplasmic transport. A pathological feature identified in iPSC-derived motor neurons and autopsy samples from patients with ALS is a defect in nuclear import, possibly through a defect in the nuclear pore (107, 114). These observations are being extensively studied and not only provide insights into the pathogenesis of ALS but may also identify additional drug targets. As an example, the cytoplasmic retention of TDP-43 is associated with loss of TDP-43 in the nucleus, resulting in changes in RNA metabolism (115, 116).

One transcript that is markedly decreased in ALS-derived iPSCs and spinal cord tissues is the transcript that encodes stathmin-2, a tubulin-binding protein thought to play a role in neurite outgrowth by affecting microtubule dynamics. Investigation into the mechanism of stathmin-2 decrease in neurons revealed the presence of a cryptic splice and polyadenylation sites within intron 1 of the stathmin-2 pre-mRNA, adjacent to which are a trio of TDP-43-binding sites (117, 118). Loss of nuclear TDP-43 results in use of cryptic splicing and early polyadenylation sites in the stathmin-2 transcript and thus decreased levels of the protein. Strategies to increase the expression of stathmin-2 could be of therapeutic utility for patients with ALS.

Another target for ASO therapy in sporadic ALS is the RNA encoding the RNA-binding protein ataxin-2, which was identified in a yeast screen as TDP-43 modifier (119). While a long trinucleotide expansion in the ataxin-2 gene causes ataxia (120), an intermediate CAG repeat (27 to 33 repeats) is found at an enhanced rate in patients with sporadic ALS (119). Genetic depletion

of ASOs to decrease ataxin-2 expression increased survival in a mouse model of TDP-43 neurodegenerative disease, providing a therapeutic rationale for targeting ataxin-2 as a potential therapy for ALS (121).

Although investigations are still in the early stages, these results show promise that disease-modifying therapies for patients with ALS will become available.

ALZHEIMER'S DISEASE

Given the high societal impact and unmet medical need, evaluating multiple therapeutic targets and strategies for the treatment of Alzheimer's disease (AD) is warranted. Two therapeutic targets heavily researched as potential therapies for AD are amyloid precursor protein (APP) and tau (122). Multiple approaches to modulate APP are being pursued in the clinic, including preventing processing of the protein and enhancing clearance of amyloid deposits (122). Initial preclinical work has tested the efficacy of APP-targeting ASOs in cell culture and mouse models of AD (123–126). Given the limited clinical success of agents that modulate the processing of APP or enhance clearance of amyloid plaques, preventing the synthesis of APP merits further investigation as a therapeutic strategy for AD.

Tau protein, which is coded for by the gene microtubule-associated protein tau (*MAPT*), has been broadly implicated in contributing to pathology in AD, primary tauopathies, and other neurodegenerative diseases (122, 127). Like most other proteinopathies associated with neurodegenerative diseases, the precise mechanism by which the protein causes disease is not well understood but appears to be linked to intracellular misfolding and aggregation of the protein and cell-to-cell spread of pathogenic forms. In contrast to amyloid deposits, the appearance of tangles of tau protein is closely linked to onset of symptoms in patients with AD (122). Multiple drugs that affect folding, prevent cell-to-cell spread, block synthesis, or enhance clearance of tau protein are being tested. ASOs can be used to block the synthesis of tau protein or to synthesize a potentially less pathogenic form of tau through alternative RNA splicing and are currently being explored as potential therapies (41, 128–131). An RNase H ASO targeting *MAPT* pre-mRNA decreased the amount of phosphorylated tau in brain tissue, preserving hippocampal neurons and enhancing survival in a mouse model expressing a pathogenic form of tau (41). In addition, lowering tau protein levels with an ASO reduced sensitivity to seizures in mice (128), consistent with genetic manipulation of tau (132). An ASO (IONIS-$MAPT_{Rx}$) that reduces the synthesis of tau protein through an RNase H mechanism of action is currently in a phase I/II clinical study of patients with early-onset AD (**https://clinicaltrials.gov/ct2/show/NCT03186989**).

Parkinson's Disease

Parkinson's disease (PD) is a progressive movement disorder and is the second-most-common neurodegenerative disease, affecting approximately 1% of the population over age 60 (133). PD can be idiopathic or familial, with both dominant and recessive inheritance patterns. Though palliative care exists, there is no disease-modifying therapy. The pathological hallmark of idiopathic PD and some familial PD is the accumulation of α-synuclein into Lewy bodies and Lewy neurites and the subsequent loss of dopaminergic neurons (134). α-Synuclein is thought to be an underlying driver of PD, as duplication and triplication of *SNCA*, the gene that encodes α-synuclein, cause autosomal dominant PD. Similarly, α-synuclein fibrils injected directly into the CNS can propagate and are directly toxic to dopaminergic neurons (135). Thus, it is not surprising that suppression of α-synuclein with ASOs has disease-modifying benefits in multiple models (136, 137). Indeed, preventing production of α-synuclein can even reverse existing pathology and prevent dopaminergic cell death (137).

Dominantly inherited mutations in leucine-rich repeat kinase 2 (*LRRK2*) are the most common cause of familial PD (138). Patients with *LRRK2* mutations are clinically and pathologically indistinguishable from those with idiopathic PD. ASO-mediated suppression of LRRK2 mRNA in an α-synuclein model of PD reduced the pathological propagation of α-synuclein and prevented motor deficits and loss of dopaminergic cells (139). Because ASOs that reduce CNS LRRK2 mRNA are delivered centrally with limited systemic exposure, they do not have the same systemic on-target liabilities that small-molecule inhibitors of LRRK2 protein do. A LRRK2 mRNA-targeting ASO is currently in an early-stage clinical study (**https://www.clinicaltrials.gov/ct2/show/NCT03976349**).

ASO drugs have the potential to be transformative disease-modifying therapies for PD. By targeting the primary underlying disease mechanism, either α-synuclein or LRRK2, ASO drugs can prevent the loss of dopaminergic cells. Suppression of these targets is clearly beneficial after established pathology. PD is a particularly interesting case for disease-modifying therapies, because good palliative therapies already exist. It is possible that intervention in later, more established cases of disease may have a greater opportunity for benefit than other neurodegenerative diseases, because combination therapy is possible. One can envision a scenario in which disease pathology and progression are halted with an ASO and established symptoms can be well managed with existing palliative care.

Huntington's Disease: Tominersen

Huntington's disease (HD) is caused by expansion of a CAG repetitive sequence in the first exon of the huntingtin gene (*HTT*) (1). The CAG codon is translated to glutamine, resulting in an expanded polyglutamine track in the amino terminus of the HTT protein. Although the normal cellular function of HTT and the mechanism(s) by which the expanded CAG tract causes HD are not well understood, most data indicate the mechanism is a dominant gain of function of the protein (140). At least three different antisense approaches have been investigated as potential treatments for HD: selectively blocking translation of the RNA containing the CAG expansion, selective reduction of the mutant RNA, and nonselective knockdown of wild-type and mutant HTT RNAs (43, 141–146). In general, blocking translation of the mutant HTT (mHTT) RNA has proven to be challenging due to the limited in vivo potency of the ASOs used. Allele-selective silencing through either RNase H or siRNA selective targeting of single-nucleotide polymorphisms linked to the disease-causing allele would be promising strategies. However, these approaches are constrained by the limited sequence space available surrounding unique single-nucleotide polymorphisms for designing ASOs, which could impact potency and safety of the antisense drug (147). In addition, the lack of a shared polymorphism among all patients with HD means that a single drug would be applicable only to a subpopulation of patients with HD (147). The most advanced approach is nonallele-selective reduction of HTT-encoding RNAs through an RNase H mechanism. Preclinical data from mouse models have demonstrated dose-dependent reduction of HTT expression yields sustained, partial disease reversal, including improvements in clinical phenotypes in several mouse models of HD, including the R6/2 mouse model of HD, in which the ASO prevented loss of brain mass (43, 45, 148).

A phase I clinical study evaluating the safety and tolerability of tominersen (also known as IONIS-HTT_{Rx} or RG6042) was completed in 2017 (**https://clinicaltrials.gov/ct2/show/NCT02519036**) (149). Tominersen is a chimeric 2′-MOE/DNA ASO designed to reduce both mutant and wild-type HTT expression through an RNase H mechanism of action. The first-in-human study was a randomized placebo-controlled dose escalation study in which subjects were administered four monthly doses of tominersen by bolus intrathecal injection. Five

dose groups were analyzed, 10, 30, 60, 90, and 120 mg. The drug was well tolerated at all dose levels, with adverse effects being mild or moderate in severity and none ascribed to the study drug. Dose-dependent trough CSF concentrations appeared to plateau at the highest-dose groups. Importantly, a dose-dependent reduction in CSF levels of mHTT protein was observed, supporting the mechanism of action of the drug. No group-wise changes in clinical outcomes were observed, which was expected given the short duration of the treatment and small number of patients in each cohort (149). A global phase III study of tominersen is currently enrolling 801 subjects who will be randomized to receive placebo, 120-mg tominersen every 2 months, or 120-mg tominersen every 4 months (Generation HD1; **https://clinicaltrials.gov/ct2/show/NCT03761849**).

Two allele-selective antisense drugs have advanced to clinical studies, WVE-120101 and WVE-120102 (**Table 1**). However, publicly available information regarding the chemistry, design, and preclinical data for these antisense drugs is limited.

Spinocerebellar Ataxias

The spinocerebellar ataxias (SCAs) are a growing class of more than 30 diseases (150). They are progressive neurodegenerative diseases characterized predominantly by cerebellar dysfunction, which is often accompanied by broader CNS involvement. Patients typically suffer from incoordination, loss of balance, speech impairments, and early mortality (151). There are no disease-modifying therapies. The most prevalent diseases in the class are dominantly inherited SCAs caused by expansion mutations with a toxic gain of function. This group includes, but is not limited to, SCA1, SCA2, and SCA3 caused by CAG expansions in the *ATXN1*, *ATXN2*, and *ATXN3* genes, respectively.

ASO-mediated suppression of the disease genes in rodent models robustly ameliorates symptoms. Transient suppression of *Atxn1* in SCA1 knockin mice results in sustained improvement of motor phenotype, improvement of neurochemical abnormalities detected by magnetic resonance spectroscopy, normalization of SCA1 disease-associated genes, and extension of survival (56). Suppression of *ATXN2* in mouse models of SCA2 improves motor function, restores Purkinje cell neural networks, and normalizes expression of SCA2-related proteins expressed in Purkinje cells (68). ASO-mediated lowering of *ATXN3* results in reversal of nuclear ATXN3 accumulation, dose-dependent clearance of soluble and high-molecular-weight species, mitigation of motor deficits, repair of cerebellar network dysfunction, and changes in gene expression (64, 152, 153).

In these disease models, partial and transient suppression of the disease gene leads to a sustained improvement in phenotype. This improvement in phenotype did not happen immediately, but once it did, it was sustained. This suggests a model in which suppression of the disease-causing gene allows the system to recover, and once recovered, these slowly progressing diseases take time to become symptomatically detrimental again. This is similar to previous observations in models of HD, another CAG expansion disease (43, 154).

CONCLUSIONS

The approval of nusinersen as a treatment for SMA validated ASOs as a viable therapeutic approach for the treatment of neurodegenerative diseases, neurodevelopmental disorders, and possibly other diseases of the CNS. Several additional clinical studies have provided proof of mechanism (e.g., modulation of the targeted protein) and encouraging evidence of clinical benefit. The ongoing larger and longer-term studies are needed to provide robust evidence of clinical benefit, which will support the registration of additional antisense drugs. Like most technologies, ASO technology continues to evolve, resulting in drugs with improved potency and safety profile. To

help support the use of ASO technology in large patient populations, more convenient methods of administration will be needed. In summary, ASOs may finally provide a therapeutic platform for developing drugs to treat severe neurodegenerative and neurological diseases.

DISCLOSURE STATEMENT

C.F.B. and H.B.K. are employees of Ionis Pharmaceuticals and receive salary and stock options from the company. D.W.C. is a consultant for Ionis Pharmaceuticals.

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