

Antisense Oligonucleotides: Concepts and Pharmaceutical Applications


Ariana Araya

David Arias

Karla Coto

María Rebeca López

Joselyn Rivera

Juan José Mora* 

Department of Industrial Pharmacy,
Universidad de Costa Rica, San José,
Costa Rica

*email: juanjose.moraroman@ucr.ac.cr

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Abstract

Antisense oligonucleotides are drugs whose mechanism is based on binding to RNA target sequences. For this purpose, they modify the protein expression through steric hindrance and exon omission. Its production involves several steps: synthesis, purification, and lyophilization. Usually, the most complicated procedure is synthesis due to the chemical reactions necessary to add the required oligonucleotide bases. BP1001, inotersen, nusinersen, eteplirsen, and golodirsen are a few antisense drugs developed for treating neurodegenerative and neuromuscular diseases. Although antisense oligonucleotides present off-target reactions, multiple studies are being performed. The following review shows information regarding the pharmaceutical characteristics for industrial production and the current state of applicability in clinical practice. In conclusion, some molecules have already been approved for commercialization (inotersen, nusinersen, ataluren, eteplirsen, and golodirsen), showing them as promising therapeutic solutions in the short and medium term for disorders developed by specific genetic factors.

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INTRODUCTION

Since the last century, pharmaceutical and biotechnology industries have developed methods and strategies for the therapeutic use of proteins. Nonetheless, patients' diverse needs have led to the search for new alternatives. One of them is ribonucleic acid (RNA). Nowadays, various therapies are employed to modulate their function in cells, such as antisense oligonucleotides (ASOs)¹.

Grineva formulated the practical application of the ASOs principles in 1967. He proposed that the union of active chemical groups in oligonucleotides caused them to be directed to a particular complementary nucleic acid fragment. The chemical reaction occurred in a specific region of the target nucleic acid near the duplex structure formed. This approach was called the complementary-addressed modification². Later, Paul Zamecnik introduced that synthetic oligonucleotides could have significant therapeutic potential to block protein translation. The first studies were done to treat Rous sarcoma, a tumor produced by a retrovirus in chickens. Considering its origin, he demonstrated that a synthetic oligodeoxynucleotide created to bind to the virus RNA blocked its translation and, therefore, its replication³.

Antisense oligonucleotides development has guided the treatments' individualization and personalization, with multiple benefits in diseases' clinical approaches. Furthermore, they possess advantages that other pharmaceutical products have not provided in chronic sicknesses, with significant reductions in early and late response⁴. Another benefit is the time it takes to produce and trade new drugs and the high cost of these procedures. Antisense therapy research can reduce production and validation time through high throughput screening (HTS). Additionally, it facilitates deciding to keep those molecules that demonstrate activity against a specific target in development⁵.

Besides, genetic factors such as oncological, neuromuscular, neurodegenerative, cardiovascular, immune, and respiratory pathologies influence most illnesses that affect humans^{6,7}. For that reason, the research and elaboration of molecules whose mechanism is based on gene therapy are relevant to achieving a possible treatment against them⁸. Given this situation, the review aims to present a general scenario regarding antisense therapy, its pharmaceutical characteristics for industrial production, and its current applicability in clinical practice for different pathologies.

OVERVIEW OF ASOs

ASOs Participation in the Genetic Process

Deoxyribonucleic acid (DNA) and RNA are macromolecules called nucleic acids. They are responsible for the storage and inheritance of genetic material. As a complement, they can recognize and bind to specific targets. The basis of both molecules is nucleotides, and these units consist of a phosphate group, a sugar, and a nitrogenous base. In the case of DNA, the sugar is deoxyribose, and its bases correspond to adenine (A), thymine (T), guanine (G), and cytosine (C). Likewise, the RNA nucleotide sugar is ribose, and they have the same nitrogenous bases, except for uracil (U) instead of T⁹.

The Central Dogma of Molecular Biology states that DNA stores genetic information, proteins perform biological functions, and RNA is a transmission bridge between molecules. Approximately 2% of genes are eventually translated into proteins in the human genome, while 90% are transcribed into non-coding RNA (ncRNA)¹⁰. Transcription is a process where messenger RNAs (mRNAs) are synthesized¹¹. This material comprises a single-strain synthesized by an RNA polymerase, which has the amino acid sequence that encodes for a protein. Obtaining a protein from mRNA nucleotides is called translation¹².

Before translation, the RNA must go through splicing, where its introns are removed¹³. This portion that does not encode for a protein must be eliminated before the messenger RNA leaves the cell nucleus¹⁴. In the case of prokaryotes, the process does not exist. For them, transcription and translation are coupled processes¹⁵. Translation leads to protein biosynthesis. Three nucleotides of the RNA sequence (codon) encode for an amino acid, and its complementary sequence (the anticodon) is in the transfer RNA (tRNA)¹⁶. The main target of ASOs is mRNAs, as shown in **Figure 1**. If they are inhibited, the proteins associated with them, often responsible for certain diseases, would not be formed¹⁷.

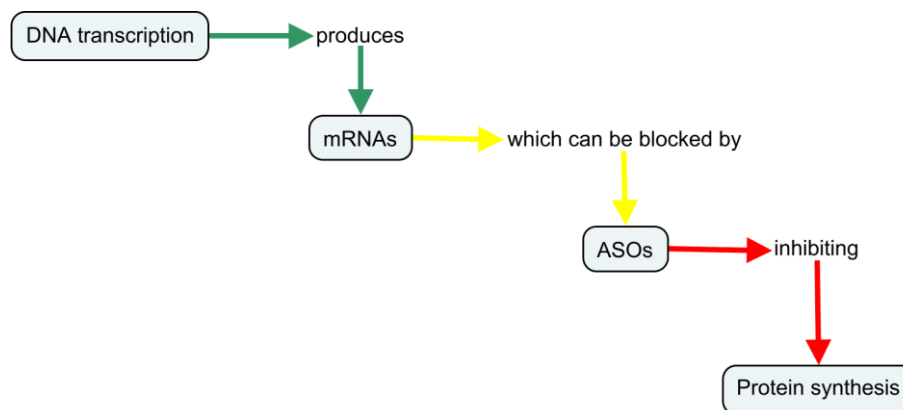


Figure 1. Diagram of protein synthesis inhibition through the ASOs

Principal Characteristics

ASOs are chemically synthesized. They are 12 to 30 nucleotides long and are designed to bind to RNA according to pairing rules (A-T and G-C)¹. Its length determined its specificity. Those with 16 to 20 nucleotides can bind exclusively to target RNA, allowing its function through cleavage, degradation, and steric blocking¹⁸. In contrast, by binding to partially complementary sites (sites with a sequence similar to the RNA of interest), adverse effects like hepatotoxicity are generated by mechanisms equal to those utilized over its target¹⁹⁻²². They are destroyed by RNase-H1, an endogenous nuclease that recognizes the duplex formed between the antisense drug and the target RNA. Thus, non-complementary junctions with non-target RNAs play a major role in addition to length²³⁻²⁵.

Antisense drugs comprise several classes of oligonucleotides. They have complementarity with target RNA molecules, including viral RNA and mRNA, and the binding to the specific sequence inhibits its function. Four major classes have been described: oligodeoxyribonucleotide (ODN), small interfering RNAs (siRNAs), RNAzymes, and DNAzymes⁴. Their characteristics are shown in **Table I**.

Table I. Principal characteristics of the antisense molecules that are currently identified⁴

Characteristics	Antisense molecule			
	ODN	siRNA	RNAzyme	DNAzyme
	Linear single-strand DNA structure, 12 to 25 base pairs long. It acts through the enzymatic activity of ribonucleases such as RNases H, L, or P.	Linear double-strand RNA structure, 21 to 25 base pairs long. It works through an RNA-induced silencing complex (RISC).	Complex RNA structure, with single- and double-band sections, comprises more than 30 base pairs. It functioned through its inherent enzymatic activity.	Single-strand DNA structure, 30 to 35 base pairs long. It acts through its inherent enzymatic activity.

Mechanism of Action

One of its features is reducing protein expression levels associated with the central mechanisms of particular illness development. They modify the expression of a target mRNA by altering splicing or recruiting RNase H. Therefore, it is a catalytic effect, and a single ASO can participate in the destruction of many RNA molecules^{4,26,27}. Other mechanisms include inhibition of translation by steric hindrance, exon skipping, destabilization of pre-mRNA in the nucleus, and targeting the destruction of miRNA that control others' expression (**Table II**). The steric hindrance and modulating splicing strategies do not use RNase H²⁷.

A drawback is that natural or unmodified nucleic acids are susceptible to nuclease degradation since they digest or cleave the phosphodiester bonds of DNA, RNA, and/or their hybrid²⁸. Also, they need better binding to plasma proteins by performing strong interactions with them. Therefore, they exhibit faster blood clearance, mainly due to blood metabolism or urine excretion²⁹. Multiple modifications to nucleotides and their linkages can improve some properties, augmenting their suitability as a drug. However, they cause changes in their pharmacokinetics and pharmacodynamics. In some cases, such a situation leads to the impossibility of employing the RNase H cleavage mechanism (desired for many ASOs). Plus, in their development as therapeutic agents, the mechanism of action depends on the target sequence and the chemistry utilized in its design²⁶.

Table II. Mechanisms of action of antisense drugs³⁰⁻³⁶

Strategy	Mechanism
Steric hindrance	Inhibition of translation by blocking the binding of protein complexes such as ribosomal units.
Exon skipping	Interruption of signal sequences to affect splicing.
Pre-mRNA destabilization	Strand invasion by its union, altering the splicing.
Targeted destruction of miRNA	Destruction by site-selective ribonucleases bound to antisense drugs. Ribonucleases destroy RNA by cutting phosphodiester bonds.

Structural Modifications

The chemical modification of the first-generation ASOs sought to reduce nuclease degradation. This strategy involved the replacement of one non-bridging oxygen atom in the phosphate group with sulfur groups (phosphorothioates), methyl groups (methyl phosphates), or amines (phosphoramidates). The phosphorothioate substitution is the oldest and most widely employed, as it supports RNase H activity against ASO binding to target RNA, renders internucleotide linkages resistant to nuclease degradation, and enhances cell penetration properties^{2,27,29}. Subsequently, new chemical modifications were developed to overcome diverse non-sequence-specific issues with improved nuclease resistance and binding affinity. The 2'-ribose changes include 2'-O-methyl (2'-OMe), 2'-fluoro (2'-F), 2'-O-methoxyethyl (2'-MOE), 2',4'-constrained 2'-O-ethyl (cEt), locked nucleic acid (LNA), morpholino oligomers (MO), and peptide nucleic acids (PNAs). These modifications developed second and third-generation ASOs^{2,27}.

Distribution

Besides the above modifications, delivery systems were developed to penetrate cell membranes. Most have a negative charge because of some adjustments, such as an oxygen substitution in the phosphodiester group by sulfur, carbon, or

nitrogen^{8,37}. Moreover, oligonucleotides can trigger the innate immune response in B cells through mechanisms like the activation of toll-like receptors^{38,39}. Both characteristics affect their incorporation into cells. Some systems established for a correct distribution correspond to liposomes, cationic polymers, and conjugated ligands⁴⁰.

Liposomes are vesicles composed of phospholipids⁴¹. When combined with oligonucleotides, they form complexes that allow better drug distribution. This structure facilitates binding to the cell membrane and is introduced by endocytosis⁴². Cationic polymers increase the oligonucleotides' introduction into the cell through electrostatic interactions with the cell membrane. The result is endocytosis stimulation⁴³. Finally, the conjugated ligand methodology binds an antisense drug receptor ligand. As a complement, the therapy can reach the specific cell receptor^{44,45}.

Pharmacokinetics

The principal administration route for systemic applications is a parenteral injection (intravenous infusion and subcutaneous injection)⁴⁶. Their pharmacokinetics have been investigated mainly in compounds modified with sulfur because, without these modifications, they are easily eliminated by various factors. They have a distribution phase of a few hours regarding phosphorothioates, followed by an elimination phase of several weeks. It is characterized by high concentrations in organs such as the liver and kidney. Its half-life is two to three days. Despite this, molecules with additional sugar modifications will have a longer half-life, going from days to weeks¹.

Furthermore, these drugs are more than 85% bound to plasma proteins. Their decrease means greater renal clearance during the distribution phase²⁹. It should be noted that the oligonucleotides have multicompartiment pharmacokinetics. The high concentration determines the transfer from the membrane to the cell interior in the liver and kidneys, the low metabolism rate, and its slow release and distribution from the tissues to the circulation⁴⁷.

Production

ASOs manufacturing process consists of five main steps: synthesis, cleavage and deprotection, purification, desalting and concentration, and lyophilization. Additional stages can be done depending on the type to be manufactured. Each phase requires equipment for that process part and associated controls to ensure reliability and batch quality. The most complicated step is synthesis, demanding sophisticated apparatus and specialized software⁴⁸. Solid-phase synthesis has been used for its manufacture through phosphoramidite monomers. They are nitrogenous bases with protecting groups that prevent the amine, hydroxyl, and phosphate groups on the nucleotides from undergoing unwanted side reactions⁴⁹. The bases are added sequentially on a controlled pore solid support during synthesis to generate the desired oligonucleotide⁵⁰. Each base addition cycle consists of four chemical reactions⁵¹, described in **Table III**.

Oligonucleotide deprotection involves removing a cyanoethyl (CNET) protecting group from the phosphate backbone, oligonucleotide chain cleavage from the support, and base deprotection. This process is generally performed in batch mode. The entire solid support is incubated with cleavage and deprotection reagents. After deprotection, the spent solid support is filtered, and the crude oligonucleotide solution is recovered⁵². Then the crude solutions are purified. Chromatographic methods achieve this part, and the selected type and its associated settings depend on the expected function. Although oligonucleotide primers require little or no purification, therapeutic products require chromatographic purification to ensure a high-quality active ingredient⁵³.

The most widely accepted option for manufacturing scale isolation is Tangential Flow Filtration (TFF). The technique employs an appropriately sized filter membrane and a pump to circulate the sample through the TFF setting, making the process more efficient. As the solution becomes concentrated, incorporating buffer or purified water desalinates the solution, removing ions and even oligonucleotide short chains formed as impurities (in case the HPLC did not produce fractions completely pure)⁵⁴. Finally, lyophilization is made through a freezer dryer (machine with a sample chamber, a condenser, and a vacuum pump). The three main steps in this stage are freezing, primary drying, and secondary drying. The final product is obtained in powder form by vacuum sublimation, providing long-term storage and more comfortable handling⁵⁵. Controlled pore glass (CPG) has been contemplated in solid-phase synthesis. It is composed mainly of silanol groups and is characterized by a narrow pore size distribution for efficient purification and a large internal surface area for oligonucleotide chains' high binding capacity. It is chemically stable to the solvents utilized during its synthesis⁵⁶.

In contrast, liquid-phase oligonucleotide synthesis (LPOS) is an alternative for large-scale manufacturing. An advantage regarding the solid phase is the absence of the process's heterogeneous nature, allowing greater accessibility of the reagents for the reaction. Also, it presents benefits in production costs, reducing the number of reagents and allowing their development in a conventional batch reactor without installing an automatic synthesizer necessary for solid-phase synthesis⁵⁷.

Table III. Reactions of the synthesis cycle of phosphoramidite monomers on the solid support⁵⁸

Reaction type	Description
Deprotection	The 5'-hydroxyl group of the first nucleoside attached to the resin is deprotected by trichloroacetic acid treatment.
Coupling	The nucleoside attached to the support is coupled with the following phosphoramidite, which is activated with tetrazole.
Blockade	The 5' hydroxyl groups of the oligonucleotide chains that did not react during the coupling reaction are blocked by acylation with acetic anhydride.
Oxidation	Oxidation of the phosphite-triester formed during the coupling reaction occurs using iodine and water.

Quality Control

Oligonucleotides and their metabolites' determination in complex matrices at a low concentration pose a challenge. Proper quality control is crucial. This concept implies obtaining reliable results through measurement traceability, uncertainties estimation, reference materials, interlaboratory comparisons, and analytical procedures validation. For ASOs, the procedures have yet to be published. Nevertheless, parameters such as accuracy, precision, the limit of detection (LOD), the limit of quantification (LOQ), and linearity are defined to obtain reliable analytical methods⁵⁹. **Table IV** lists the definitions of these validation parameters. By its nature, there is no specific regulatory guidance for ASOs. There needs to be more quality expectations and standards throughout the phases of development. Considering the guidelines of the International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use (ICH) is recommended. Quality control strategies should be based on the ICH Q8, Q9, and Q10 standards, oriented to pharmaceutical development, quality risk management, and pharmaceutical quality system, respectively⁶⁰.

Quality control systems for articles obtained through biotechnological processes are similar to those routinely used in conventional pharmaceutical products. The difference lies in the methods for finding the identity, uniformity, and impurity profile, and the methodology complexity is related to its size, characteristics, and manufacture. The United States Pharmacopeia (USP) establishes that the sterility assay, the safety product in experimental animals, and the potency must be performed within the tests. Likewise, the chapters corresponding to injections, pH, injection particles, bacterial endotoxins testing, and impurities should be consulted. Analysis of biotechnology products relies on sophisticated methods to demonstrate structural identity and homogeneity and assess shelf life and stability⁶¹.

Mass spectrometry is one of the most popular tools for oligonucleotide analysis, and it enables identification and quantification with high precision. Molecular mass measurements can be employed to characterize their purity and appreciate low-molecular-weight impurities, metabolites, or post-synthesis modifications⁷. When coupled with liquid chromatography, it achieves a selective separation of the components and a precise quantitative and qualitative determination of complex oligonucleotide mixtures. Together, they have become the primary tool for its analysis⁶².

Moreover, due to the growing diversity of products and their applications, it is necessary to seek input from the global regulatory agency throughout the development on a case-by-case basis. In 2018, the ICH considered developing a new quality guideline focused on oligonucleotides to provide harmonized quality management recommendations. The document would be applicable internationally and facilitate access to new therapeutic candidates in a rapidly growing field. The same advance is expected in the future⁶⁰.

Table IV. Definitions of the parameters required for an analytical validation^{63,64}

Parameter	Definition
Accuracy	Closeness between an accepted value (either from a standard or reference material) and the one found in the experiment when applying an analytical method.
Precision	Agreement degree between a series of individual measurements obtained from multiple samples of the same analyte.
LOD	Lowest analyte amount that can be detected in a sample, although it cannot be quantified with an exact value.
LOQ	Lowest analyte amount that can be quantitatively determined in a sample, with acceptable precision and accuracy, under established experimental conditions.
Linearity	Verification of a linear relationship between the analyte amount or concentration and the analytical signal determined in the method.

Advantages and Disadvantages

Therapies with ASOs have advantages and disadvantages to be defined. Some of them, as shown in **Table V**. Some solutions for the limitations exposed are the phosphate bond modifications between the nucleotides and modifications to the sugar rings, altering the resistance to the nucleases degradation, the binding to plasma proteins to maintain stable concentrations, and the activation of the immune system.

Table V. Advantages and disadvantages associated with the ASOs

Advantages	Disadvantages
<ol style="list-style-type: none"> 1. The target specificity depends on the nucleotide sequence, and the pharmacokinetic characteristics are more associated with the chemical structure. Therefore, variations or improvements in these aspects can be made separately⁶⁵. 2. The mechanistic characteristics give the ability to direct them towards objectives inaccessible through conventional methods, counting on low toxicity because of the limited systemic exposure⁶⁶. 	<ol style="list-style-type: none"> 1. Nucleases degrade these molecules⁶⁷. 2. They cannot cross the cell membrane due to their high negative charge⁶⁷. 3. ASOs can trigger immune responses⁶⁷. 4. They have problems crossing the blood-brain barrier, requiring an invasive administration through intrathecal or intraventricular routes⁶⁶.

APPLICATIONS

Numerous studies have shown that the dysfunction of determined proteins is the leading reason for distinct diseases. To date, there is a limited number of treatments for such conditions. More efficient strategies have emerged recently, such as modifying the pathology proteins using ASO therapies⁶⁷. Some of them are listed below:

Neurodegenerative Diseases

Huntington diseases

Huntington's disease is caused by the expanded repeat of the CAG trinucleotide in exon 1 that encodes for the huntingtin protein. It is found on chromosome 4. This mutation generates a polyglutamine (PolyQ) tract⁶⁸⁻⁷⁰. As a result, a triad of cognitive, motor, and psychiatric problems is triggered. Currently, there is no approved treatment to slow the condition's progress. In addition, its pathophysiology makes it an excellent candidate for developing therapies related to its genetic origin⁶⁹.

Studies developed in mouse models have shown the efficacy of non-allele-specific ASOs for disease treatment. Thus, progressing to clinical studies has been achieved. An example is IONIS-HITTRX. This oligonucleotide binds to the RNA of HTT, promoting its destruction and reducing the protein's excess amount^{68,69}. Phase I and II studies have demonstrated its safety and tolerability, being administered intrathecally⁷⁰. Unfortunately, a Phase III investigation does not benefit patients who received the molecule.

Hereditary transthyretin amyloidosis (AHTTR)

It is a pathology characterized by the amyloid extracellular deposit and the progressive destruction of the peripheral nervous system, affecting kidneys, liver, and gastrointestinal tract, leading to autonomy loss and eventual death. In most cases, it originated through a point mutation in the TTR gene by substituting valine for methionine at position 30 of the mature protein. It requires a multidisciplinary approach to reduce the number of deposits and control cardiac, renal, and ocular complications⁷¹.

Inotersen is a second-generation ASO aimed at reducing the production of hepatic TTR⁷². This ASO is complementary to a region in the 3'-UTR of the human TTR mRNA, lowering its production. The deposits' formation is reduced by limiting their amount, stopping the disease progression⁷¹. Studies in Phases II and III have indicated that it benefits the patient, as in preclinical trials performed in mice and cynomolgus monkeys⁷³. Still, platelet levels and renal function should be monitored when administered due to thrombocytopenia and glomerulonephritis⁷⁴. The European Medicines Agency (EMA) and the Food and Drug Administration (FDA) have approved it for AhTTR in Stages 1 and 2⁷¹.

Amyotrophic lateral sclerosis (ALS)

It is a progressive and fatal illness that affects motor neurons. Its fatality is mainly associated with the respiratory paralysis that it can produce over time. Approximately 90% of the cases are sporadic (mutation origin is unknown with certainty),

while the remaining percentage belongs to familial cases. Various genes are associated with an augmented pathology risk. Most cases have mutations in the C9ORF72 gene. Nonetheless, the first identified was SOD1, found mainly in hereditary cases. Other altered genes include FUS-P525L, PFN1, and SOD1-A4V. The FDA approves two drugs as a traditional treatment. One is riluzole, which suppresses excessive excitation of motor neurons, slows disease progression and increases survival. As a complement, edaravone seeks to suppress oxidative stress. However, no existing therapy is genuinely curative^{75,76}.

Antisense therapy targets the principal and most well-known mutations. The developed ASOs seek to bind to SOD1 and C9ORF72 mRNA. The SOD1 gene encodes the enzyme superoxide dismutase 1, and its mutations lead to a protein with toxic characteristics and prone to aggregation. Subsequently, the C9ORF72 gene encodes a protein present in numerous brain regions. Although its function remains unknown, the formation of an aberrant product by the hexanucleotide repeat expansion (GGGGCC) in the noncoding gene region has been associated with possible neuronal cell death and symptoms such as ataxia^{30,76}. One preclinical *in vivo* study directed at SOD1 reversed the disease course due to its suppression gene in rats and mice. This data demonstrated its great potential, as it stopped the disease and withdrew some of its aspects⁷⁷.

In the first human study with SOD1 antisense therapy, increasing doses of tofersen (ISIS 333611, BIIB067, or NCT02623699) were considered. It is an ASO that binds to SOD1 mRNA and originates its degradation through the activation of RNase H1. Plus, it prevents the mRNA reading and the formation of potentially toxic proteins. The substance was administered intrathecally since, as observed in animal models (rodents and nonhuman primates), this route allows a generalized distribution in the brain and spinal cord^{77,78}. Their main objective was to assess their safety and tolerance. The results indicated that the treated patients had fewer adverse effects than those who received a placebo and the drug was generally tolerated. The small number of subjects and the low concentrations administered limited the results presented⁷⁸. A couple of years after the publication of this study, the clinical investigation of tofersen started, and a Phase III study was completed in 2021⁷⁷.

Regarding the C9ORF72 gene, some preclinical studies have indicated that protein synthesis reduction to 50% prevents the course of the disease. Despite this, its total absence has unwanted effects, such as splenomegaly and enlarged lymph nodes, although it does not seem to generate motor deficiencies. For these reasons, the therapy has to attack the RNA with aberrant expansion but preserve its levels for encoding the protein. Regarding human studies, a clinical study began in 2018 to assess the safety, tolerance, and pharmacokinetics of BIIB078⁷⁹.

Neuromuscular Diseases

Spinal muscular atrophy

Spinal muscular atrophy is an autosomal recessive disease caused by a loss of survival motor neuron (SMN) protein through deletions or other mutations in the SMN1 gene. The pathology is characterized by progressive symmetrical muscular atrophy and weakness⁸⁰. People have a nearly identical gene called SMN2, which differs only by one nucleotide at the beginning of exon 7. It weakens the splice site's signal, excluding this exon from most SMN2 transcripts and producing an unstable protein. Even so, it can generate a low amount of SMN protein. ASO therapy focuses on correcting the splicing defect of the SMN2 gene to produce a more significant amount of functional protein. The treatment used is called nusinersen. It binds to the regulatory sequence of intron 7, suppressing the splicing of exon 7, favoring its inclusion, and increasing the amount of functional protein⁶⁷.

In preclinical studies, nusinersen was shown as a potent inducer of SMN2 exon 7 inclusion and SMN protein expression. Furthermore, the molecule improved function and survival in murine models with spinal muscular atrophy. Central nervous system distribution, pharmacokinetics, and pharmacodynamics were evaluated in mice and nonhuman primates. The intracerebroventricular bolus injection was the most efficient administration form in rodents and produced long-lasting pharmacological effects. Intrathecal bolus injection was distributed throughout the spinal cord and had pharmacologically active accumulation levels in primates. Thus, it was possible to extrapolate the sustained correction in the SMN2 splicing in mice to a low-frequency dosing regimen in patients⁸¹.

In the Phase II clinical study, the safety, tolerability, pharmacokinetics, and efficacy of multiple intrathecal doses of nusinersen were evaluated in pediatric patients. The administration had no major safety problems. All patients had mild or moderate adverse effects, while the severe ones were not drug-associated. Only one of the 19 participants had transient mild

drug-associated neutropenia. In the remaining cases, no significant changes were observed in laboratory tests, vital signs, electrocardiogram parameters, and cerebrospinal fluid (CSF)⁸². Clinical efficacy was studied through two assessments: the Hammersmith Infant Neurological Exam-Part 2 (HINE-2) and the Children's Hospital of Philadelphia Infant Test of Neuromuscular Disorders (CHOP-INTEND). They showed improvements in motor function and increased amplitude of the ulnar nerve's compound muscle action potential and the peroneal nerve⁸².

The pharmacokinetics studied in CSF and plasma showed that their elimination was consistent with their average turnover. Mean peak plasma concentrations were observed one hour after dosing and decreased within 24 hours⁸². During Phase III clinical studies, the therapy's effectiveness was demonstrated in patients with spinal muscular atrophy, and its early utilization was supported. Therefore, it was approved by the FDA on December 23, 2016, for treatment in adults and children⁸³.

Duchenne muscular dystrophy (DMD)

It is an X-linked degenerative neuromuscular disorder related to mutations in the DMD gene that encodes for the protein dystrophin. It occurs mainly in males, and in most patients, the mutations present are exon deletions or duplications, whereas missense or displacement point mutations occur less frequently. Excluding one or more exons in the mRNA produced little dystrophin during transcription. The molecule allows the anchorage between the actin cytoskeleton and the connective tissue. By being diminished, the muscle fibers become more susceptible to damage by contraction. This progressive damage generates weakness and function loss, and even death from respiratory or heart failure^{80,84}.

Its treatment is based on physiotherapy and glucocorticoids. These drugs have shown a delay in gait loss and the functioning preservation of the respiratory system. The first one approved by the FDA was deflazacort. Aminoglycosides such as gentamicin have been studied as well. Nevertheless, its use increases the risk of bacterial resistance, so it is not advisable. In addition, nutritional monitoring should be given since loss of swallowing function and complications associated with weight gain or loss may occur^{85,86}.

Clinical studies have evaluated potential treatments, including anti-inflammatory and antioxidant molecules, vasodilators, compounds to reduce fibrosis, and drugs targeting myostatin. In the European Union, there is approval for employing an orphan drug called ataluren^{85,86}. The classification as an orphan is attributed to its indication for rare diseases (presented in a small population)⁸⁷. It is an RNA-targeted therapy where the reading of a premature stop codon is restored. It is helpful for approximately 11% of patients whose pathology is caused by a codon reading stop in the DMD gene^{85,86}.

Other ASOs are based on exon skipping. When the mRNA is read, the defective exon or exons are skipped, reducing the severity of the symptoms⁸⁸. Most causative mutations occur in the hot spot region, comprising exons 45 to 55. Approximately 14% of patients present mutations in exon 51, becoming a key target in the treatment search. There are two molecules for this purpose: drisapersen and eteplirsen^{84,88}. Drisapersen has a 2'-OMePS modification. Initial studies yielded promising results, observing increased dystrophin expression in muscle fibers and improvements in infants' gait. However, the FDA and the EMA refused approval due to adverse effects such as proteinuria^{80,84}.

Related to eteplirsen, it received FDA approval as an orphan drug. It is a phosphorodiamidate morpholino oligomer (PMO). Like drisapersen, it has shown an improvement in functional dystrophin expression and occasioned a delay in ambulation loss. This drug is neutral, so it shows better tolerance than charged molecules. Despite this, PMOs' disadvantage is the requirement of an increase in dosage frequency or amount to achieve their function^{84,86}. Finally, one of the most recently developed alternatives for this pathology is golodirsen. Unlike the previous two, it looks for exon 53 skipping. This therapy could benefit approximately 8% of patients⁸⁶. Their Phase I and II studies indicated positive results for its utilization, and Phase III studies are currently underway⁸⁹. This therapy has already been approved as an orphan drug by the FDA. Still, there is controversy since there are reports of nephrotoxicity⁸⁶.

Ulcerative Colitis (UC)

It represents a disease that affects the quality of life of many patients. Likewise, surgical treatment complications have become a growing problem, especially pouchitis (inflammation that develops after the anal ileal pouch-anal anastomosis). Ileal pouch-anal anastomosis after total proctocolectomy is considered the standard surgical procedure for patients with UC. The intercellular adhesion molecule 1 (ICAM-1) is responsible for leukocyte migration to inflammation areas and is an active

component in inflammatory bowel disease's pathophysiology. This molecule is an inducible transmembrane glycoprotein expressed on the vascular endothelium, the colon membranes, and some leukocytes' cell surfaces. Its expression is upregulated in response to proinflammatory mediators, and it binds to ligands on the leukocyte's surface, such as β 2 integrins, Mac-1, and leukocyte function-associated antigen 1. Some studies showed augmented ICAM-1 expression and higher circulating blood concentrations within the inflamed gut⁹⁰.

Alicaforsen is one of the most promising agents for treating UC and refractory pouchitis. It is an ASO that negatively regulates the development and expression of the molecule on the cell surface. After ICAM-1 mRNA transcription, alicaforsen binds to it, and this process makes the genetic material ineffective and causes its destruction by cleavage. As a result, translation and further expression of the protein are inhibited. Finally, leukocyte migration and trafficking are diminished, substantially reducing the inflammatory cascade associated with the disease⁹¹.

In a clinical study, a six-week course of alicaforsen was administered to 12 patients. The drug was safe and effective in improving the UC and pouchitis clinical picture, which was prolonged in most participants. In this investigation, 11 patients received the entire course of 240 mg of alicaforsen once daily, while, in one case, it was discontinued early due to a lack of efficacy. After three months, they showed a significant reduction in the disease manifestations. Nonetheless, a relapse occurred in seven patients. The mean duration of clinical improvement was 18 weeks, and it was longer than nine months in three patients with a sustained response⁹¹.

Cancer

Advances in antisense technology have resulted in higher potency and better tolerability, translating into a more significant clinical benefit⁹². ASOs target distinctive cancer pathologies at the RNA level through sequence-specific binding to modulate proto-oncogenes expression⁹³. These genes participate in the regulation of growth and the cell cycle. When they undergo some mutation, they become oncogenes, favoring cancer formation⁹⁴. Explicit efficiency modulates gene expression by targeting a causative gene, limiting the required doses. Unlike traditional therapies such as chemotherapy, this reduces side effects and treatment costs. Because cancer is a multifactorial disease, it is difficult to establish the target sequence or sequences to develop adequate therapy. Certain angiogenic factors (Table VI) have recently been identified as potential targets for ASOs-mediated intervention⁹⁵.

Preclinical studies have been designed using ASO for small-cell lung cancer. One of the pathways for developing this tumor is the abnormal functioning of the Ser/Arg repetitive matrix 4 (SRRM4), which is a splicing activator. Damage to SRRM4 also affects the RE1-Silencing Transcription Factor (REST) activity, which acts as a tumor suppressor by regulating cell division. When detecting any abnormality in this cycle, it usually suppresses cell division with carcinogenic potential. That is why SRRM4 has been employed as a therapeutic target. In *in vivo* studies with mice, tumor shrinkage has been evidenced by suppressing the synthesis of the said matrix⁹⁵.

One drug utilized is custirsen. It corresponds to a chimeric 2'-O-methoxyethyl modified ASO targeting clusterin, an antiapoptotic protein⁹². The drug increases efficacy against cancer by inhibiting clusterin production by binding to the protein's mRNA. Promising results were sought in several Phase II studies and are currently being evaluated in Phase III clinical trials to treat prostate and lung cancers⁹⁶. AZD9150 is another next-generation ASO antisense. It contains 2'-4' cEt modified residues, giving a high affinity⁹⁷. The molecule consists of 16 nucleotides designed to attack and indirectly decrease the expression of the human signal transducer and activator of transcription 3 (STAT3). Its constitutive activation increases the levels of tumor-associated signaling molecules⁹². This ASO has provided preclinical activity in cell line models and in lymphoma patient-derived tumor xenograft (PDX)⁹⁷ models in which cells from cancer patients are implanted into a distinct host such as mice⁹⁸.

A Phase Ib clinical study was developed in patients with relapsed or refractory lymphoma. This safe drug appeared to benefit some patients with diffuse large B-cell lymphoma⁹⁹. Besides, apatorsen (OGX-427) is a second-generation phosphorothioate ASO, which inhibits the heat shock protein 27 (Hsp27) expression. It is an ATP-independent cytoprotective chaperone expressed in prostate cancer. In many other human cancers, it is induced by cellular stress, and its detection correlates with poor clinical outcomes¹⁰⁰.

In a Phase I study in patients with previously treated castration-resistant prostate (CRPC), breast, non-small-cell lung, or ovarian cancer, doses up to 1000 mg were used. These were well tolerated when given alone or with docetaxel

chemotherapy²⁷. Additionally, an open-label, randomized Phase II trial was conducted to evaluate the antitumor activity of apatersen plus prednisone versus prednisone alone in men with metastatic CRPC. The drug combination did not change the proportion of patients with CRPC without disease progression but was associated with significant decreases in prostate-specific antigens (PSA)¹⁰⁰. They are produced by secretory epithelial cells and are androgen-regulated serine proteases expressed in benign and malignant prostatic tissue¹⁰¹.

Another study drug is BP1001. It is an antisense ODN directed to the translation initiation site of the growth factor receptor-bound protein 2 (Grb2). This protein is crucial for transducing oncogenic tyrosine kinase signals. Its inhibition suppresses fibroblast transformation, hematopoietic cell proliferation, and leukemia-like disease in mice. Preclinical studies showed that it effectively decreased the proliferation of leukemic cell lines positive for the BCR-ABL gene. Therefore, Phase I/Ib studies were performed in patients with refractory or relapsed leukemia. It was well tolerated, and early evidence of antileukemic activity combined with low-dose cytarabine was obtained¹⁰².

Despite the many preclinical data generated, the efficient approach to the challenges presented for targeting cancer treatment still needs to be better understood. One arises from some oligonucleotides' nonspecific mechanism since, when modified, they suffer an affinity decrease for the target sequence, with its consequent accumulation in diverse tissues¹⁰³. Another problem is drug distribution. Tumor tissues present specific microvascular characteristics, lymphatic drainage deficiency, and variable effects of interstitial pressure. Different areas can occur in the same tumor (necrotic nucleus, seminecrotic region, and active angiogenic front)¹⁰⁴. These aspects hinder the drug's accessibility. By not accumulating to a significant degree in tumor tissue, the functional efficiency is affected, leading to some cells' evasion of the anticancer action¹⁰³.

Table VI. Angiogenic factors identified as potential targets for the development of ASOs against cancer

Angiogenic factor	Function in cancer development
Epidermal growth factor (EGF) ¹⁰⁵	Expression levels of EGF and its receptor (EGFR) are correlated with progressive tumor growth and metastasis.
Platelet-derived growth factor (PDGF) ^{93,106}	It promotes the blood vessels' vascularization and induces vascular wall maturation.
Basic fibroblast growth factor (bFGF) ^{93,106}	It favors cell survival and mitogenic activities.
Transforming growth factor α (TGF- α) ⁹³	It facilitates the epithelial-to-mesenchymal transition process, which is crucial for metastasis and poor prognosis.
Transforming growth factor β (TGF- β) ⁹³	Failure in its functionality triggers tumor growth and invasiveness.
Vascular endothelial growth factor A (VEGF-A) ¹⁰⁷	It promotes the growth of a network of blood vessels surrounding the tumor.
Endoglin ⁹³	It acts as a membrane co-receptor for TGF- β , favoring tumor growth proliferation and metastasis.
Angiopoietins 1, 2, 3, and 4 ⁹³	They stabilize blood vessels and cause vascular remodeling.

CONCLUSION

Since their discovery, ASOs have taken on great importance in developing antisense drugs for the possible treatment of neurodegenerative, neuromuscular, intestinal, and oncological pathologies, among others. Its development difficulties comprised its charge, easy degradation, complex distribution to cells, and unspecific binding to RNAs distinct to the therapeutic target, promoting adverse effects such as hepatotoxicity. Solutions have been promoted for these problems, including structural modifications and distribution methodologies to increase their therapeutic potential and size diminishing to increase their specificity. Therefore, several ASOs are examined in clinical studies for various diseases. Some have already been approved for commercialization (inotersen, nusinersen, ataluren, eteplirsen, and golodirsen), showing them as promising therapeutic solutions in the short and medium term for disorders developed by specific genetic factors.

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AUTHORS' CONTRIBUTION

All authors have an equal contribution in carrying out this study.

DATA AVAILABILITY

None.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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