Student Paper

Antisense Oligonucleotides (ASOs)

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Introduction

Antisense oligonucleotides (ASOs) are short, synthetic oligonucleotides, typically 15-20 nucleotides in length, engineered to bind selectively to complementary RNA sequences in cells. Their target-specific binding by duplex formation can lead to reduced levels of full-length mRNAs, thereby decreasing the expression of harmful proteins. There are multiple mechanisms through which ASOs exert their effects; including RNase H-induced cleavage of mRNA or modulation of RNA splicing to restore proper expression.[1], [2]

Antisense oligonucleotides offer potential treatments for a wide range of genetic, infectious, and neurodegenerative diseases that can be linked to a specific genetic sequence. With the 15th ASO therapy approved at the end of 2023, and numerous others under development, ASOs in the therapeutic landscape is increasing.[3] Potential is seen for rare diseases, as approximately 80% of rare diseases are believed to be caused by single-gene mutations.[1], [4]

This paper outlines the mechanisms of action of ASOs, their therapeutic applications, the medicinal chemistry background, and the challenges they face.

Mechanisms of Action

In comparison to small molecule RNA inhibitors, oligonucleotides have the advantage of primarily functioning by binding to their target RNA via Watson-Crick base pairing. This significantly increases ease of drug design, as well as the specificity for the targeted sequences. The hybridization to the target sequence can elicit various biological outcomes, depending on the oligonucleotides' chemical design.
Some modes of action will be briefly discussed for therapeutic oligonucleotides below: transcript knockdown using gapmer ASOs, small interfering RNAs (siRNAs), steric hindrance via direct blocking of the mRNA or microRNA (miRNA), and splice modulation using splice-switching ASOs (ssASOs). Figure 1 visually presents a brief overview of mechanisms of action.[2], [1], [5]

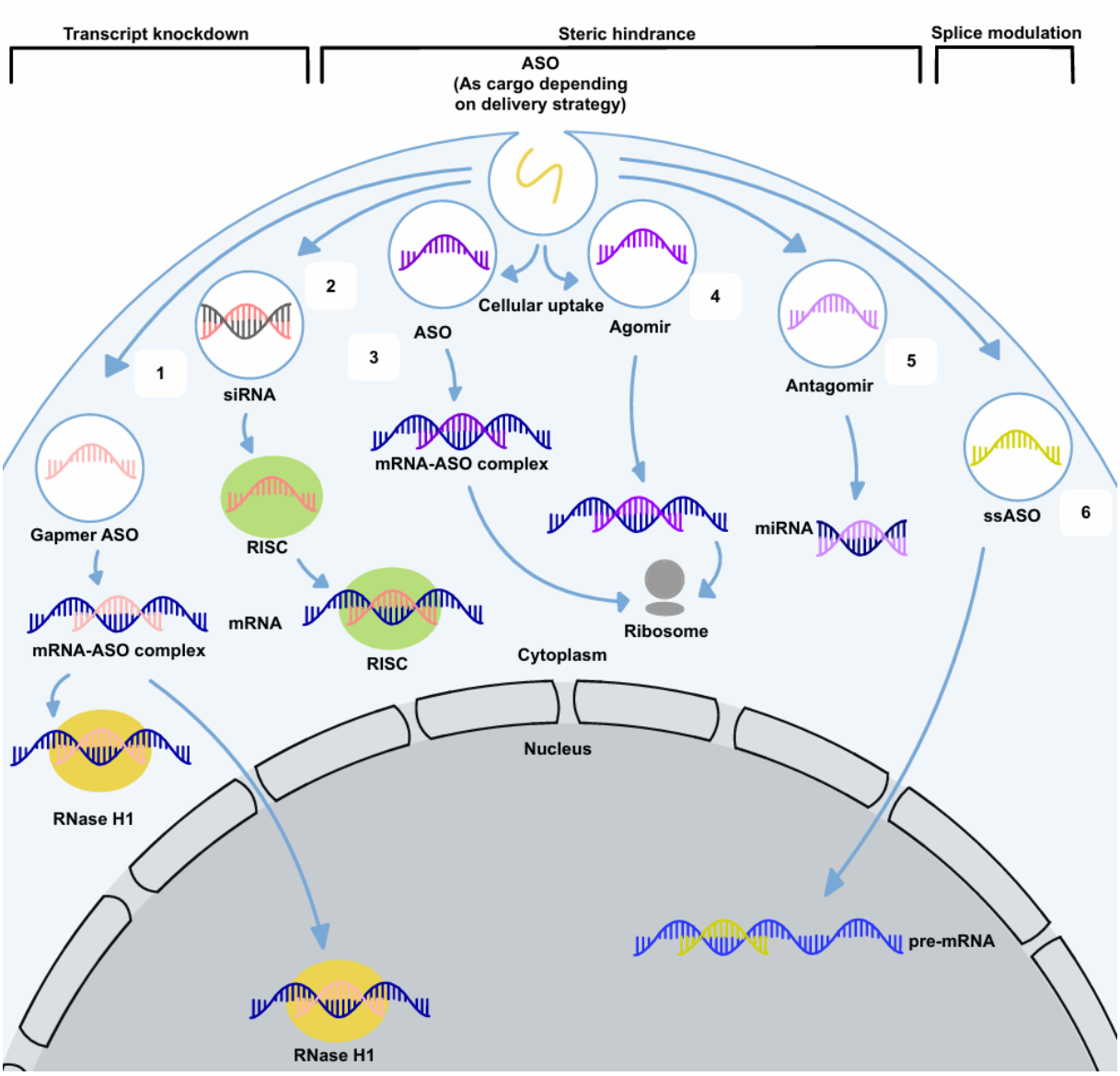


Figure 1. Overview of mechanisms of action of ASOs: ASOs act through transcription knockdown via RNase H1-mediated degradation (gapmers **(1)**) or RNA interference (siRNAs **(2)**), steric hindrance using ASOs **(3)**, agomirs **(4)**, or antagomirs **(5)**, and splice modulation with ssASOs **(6)**.

1. Transcript knockdown

RNase H-Mediated Degradation (1): Gapmer ASOs with DNA-like backbones and modified nucleotides form a heteroduplex with the targeted RNA, which recruits RNase H1, an endogenous enzyme present in the cytoplasm and cytosol. RNase H1 cleaves RNA strands in RNA-DNA hybrids. Gapmer ASOs are chemically designed to resist degradation by RNase H1 and to increase binding affinity to the enzyme. This degradation effectively reduces the levels of the target RNA.

RNA Interference (RNAi) (2): Double-stranded siRNAs 21-23-nucleotides in length also reduce target RNA levels but via the endogenous RNA interference pathway. The siRNA duplex is incorporated into the RNA-induced silencing complex (RISC), which unwinds and separates the duplex into sense and antisense strands. The antisense strand binds complementarily to the target mRNA within the RISC, leading to gene silencing.

In comparison, RNase H1 in the RNase H1 pathway binds to the ASO-RNA duplex, while RISC associates with siRNA before forming the RNA duplex in the RNAi pathway. The RNAi pathway

usually provides more potent downregulation but has a higher tolerance for mismatches, increasing off-target effects.

2. Steric hindrance

ASOs can also lead to lower protein production by blocking mRNA-ribosome interaction when an mRNA-ASO heteroduplex is formed **(3)**. Additionally, ASOs can engage with miRNA by sequestering miRNA, acting as antagomirs **(5)** or by generating miRNA mimics (agomirs) **(4)**.

3. Splice Modulation (6):

ASOs can alter pre-mRNA splicing by blocking splice sites or enhancing exon inclusion through splice-switching ASOs (ssASOs). These ASOs influence pre-mRNA splicing by targeting and obstructing the recognition of splice-regulatory elements. Designed to enter the cell nucleus, ssASOs bind specifically to pre-mRNA targets and block regulatory elements, effectively masking them from the spliceosome—the cellular machinery responsible for processing pre-mRNA.

These elements include canonical splice sites (normal sequences at exon-intron boundaries), cryptic splice sites (hidden sequences sometimes mistakenly used for splicing), branchpoints (specific intronic motifs assisting in splicing), as well as splicing enhancers and silencers within exons and introns. By blocking these regulatory elements, ssASOs can induce the inclusion or exclusion of specific exons, enabling targeted adjustments to mRNA. This process can restore disrupted reading frames to increase functional protein production or suppress the production of harmful proteins.

Therapeutic Applications

Antisense oligonucleotides (ASOs) have gained attention for their potential across a variety of therapeutic areas, including neurological diseases, oncology, infectious diseases, and rare genetic disorders. In neurology, ASOs offer hope for conditions such as Huntington's disease[6] and amyotrophic lateral sclerosis[7], as they can cross the blood-brain barrier when paired with effective delivery systems. Similarly, in oncology, ASOs can inhibit oncogene expression or sensitize cancer cells to chemotherapy, underscoring their growing importance in cancer treatment.[8] Infectious diseases also present a promising avenue; ASOs targeting viral RNA are being explored as antiviral therapies in clinical trials, including those targeting hepatitis B virus and SARS-CoV-2.[9]

A particularly impactful application of ASOs lies in addressing rare diseases, a field where over 90% of conditions lack targeted treatments.[10]

Duchenne muscular dystrophy (DMD), one of the most studied rare diseases in this context, exemplifies the potential of ASOs. DMD is a fatal, X-linked recessive disorder and the most common inherited muscle disease, affecting approximately 1 in 5500 males. Characterized by progressive muscle wasting from early childhood, patients with DMD typically face severe disability and a reduced lifespan, often dying of the symptoms in their 20s.

Despite being incurable, advances in medical care and physical therapy, such as ASOs therapies, have gradually extended the life expectancy of individuals with DMD. The genetic basis of the disease was elucidated in 1987 when the DMD gene was cloned. The gene is encoding the protein dystrophin, and was found to contain mutations leading to dystrophin deficiency. Dystrophin is a critical structural protein that connects the cytoskeleton of muscle fibers to the extracellular matrix, providing mechanical stability during muscle contraction. Its

absence leads to progressive muscle fiber damage, inflammation, and loss of muscle function.[11] Exon deletions disrupt the reading frame of dystrophin mRNA, resulting in severe DMD.

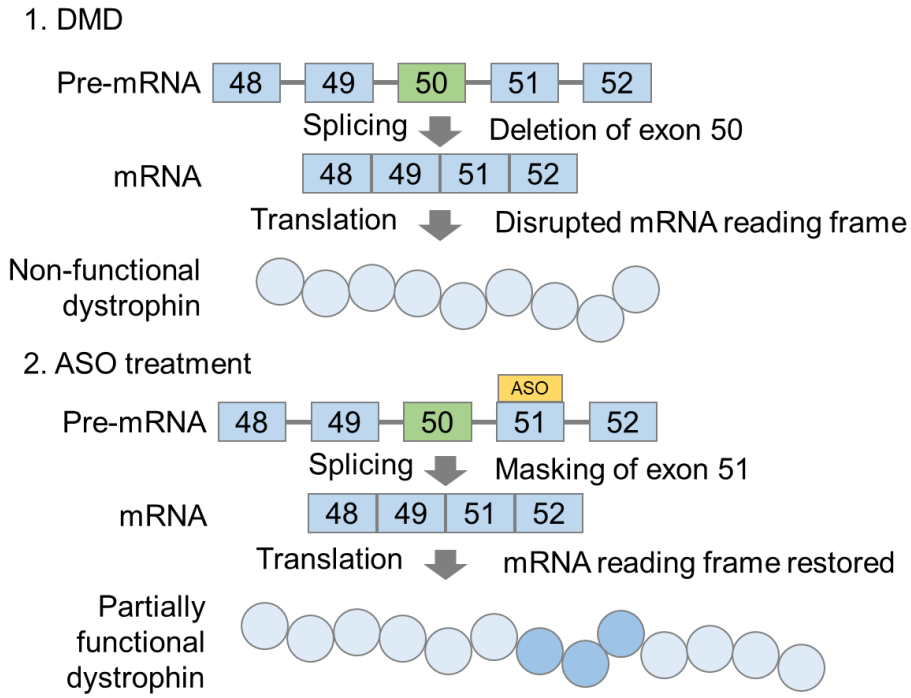


Figure 2. 1. Mechanism of disease of DMD, and 2. Mechanism of action upon ASO treatment via exon splitting.

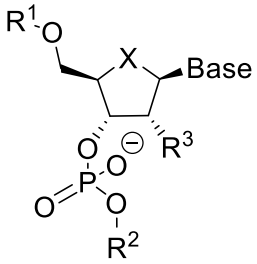
The ASOs for DMD primarily employ splice modulation through exon skipping, as visualized in Figure 2. By skipping flanking exons during splicing, ASOs restore the mRNA reading frame, enabling the production of a partially functional dystrophin protein. This mechanism has underpinned the development of FDA-approved ASO therapies for DMD, with three treatments currently approved and others nearing regulatory review. These therapeutic breakthroughs highlight the transformative potential of ASOs in treating rare genetic disorders and emphasize their expanding role in modern medicine.[12]

Chemical Modifications

As with any drug, the chemical structure dictates the biological function. The differences between various types of antisense oligonucleotides (ASOs), such as siRNA and gapmers, illustrate the remarkable flexibility of ASO therapeutics. To optimize properties like *in vivo* stability against nucleases, specificity, and pharmacokinetics, ASOs undergo extensive chemical modifications. A well-structured, comprehensive review on the medicinal chemistry of oligonucleotide therapeutics is provided in the 2016 JMC review by W. Wan and P. Seth.[13] Four main categories of modifications are typically employed in medicinal chemistry: backbone modifications, base modifications, sugar modifications, and alternative chemistry. The following paragraphs offer an overview of the most commonly used modifications in ASO development.

Alternative chemistry
Modulation of flexibility
Modulation of binding affinity

Backbone modification
Enhancement of nuclease stability
Enhancement of pharmacokinetics



Base modification
Enhancement of RNA affinity
Modulation of specificity

Sugar modification
Enhancement of RNA affinity
Enhancement of nuclease stability

Figure 3. Modifications and complementary sites of ASOs often used in drug discovery and development.

1. Backbone modifications

Backbone modifications in ASOs are crucial for improving stability and resistance to exonucleases, which degrade unmodified oligonucleotides *in vivo*. The most common modification involves the substitution of the phosphodiester backbone with a phosphorothioate (PS) group. This modification replaces one of the oxygen atoms in the phosphodiester linkage with a sulfur atom, significantly enhancing the oligonucleotide's resistance to nucleases while maintaining its binding affinity to complementary RNA sequences. Additionally, other modifications such as phosphorodiamidate and peptide nucleic acid (PNA) backbones have been explored to further increase stability and specificity.

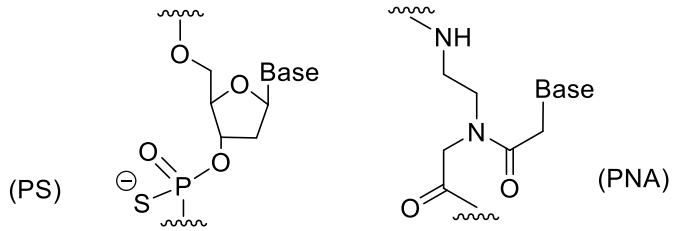


Figure 4. Phosphate modification – Phosphorthioate (PS) on the left and Peptide nucleic acid (PNA) on the right.

2. Base modification

Base modifications alter the nucleobases to improve the affinity of ASOs for their target RNA while minimizing off-target effects. One of the most common base modifications is the use of 5’-methylcytosine (5mC) instead of cytosine to reduce immune stimulation while maintaining base-pairing fidelity.

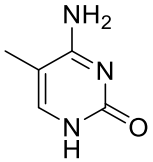


Figure 5. Base modification – 5-methylcytosine (5mC).

3. Sugar modifications

Sugar modifications are a powerful tool for modulating the biophysical and biological properties of ASOs. These modifications can be used to enhance RNA-binding affinity, increase stability against nucleases, and optimize functional activity. The sugar moiety in ASOs, typically a ribose, can be modified at various positions. A well-known modification is the 2′-O-methyl (2′-OMe) group, which improves nuclease resistance and is commonly used in the design of ASOs aimed at RNA splicing modulation. Another widely used modification is the 2′-O-methoxyethyl (2’-MOE) group, which introduces superior stability and steric hindrance, enhancing resistance

to nucleases while maintaining strong RNA affinity. Furthermore, the 2′-fluoro (2’-F) modification, which substitutes the 2′-OH group with a fluorine atom, has shown potential in improving duplex stability and base-pairing fidelity in ASOs. Another significant sugar modification is the incorporation of locked nucleic acids (LNA), which involve a methylene bridge connecting the 2′- and 4′-positions of the sugar ring. This modification locks the sugar into a North conformation, enhancing the binding affinity of the ASO for complementary RNA.

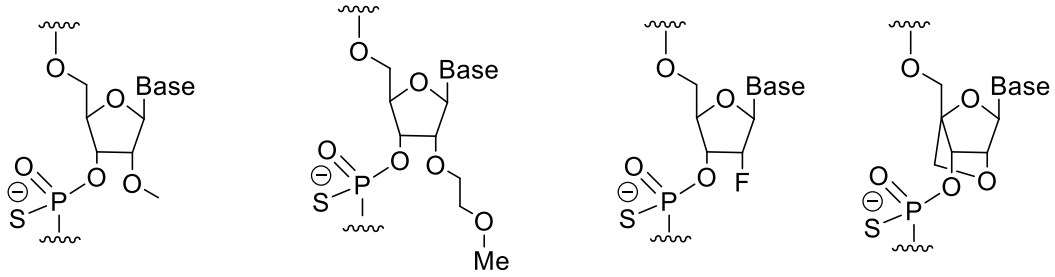


Figure 6. Sugar modifications; 2’-OMe, 2’-MOE, 2’-F, LNA (from left to right).

4. Alternative chemistry

Beyond the traditional backbone, base, and sugar modifications, alternative chemistries have been explored to further enhance the properties of ASOs. One such approach is the incorporation of novel linkers or side chains that can introduce steric hindrance or promote specific interactions with target molecules. Examples include the use of peptide nucleic acids (PNAs), which feature a backbone that mimics the sugar-phosphate backbone of DNA but lacks the phosphate component entirely. This structure allows PNAs to bind more tightly to complementary RNA or DNA sequences. Additionally, other non-natural nucleic acid chemistries, such as the use of cyclic oligonucleotides or conjugation to therapeutic agents, have been investigated for their potential to target disease pathways with high specificity and efficiency.

These four parts of the toolbox that medicinal chemistry provides hold promise for advancing the field of ASO therapeutics, offering new strategies for the development of effective, targeted treatments.

Delivery Strategies

The therapeutic targets of ASO therapeutics are diverse, and chemical modifications play a crucial role in improving their solubility and permeability, enabling efficient delivery to the site of action. However, systemic administration of ASOs has often proven inefficient due to poor tissue uptake. Cellular uptake of ASOs primarily relies on endocytosis, and the lysosomal environment often degrades a significant portion of the therapeutic, leading to reduced efficacy. To address these challenges, various delivery approaches have been developed, including direct conjugation to carriers and incorporation into nanoparticle systems. These strategies often involve direct administration to the target site, such as injections into the brain, spinal cord, or eye, or intranasal delivery to the central nervous system.[14], [1]

1. Chemical conjugate-bound delivery

Chemical conjugation is a widely used strategy to enhance the ADMET (absorption, distribution, metabolism, excretion, and toxicity) properties of ASOs. Common conjugation partners include polymers, peptides, lipids, receptor ligands, and aptamers. Conjugation facilitates both passive targeting, through cell-penetrating peptides, fatty acids, and polymers, and active targeting, using antibodies, specific ligands, or aptamers. For successful

conjugation, ASOs typically require full chemical modification to introduce a linker and attach the conjugate.

Polymer-Based Delivery

Polyethylene glycol (PEG) is the most commonly used polymer in ASO conjugation. PEG is a flexible, hydrophilic polymer with reactive end groups that serve as conjugation handles. It forms a hydration shell around the ASO, sterically blocking biomacromolecules and prolonging circulation time while enhancing stability. The properties of PEG-modified ASOs are strongly influenced by the polymer's molecular weight, end-group modifications, and linear or branched architecture.

Peptide-Drug Conjugates (PDCs)

Peptide conjugates are increasingly utilized for targeted delivery of ASOs. These peptides can interact with membrane proteins, enabling translocation across cell membranes. Peptides used in ASO delivery are often cationic or amphipathic, which precludes their use with charged ASOs. While peptide conjugates are primarily classified as passive targeting agents due to their role in enhancing cellular uptake, active targeting is also possible in tissues overexpressing specific membrane proteins. Peptides can engage in protein-protein interactions, making them valuable for targeting previously considered "undruggable" surfaces.

Receptor Ligand-Based Delivery

Among receptor ligands, *N*-acetylgalactosamine (GalNAc) is a key player, particularly for FDA-approved ASO therapeutics. GalNAc binds with high affinity to the asialoglycoprotein receptor (ASGPR), which is highly expressed on hepatocytes. This selective binding makes GalNAc conjugation an ideal strategy for delivering ASOs to the liver.

2. Carrier based delivery

While the nature of the ASO is highly important for chemical conjugation, carrier-based delivery is compatible with negatively charged ASOs and therefore most therapeutics. The charge also reduces the need for chemical stabilization, and the ADMET properties do not rely on the ASO but rather on the carrier system. Carrier systems can be subdivided into active and passive targeting. In passive targeting, tissues with higher cellular uptake are targeted, whereas active targeting relies on the presence of targeting ligands on the surface of the carrier to directly target specific cells. The most commonly used delivery systems are lipid-based and polymer-based, though peptide- and antibody-based systems are also known.

Lipid Nanoparticles (LNPs)

Lipid nanoparticles (LNPs) is a generic term, as these nanoparticles exhibit high structural complexity but share the essential characteristic of being loaded with ASO therapeutic molecules. LNPs are primarily subdivided into ionisable LNPs and lipid-polymer nanoparticles. Ionisable LNPs, are clinically relevant due to their ability to stabilize ASOs during formulation and improve delivery efficiency by leveraging pH-responsive behavior. Lipid-polymer nanoparticles combine the serum stability and mechanical strength of polymers, such as PLGA, with the biocompatibility and high loading capacity of lipid systems. This hybrid approach ensures prolonged circulation and targeted delivery, optimizing therapeutic outcomes.

Polymer-Based Delivery Systems

Polymer-based delivery systems, though less clinically advanced than lipid-based systems, offer distinct advantages due to the chemical flexibility of polymers. Synthetic polymers like polyphosphazenes can be tailored to respond to external stimuli such as local pH, enabling controlled ASO release at the target site. Additionally, naturally derived polymers like chitosan,

often combined with PLGA or alginate, provide high biocompatibility and efficient ASO encapsulation via electrostatic interactions. These versatile systems continue to gain attention for their stability and targeted delivery capabilities.

Challenges and Limitations

ASO therapies have revolutionized the landscape of genetic and rare disease treatment, yet several challenges remain that limit their broader application and therapeutic potential.[1],[15],[16],[17]

1. Off-Target Effects

Despite careful sequence design, ASOs can exhibit off-target activity due to partial complementarity with unintended RNA sequences. This may result in the silencing or modulation of non-target genes, potentially leading to unforeseen side effects. Enhancing specificity remains a critical area of focus for mitigating these risks.

2. Immune Activation

Certain ASO chemistries, such as phosphorothioate backbones, can inadvertently stimulate innate immune responses. Toll-like receptor (TLR) activation is a well-documented concern, as it may trigger inflammatory pathways and compromise patient safety. Engineering ASOs with reduced immunogenicity is an ongoing effort in the field.

3. Stability and Degradation

Although chemical modifications have enhanced ASO stability, degradation by nucleases remains a challenge. Unmodified or partially modified ASOs may have limited half-lives, reducing their efficacy and necessitating frequent dosing.

4. Regulatory challenges

The integration of gene-based therapies, such as antisense oligonucleotides (ASOs), into clinical treatment has presented novel regulatory challenges for their development and commercialization. As ASOs target specific mRNA sequences, genetic variability among patients can significantly influence pharmacokinetics and pharmacodynamics, necessitating careful evaluation of even minor base modifications. The unique nature of ASO therapies, including their molecular interactions and genetic dependencies, calls for comprehensive regulatory frameworks to ensure both safety and efficacy while addressing the specific needs of small patient populations.

5. Cost and Accessibility

The cost and accessibility of ASO therapies present significant challenges, particularly in the context of rare diseases, where the development costs per patient are often higher due to smaller patient populations. While this is a broader issue for rare disease therapies, it exacerbates inequities in healthcare access, potentially restricting life-saving treatments to only those in the highest socioeconomic strata. Given that the advances of precision medicine should be available to all sectors of society, addressing the affordability of these therapies becomes crucial. AONs targeting rare diseases are particularly costly, and the process of reimbursement can be complicated, with access often hindered by the lengthy approval process and varying marketing requirements across European Union member states. Furthermore, many ASO therapies require hospital administration, such as intrathecal or intravenous infusions, adding to healthcare system burdens. Developing scalable and cost-effective manufacturing processes is essential for democratizing access to ASO therapies.

Future Perspectives

The scope of ASO therapies has rapidly increased over the past two decades, driven by the growing demand for personalized medicine and therapies for rare diseases. Medicinal chemists are working to expand their efficacy, and with solutions emerging for current challenges such as delivery systems and sustainable production, the pharmaceutical industry is poised for significant growth in this area. The ability to target complex diseases with combinatorial approaches and broaden the scope of treatment for previously untargeted conditions highlights the transformative potential of ASOs. Achieving wider application will require continued collaboration between scientists, clinicians, and policymakers, but if these efforts succeed, ASOs will redefine therapeutic possibilities and exemplify the power of modern molecular medicine.

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