Scientific Research Publishing

Journal of Biosciences and Medicines, 2024, 12, 22-39
https://www.scirp.org/journal/jbm
ISSN Online: 2327-509X
ISSN Print: 2327-5081

Rare Diseases and Antisense Oligonucleotides: A Mirage or Miracle

Harika Vaddadi, Terry Oroszi*

Department of Pharmacology and Toxicology, Boonshoft School of Medicine, Wright State University, Fairborn, OH, USA
Email: *terry.oroszi@wright.edu

How to cite this paper: Vaddadi, H. and Oroszi, T. (2024) Rare Diseases and Antisense Oligonucleotides: A Mirage or Miracle. *Journal of Biosciences and Medicines*, **12**, 22-39.
https://doi.org/10.4236/jbm.2024.1212003

Received: March 20, 2024
Accepted: November 29, 2024
Published: December 2, 2024

Abstract

Gene therapy and antisense oligonucleotides (ASOs) are promising approaches to treating rare diseases by targeting specific genes. However, ASOs can have off-target effects that need careful consideration during development. Researchers can add moieties like peptide nucleic acid or methoxyethyl-modified ribose sugars to enhance specificity and reduce toxicity. Current research suggests that challenges such as nonspecific action, interference at various stages, adverse reactions, and nuclease degradation may soon be manageable with advanced technologies. ASOs show particular promise in treating rare conditions like Duchenne Muscular Dystrophy (DMD) and Timothy syndrome. Stereopure ASOs with repeated left-right patterns offer increased potency and half-life due to their resistance to nuclease activity and improved cellular uptake. This review explores how technological advancements can enhance the use of ASOs to manage various rare disease conditions effectively. Despite challenges in development and application, ASO therapy holds the potential to become a viable treatment option for a wide range of rare diseases. Advances in technology offer the possibility of increasing specificity and reducing toxicity, making ASO therapy a more effective and safe treatment option for patients with rare diseases.

Keywords

Antisense Oligonucleotides, Rare Diseases, Gene Therapy, Off-Target Effects, Duchenne Muscular Dystrophy, Timothy Syndrome

1. Introduction

Antisense oligonucleotides (ASOs) are primarily high molecular weight compounds with nucleotide base pairs capable of binding to mRNA and impeding protein translation or splicing [1]. ASOs represent a new era of gene therapy

(transcript-targeted therapy) for the treatment of genetic disorders that are otherwise impossible to treat [2]. Rare diseases often have fewer treatment options due to their lower frequency, improper diagnosis, and limited knowledge about their pathological basis. Gene therapy shows promise in treating rare diseases, although clinical challenges must be overcome to realize its therapeutic benefits fully. Current research on ASOs and FDA approval of drugs like Nusinersen, Eteplirsen, and Inotersen shed new light on the potential of this approach. However, the molecular mechanisms involved in the pathophysiology of rare diseases are so complex that the nonselective action of ASOs can cause more harm than benefit. Therefore, ASO therapeutics can be a miracle or a mirage unless they are rigorously tested and approved. This paper emphasizes the importance of ASOs in the treatment of rare diseases by weighing their advantages and disadvantages and citing recent studies conducted with ASOs.

2. Antisense Oligonucleotides

2.1. Background of ASOs

Antisense oligonucleotides (ASOs) are primarily high molecular weight compounds with nucleotide base pairs capable of binding to mRNA and impeding protein translation or splicing [3]. ASOs represent a new era of gene therapy (transcript-targeted therapy) for the treatment of genetic disorders that are otherwise impossible to treat. Rare diseases often have fewer treatment options due to their lower frequency, improper diagnosis, and limited knowledge about their pathological basis [4]. Gene therapy shows promise in the treatment of rare diseases, although clinical challenges must be overcome to realize its therapeutic benefits fully. Current research on ASOs and FDA approval of drugs like Nusinersen, Eteplirsen, and Inotersen sheds new light on the potential of this approach. However, the molecular mechanisms involved in the pathophysiology of rare diseases are so complex that the nonselective action of ASOs can cause more harm than benefit [5] [6]. Therefore, ASO therapeutics can be a miracle or a mirage unless they are rigorously tested and approved. This article emphasizes the importance of ASOs in the treatment of rare diseases by weighing their advantages and disadvantages and citing recent studies conducted with ASOs.

2.2. Chemistry of ASOs

Antisense oligonucleotides (ASOs) are short, single-stranded DNA molecules that can be chemically modified to bind to specific cell sequences [7]. However, one of the significant limitations of using ASOs is their rapid degradation by enzymes, such as nucleases, in the body. To overcome this, researchers have developed chemical modifications to the oligonucleotides to increase their stability and effectiveness. One of the most commonly used modifications is the phosphorothioate linkage, which replaces a non-bridging oxygen atom in the oligonucleotide chain with sulfur [8]. The phosphorothioate linkage modification increases the stability of oligonucleotides in cells and tissues, but also has some drawbacks, such

as decreased melting temperature and helix destabilization. Despite these limitations, phosphorothioates are effective in activating RNase H activity and have been used in many studies for both tissue culture and *in vivo* experiments, leading to their introduction in clinical therapeutic trials. An example of an ASO being evaluated in clinical trials is G3139, an 18-mer targeted at the initiation codons of the bcl-2 mRNA, which is currently being tested as a treatment for melanoma and chronic lymphocytic leukemia [5].

To improve the efficacy of oligonucleotides, researchers have developed newer versions, such as those with 2'-O-alkyl modifications, which create strong bonds with targeted mRNA and work through alternative mechanisms not dependent on RNase H. Despite their drawbacks, phosphorothioates are still considered viable options for clinical use and may remain preferred [9]. Different modifications have been made to traditional phosphorothioate oligonucleotides to improve their properties as antisense molecules. For example, PNAs and morpholino oligonucleotides have been created with alternate backbones that are more stable and specific in binding to target nucleic acids.

PNAs use an uncharged polyamide backbone, while morpholino oligonucleotides use a morpholine ring and an uncharged phosphorodiamidate linkage. PNA and morpholino oligonucleotide modifications not only make the oligonucleotides less affected by cellular nucleases and more specific in binding to their target, but they also make them less able to bind to cationic lipids for delivery. Techniques such as scrape loading and permeation with streptolysin O have been used to overcome this issue and maximize the antisense effects of these modified oligonucleotides. Phosphorothioates are the most commonly used modified oligonucleotides, but their limitations decrease their effectiveness as antisense molecules. Researchers have developed new modifications, such as 2'-O-alkyl groups, to improve the stability and specificity of the oligonucleotides. In laboratory and animal studies, these second-generation oligonucleotides, such as those with N3'-P5' PN modifications, have shown specific and selective antisense activity. Additionally, combining different types of modifications can further increase specificity and effectiveness [10].

2.3. Mechanism of ASOs

ASOs in their parent form may not show significant therapeutic effects because of their degradation by nuclease, poor affinity, and poor cellular permeation. Therefore, a combination of synthetic and modified ASOs is used as chimeric molecules or gapmers that can overcome the demerits of ASOs [11]. The main principle of ASOs is to down-regulate gene expression. The mechanism of action varies with the degree and nature of modification of ASO, which are detailed below.

2.3.1. Phosphate Linkage Modifications

Phosphate linkage modifications in the ASOs with nonionic phosphorodiamidate linkage will resist nuclease degradation, offer more affinity, and have a broader scope of therapeutic action. These agents act through the RNase H-mediated

translation correction process by active cellular uptake through selective transporters [3].

2.3.2. Ribose Sugar Modifications

Eteplirsen, an FDA-approved ASO for the treatment of DMD, is developed by modifying the ribose sugar in the backbone of nucleotides [12]. Compounds produced by ribose sugar modification are called morpholinos, which exert their action by exon skipping through an exonic splicing enhancer. Methoxyethyl modifications of the ribose sugar in the ASOs increase their cellular uptake, nuclease resistance, and elimination half-life. These molecules cause exon inclusions and are therefore termed intronic splicing silencers [3].

2.3.3. Locked Nucleic Acids

Adding a methyl group between the second oxygen and fifth carbon atom renders the nucleotide rigid and constrained, known as locked nucleic acid. Such a chemical modification makes the ASO more specific, with higher affinity and lower degradation. The mechanism of action involved RNase. However, there is a trade-off in terms of more untoward reactions, especially hepatotoxicity. Current research promotes a hybrid approach with methoxyethyl modification and a locked nucleic acid approach to achieve balanced therapeutic action with fewer adverse effects [3] [13].

2.3.4. Stereomeric ASOs

The stereoisomers of ASOs have different pharmacokinetic and pharmacodynamic properties. Stereo pure ASOs with repeated left-left-right (SSR) patterns have significant nuclease resistance benefits, better cellular uptake, increased potency, and half-life. Stereo pure ASOs are indicated in the treatment of Huntington's disease, where ASOs target the HTT gene expression [3] [14].

2.3.5. Peptide Nucleic Acid ASOs

This group of ASOs has a nucleic peptide in the place of the ribose sugar. Contrary to other forms of ASO, these compounds do not need RNase H to exert their therapeutic action. The mainstay of the action of these compounds is exon skipping. Peptide nucleic acid ASOs are used in the treatment of DMD, although their therapeutic efficacy is poor compared to other forms of ASO [3] [15].

2.3.6. Modification of Cytosine

Methylation of the 5'cytosine group in the nucleotide can increase the specificity of the ASO due to its steric hindrance and hydrophobic nature. Increased specificity comes at the cost of increased toxicity and immunogenic response [3].

2.3.7. Gapmers

This group of ASOs has additional moieties on locked nucleic acids or methoxyethyl-modified ribose sugars. The gapmer approach increases target specificity and reduces toxicity due to RNAse H-mediated therapeutic action [3].

2.3.8. Classification of ASOs

First-generation ASOs are built on the premise of the phosphorothioate group (Fomivirsen), used in treating Cyto-Megalo-Virus (CMV)-retinitis. These ASOs attach to the UL123-RNA and impair IE2 protein translation [3].

Second-generation ASOs are based on the Gapmer structure with chemical modifications to the ribose group in the nucleotides. Examples include Inoteresen, Mipomersen, and Nusinersen, which are used to treat hATTR, familial hypercholesterolemia, and SMA [15].

The third and current generation of ASOs, known as phosphorodiamidate morpholino oligonucleotides (PMOs), includes Eteplirsen, which is indicated for the treatment of Duchenne Muscular Dystrophy (DMD) [16].

2.4. Rare Diseases and ASO

ASO therapy is currently being advocated against rare diseases such as Timothy syndrome, Schuurs-Heoijmakers syndrome, Rett Syndrome, MECP2 duplication syndrome, Angelman Syndrome, Duchenne Muscular Dystrophy (DMD), KCNT encephalopathy, gain-of-function epilepsy, Spinocerebellar ataxias, familial amyloid polyneuropathy. The list of rare diseases treatable by ASOs is increasing daily as new ASO research is being explored against the protein translation or splicing associated with rare diseases [11].

3. ASOs as a Miracle Treatment

ASOs play a prominent role in the treatment of genetic diseases, and a "miracle" approach was once seen as just a mirage. A thorough literature review, analysis of ongoing clinical trials, and revisiting the approvals of drug regulatory agencies reaffirmed the promising benefits of the ASOs.

An autosomal dominant disease called hereditary transthyretin amyloidosis (hATTR) occurs due to mutations (single nucleotide substitution) in the TTR gene on chromosome 18q12.1. hATTR is a progressive condition with a poor prognosis and a higher mortality rate, indicating that current approaches are ineffective in their management. Transcript-targeted therapy using ASO inotersen can reduce TTR production by interfering with the mRNA responsible for its turnover. The clinical trial results showed that the patients receiving inotersen had improved their quality of life. However, the study also indicated adverse effects such as glomerulonephritis, blood dyscrasias, and death [17].

Familial chylomicronemia is an autosomal recessive disorder caused by the mutation of the lipoprotein lipase gene present on chromosome 19q13.32. Volanesorsen, a second-generation ASO, is found to be effective in reversing this condition by specifically targeting apolipoprotein C1 (APOC2) mRNA. However, the therapeutic benefits of this study are outweighed by adverse drug reactions, especially the high reduction of thrombocytes [18].

Non-arteritic ischemic optic neuropathy (NAION), characterized by ischemia of the posterior ciliary arteries, is responsible for optic disc compartment syndrome

and blindness. Chemically modified ASO called QPI-007 (developed by Quark Pharmaceuticals) can interfere with Caspase-2 expression and prevent NAION. QPI-007 produced a significant neuroprotective effect in animal and preclinical studies [19].

Familial hypercholesterolemia (FH) is a genetic disorder characterized by an increase in blood LDL level (>190 mg/dL). An experimental ASO, Mipomersen, can inhibit Apo B-mRNA and check the translation [20]. A similar ASO Inclisiran developed by Novartis is approved for the treatment of HHF. The therapeutic benefits of Mipomersen are similar to those of inclisiran and can be promising in managing FH with fewer adverse effects [21].

Amyotrophic lateral sclerosis (ALS) caused by the SOD1 mutation is a highly debilitating condition with a poor prognosis. Tofersen-elastic experimental ASO therapy is currently in clinical trials for the treatment of ALS. Tofersen exerts its action through RNase H-dependent degradation of the SOD1 protein, which helps counter the effects of ALS. However, the initial findings of the clinical trial revealed an increased risk of Cerebrospinal Fluid (CSF) pleocytosis [22].

Spinal muscular atrophy (SMA) occurs due to the loss of lower motor neurons and is caused by a mutation in the survival motor neuron (SMN2) gene that encodes the SMN2 protein. A second-generation Nusinseren ASO can inhibit the SMN2 gene splicing and block the intronic splicing silencer. In early clinical trials, Nusinseren improved neurological function and life expectancy, and therefore, it could be a miraculous treatment if adverse effects are ruled out [23].

Milasen is another promising ASO that has been experimentally used to treat a rare neurodegenerative disease that can lead to death in adolescence. Milasen inhibits the expression of MFSD8 and combats the disease by preventing neurodegeneration. Milasen is one of the finest examples of personalized medicine approved by the FDA [24]. Custirsen is another ASO that can negatively regulate the production of BCL2 and NfkB proteins that help the apoptosis of cancerous cells. It binds to clusterin mRNA and prevents tumor cell proliferation of tumor cells [25].

Rett syndrome is a genetic disorder caused by mutations in the MECP2 gene, and potential treatments include gene therapy and gene editing. However, the main challenge is to find the correct dosage of the wild-type MECP2 gene, as too much could cause dysfunction in wild-type cells. CRISPR-Cas9 has been proven to edit genes in neurons of living animals, but studies have not yet investigated its effectiveness in reversing the symptoms of Rett syndrome. Another research approach is to use antisense oligonucleotides to silence the different alleles of the MECP2 gene in mice and human cells, which has been shown to reverse molecular, behavioral, and synaptic defects. The challenge with this approach is to get the dosage right, as too low could cause Rett syndrome. Another option is to use the chemical fostriecin to inhibit protein phosphatase 2A, which reduces MECP2 in the central nervous system and improves motor abnormalities in mice with MECP2 duplication syndrome. This approach has the advantage of fine-tuning

the dose so that the amounts of MECP2 do not drop below a threshold that would cause deficiency symptoms [26].

Ataxia telangiectasia (AT) is a genetic disorder that affects the nervous system and causes a progressive deterioration of motor skills. It is caused by mutations in the ATM gene located on chromosome 11. The ATM protein, primarily found in the nucleus, is important for controlling cell cycle checkpoints, DNA repair, and responses to stress, and it plays a role as a tumor suppressor. The disorder usually appears in early childhood and affects approximately 1 in 40,000 to 1 in 100,000 individuals. Many mutations in AT are related to splicing, which can result in the deletion of part of an exon or the insertion of an intron, producing a shortened, non-functional ATM protein. A new mutation in the ATM gene that causes a splicing defect was identified in a study by Cavalieri and colleagues [27]. The study aimed to correct a genetic defect in intron 11 of a gene known as ATM, leading to a disorder called Ataxia-Telangiectasia (AT). The researchers used PMO to mask a cryptic splice site in the gene, resulting in an increase of 26% in correctly spliced mRNA in the patient's cells. They also used a similar oligonucleotide called vivomorpholino, which led to an exon skipping efficiency of 50% - 95%, depending on the concentration used. However, these concentrations also had a cytotoxic effect on patient cells and reduced the functional protein activity to 50% of wild-type levels. The researchers suggest that even minimal rescue of functional ATM protein levels could provide therapeutic benefits to AT patients, as patients with 5% - 20% functional ATM have a mild phenotype, and ATM heterozygotes with 40% - 50% of normal ATM protein levels do not show any signs of disease.

The use of antisense oligonucleotides (ASOs), specifically phosphodimethyloluene (PMOs), is used as a treatment for Congenital Disorders of Glycosylation (CDG). CDG is a group of genetic disorders caused by gene mutations involved in glycoprotein synthesizing. These disorders lead to multiorgan failure, including neurologic deficits such as cognitive impairment, ataxia, pigmentary retinal degeneration, and neuropathy. The treatment targets a specific mutation, c.640-15479C > T, which activates a cryptic 5' splice site in intron 7 of the PMM2 gene. To correct this splicing defect, Vega *et al.* [28] used PMOs to target the 5' or 3' cryptic splice sites of the pseudoexon in intron 7. Using the Endo-Porter reagent, PMOs were transfected into patient fibroblasts carrying the c.640-15479C > T mutation. The treatment completely restored correctly spliced mRNA and increased PMM2 protein levels from 9% to 23% of the amount detected in the control cell line. The enzymatic activity was also restored to almost 50% of that of the control fibroblasts. The use of ASOs, specifically PMOs, shows promise as a treatment for rare genetic disorders by targeting specific mutations.

FTDP-17, or Frontotemporal Dementia and Parkinsonism linked to chromosome 17, is a rare neurodegenerative disorder caused by mutations in the MAPT gene located on chromosome 17q21. The disorder manifests through cognitive impairment, changes in behavior and personality, and motor symptoms. More

than 100 families with 44 different mutations in the MAPT gene have been identified throughout the world, and the symptoms and onset age of FTDP-17 can vary greatly between families and even within families carrying the same mutation. The pathological hallmarks of the disease are the aggregation of tau protein in neurofibrillary tangles (NFTs) in neurons and/or glial cells. The mechanisms underlying the disorder are related to changes in the proportion of tau isoforms or the ability of tau to bind to microtubules. Currently, there is no cure for FTDP-17, and treatment is only symptomatic and supportive, with a variable rate of progression and life expectancy [29].

Tau is a protein in the central and peripheral nervous systems and plays a role in the formation and stability of microtubules, as well as in processes such as neurogenesis and axonal maintenance. The MAPT gene on chromosome 17 produces tau, and its expression can be regulated by alternative splicing of exons. In the human brain, specific exons are excluded or included in the final protein, leading to tau's microtubule-binding domain variations. Kalbfuss and colleagues [30] researched the E10 5' splice site, which Peacey and colleagues [34] later extended. They targeted this site with a bipartite 2'OMe-PS AON and compared its efficiency in inducing E10 skipping to that of Kalbfuss' continuous 5' splice site-targeting 2'OMe-PS oligonucleotide [31]. In addition, researchers have developed a type of ASO called bipartite 2'OMe-PS, designed to interact with specific regions of a gene known as exon ten and intron ten. This interaction results in a reduction in a specific form of the protein tau known as 4R and an increase in another form known as 3R. When tested in different forms of the minigene, the ASO reduced the expression of 4R tau and increased the expression of 3R tau. The effects of AON on endogenous tau mRNA were also tested in HEK 293 cells, resulting in a 42% reduction in E10 inclusion.

Niemann-Pick disease Type C (NPC) is a genetic disorder caused by mutations in the NPC1 or NPC2 genes and affects about 1 in 150,000 people. The disease is linked to a significant decrease in life expectancy and causes progressive neurological deterioration, psychiatric symptoms, and liver problems. Symptoms can vary greatly depending on the age of onset and can include muscle weakness, developmental delays, cognitive impairment, and psychosis. Mutations cause NPC in the NPC1 gene, many of which are missense mutations. However, some splicing mutations have also been identified. For example, a mutation in intron 9 of the NPC1 gene creates a pseudoexon, leading to abnormal splicing. Researchers have used a PMO (phosphorodiamidate morpholino oligomer) to correct this mutation by targeting the cryptic 5' splice site and blocking the access of the splicing machinery to the premRNA. Delivering the treatment to patient fibroblasts via the Endo-Porter system restores normal splicing. The PMO successfully eliminated aberrant splicing after 48 hours of treatment at a concentration of 10 mM [32]. The PMO was able to eliminate aberrant splicing after 48 hours of treatment at a concentration of 10 mM [32].

Neurofibromatosis type 1 (NFT 1) is a genetic disorder passed down through

families and is characterized by certain physical traits and an increased risk of certain types of tumors. It affects about 1 in 3000 people and often affects young children [33]. About 44% of these mutations are splicing mutations. Researchers have used a specific antisense oligonucleotide (PMO) to restore normal splicing in primary cell lines derived from NFT1 patients with deep intronic mutations. PMOs were designed to target the mutant 5' cryptic splice sites and delivered via an Endo-porter reagent. Treatment with PMO effectively restored normal splicing at the mRNA level for the three mutations studied in different cell lines, and it was found that the PMOs had an immediate effect on fibroblasts that lasted for several days. The researchers also found that PMOs positively affected neurofibromin function by reducing Ras-GTP levels, which is consistent with the restoration of neurofibromin function [34].

Neurofibromatosis type 2 (NFT 2) is a genetic disorder that causes tumors in the nervous system and ocular abnormalities. It affects approximately 1 in 33,000 newborns and is caused by mutations in the NF2 gene, which is responsible for producing the tumor suppressor protein Merlin. The presence of tumors such as bilateral vestibular nerve schwannomas and other types of tumors such as meningiomas, ependymomas, and tumors in other cranial, spinal, and peripheral nerves characterizes this disorder. A recent study by Castellanos *et al.* found a patient with a deep intronic mutation in the NF2 gene, which leads to the inclusion of a pseudoexon and results in a truncated Merlin protein. The study used PMO, a therapeutic approach, to target the deep intronic mutation and inhibit the inclusion of the pseudoexon in patient-derived fibroblasts, which increased Merlin protein levels [35].

Megalencephalic leukoencephalopathy with subcortical cyst type 1 (MLC1) is a rare genetic disorder that affects the brain and is caused by mutations in the MLC1 gene. The main symptoms of this disease include a large head, motor delays and disability, seizures, and cognitive decline. The MLC1 protein is found mainly in the plasma membrane of glial cells and neurons. Recent research has found a specific mutation in the MLC1 gene that reduces certain transcripts. The troublesome exon was completely removed when this mutation was targeted in a patient's cells with PMO therapy [36].

Pelizaeus-Merzbacher disease (PMD) is a rare genetic disorder that affects the nervous system and is passed down through families. It is caused by mutations in the PLP1 gene, which produces the proteolipid protein (PLP), which plays an essential role in the development of myelin in the central nervous system. Symptoms typically appear in early childhood, including nystagmus and hypotonia, ranging from mild to severe. A recent study found a mutation in the PLP1 gene that leads to the introduction of regulatory motifs that affect splicing, resulting in a loss of the significant PLP transcript. A therapeutic approach using PMO was used to target the mutation, and the results showed an increase in the ratio of PLP/(DM20 + PLP) mRNA. However, it remains to be seen whether the recovered PLP protein is functional [37].

4. Assessment of ASOs Performance

There are no established guidelines for testing the safety of antisense oligonucleotides (ASOs) outside clinical trials. Since these compounds are chemically synthesized, the safety testing guidelines for small-molecule drugs are typically used. Repeat-dose toxicity studies are generally required in rodents and non-rodents, using a model relevant to the compound's pharmacology. Nonhuman primates are the preferred model for these studies, but the Göttingen Minipig is a suitable alternative due to its similar safety profile. The genome of the Göttingen Minipig has been sequenced, allowing the development of homologous ASOs that cross-react in swine and evaluate adverse effects related to the pharmacological target [38]. However, only a small percentage of ASOs have been tested in minipigs for non-rodent toxicity studies.

ASOs are also being explored to treat pediatric diseases, such as neuromuscular diseases and retinopathy of prematurity, so more repeat-dose toxicity studies may be required in juvenile animals before starting pediatric clinical trials [39]. The juvenile minipig is a promising model for these studies due to its similarity to humans, technical feasibility, and welfare considerations. However, data on the pharmacodynamic, toxicological, and metabolic profiles of ASOs in juvenile minipigs are currently limited. To determine whether the juvenile Göttingen Minipig is a viable model for pediatric evaluation of ASOs, more information is needed about the ontogeny of the key nucleases that play a role in the metabolism and pharmacologic activity of ASOs, as well as an understanding of the functional immaturity of these nucleases.

A high level of complementarity between the oligonucleotide and mRNA is necessary to target an mRNA with an antisense oligonucleotide successfully. However, this can be difficult to achieve due to secondary or tertiary RNA structures or proteins bound to the RNA. Therefore, various methods have been developed to identify the best binding sites, such as using algorithms and thermodynamic properties to predict the structure of the RNA, measuring the melting temperatures or free energy of the oligonucleotide/RNA duplexes, or using combinatorial oligonucleotides to directly locate the hybridization sites within the RNA through techniques such as RNase H cleavage, microarrays, or mass spectrometry. These methods can be complex but have the potential to pinpoint optimal binding sites for antisense oligonucleotides [5].

4.1. Mirage to Overcome

The main challenge in developing ASO to correct genetic errors is nonspecific action (poor affinity to target sites), interference at several stages, unwanted reactions, drug delivery, and nuclease degradation [40]. To fully appreciate the therapeutic efficiency, these challenges need to be addressed. Current research and sophisticated equipment could make this possible in the near future, making ASO therapy a miracle in the treatment of rare diseases [41].

4.2. Nonspecific Action

For an antisense oligonucleotide to bind effectively to its target mRNA, there must be a high level of complementarity between the two. However, secondary or tertiary RNA structures and proteins can make it difficult for the oligonucleotide to bind. Various prediction methods have been developed to find the best binding sites. These include using algorithms to predict the structure of the RNA, determining the melting temperatures or free energy of the oligonucleotide/RNA duplexes, and using combinatorial oligonucleotides to identify hybridization sites within the RNA through techniques such as RNase H cleavage, microarrays, or MALDI-TOF mass spectrometry. Although these methods can be complex, they have the potential to identify optimal binding sites for antisense oligonucleotides [5].

4.3. Drug Interactions

ASOs can interact with other drugs in several ways. For example, if an ASO is designed to target a specific gene targeted by another drug, the two drugs may interact and potentially enhance or inhibit each other's effects. Additionally, some drugs can affect the pharmacokinetics or pharmacodynamics of an ASO, such as altering its absorption, distribution, metabolism, or excretion. Other drugs may also potentially degrade an ASO, such as nuclease enzymes that can break down the structure of the ASO. It is important to consider potential drug interactions when developing or administering ASO therapeutics. Drug interactions should be evaluated in preclinical studies and clinical trials [42].

4.4. Off-Target Effects

ASOs aim to ensure they only target their intended mRNA when developing ASO therapies. One way to do this is by using quantitative PCR to measure mRNAs' expression with sequences similar to the ASO candidate and ensure that their expression is not affected. Another method is to analyze the transcriptome of tissues from mice lacking the targeted mRNA after treatment with the ASO candidate. Any differently expressed genes would indicate off-target effects or potential cytotoxicity. Different chemical modifications have been used to improve the properties of ASOs, such as phosphorothioate or phosphorodiamidate backbones, modifications at the 2' position of the sugar, or conformationally constraining the sugar at the 2' and 4' positions. Modifications to ASOs, except for phosphorothioate, inhibit RNase H cleavage, but these limitations can be overcome by using a "gapmer" design. Although several ASO therapies exist in preclinical development and clinical trials for neurodegenerative diseases, only two therapies have been FDA-approved: nusinersen and eteplirsen. However, the success of these therapies and promising data from ongoing clinical trials for other ASO therapies for Huntington's disease and ALS suggest a positive future for new effective treatments for neurodegenerative diseases [3].

4.5. Nuclease Degradation

One of the main challenges in developing ASO therapies is to ensure that the oligonucleotides remain stable and effective in the body, as they are prone to degradation by nucleases, enzymes that break down nucleic acids. Nucleases are present in various body fluids and tissues and can rapidly degrade ASOs, reducing their efficacy and increasing the risk of off-target effects. Several nucleases can degrade ASOs, including exonucleases, which degrade the ends of nucleic acid molecules, and endonucleases, which break down nucleic acids within the molecule. Additionally, RNases, which are specific to RNA molecules, can also degrade ASOs. To overcome the issue of nuclease degradation, several chemical modifications have been developed to improve the stability of ASOs [16].

4.6. Drug Delivery

How antisense oligonucleotides enter cells is not well understood. Oligonucleotides are thought to be taken up by active transport, which depends on factors such as the temperature, the structure and concentration of the oligonucleotide, and the cell line. The main internalization mechanisms are adsorptive endocytosis and fluid phase pinocytosis, with the amount of internalized material depending on the oligonucleotide concentration. At lower concentrations, internalization is believed to occur through interaction with a membrane-bound receptor, partially characterized by Diesbach *et al.* [43].

Naked oligonucleotides are poorly internalized by cells and tend to localize in endosomes/lysosomes, where they cannot function as antisense molecules. Various techniques and transporters have been developed to improve cellular uptake and oligonucleotide activity. These include liposomes, cationic polymers, and basic peptides. Liposomes are vesicular colloid vesicles composed of phospholipids and cholesterol, which can encapsulate oligonucleotides, while cationic polymers and basic peptides can be used to increase the penetration of oligonucleotides through the plasma membrane. Furthermore, transient plasma membrane permeabilization can allow naked oligonucleotides to enter cells. While techniques such as liposomes, cationic polymers, and basic peptides have been used successfully in in vitro studies, their relevance for *in vivo* use remains uncertain [5].

5. Recommendations for the Use of ASOs in Gene Therapy

5.1. Chemical Modification—A Prerequisite

ASOs are versatile compounds that can be modified to improve their stability and specificity. To ensure the safety and efficacy of an ASO, in vitro and *in vivo* tests are necessary to understand its half-life and knockdown efficiency. Adding compounds such as peptide nucleic acid or modified ribose sugars can increase specificity and reduce toxicity. However, choosing the ASO sequence from a panel of candidates is important because only a small percentage of putative ASOs are active. To confirm the activity of an ASO, pilot experiments and various controls, such as the use of isosequential oligonucleotides with different backbones, multiple oligonucleotides

with different sequences that target the same mRNA, or the introduction of mutations to the target gene, can be used. Additionally, antisense RNA can also be used as a control for the inhibition of the expression of a target gene [44].

5.2. Evaluating the Effectiveness of ASOs in Targeting Specific mRNA

Using an observed biological endpoint to claim the effectiveness of antisense oligonucleotides is not considered acceptable. ASOs, particularly those with phosphorothioate backbones, can have sequence-nonspecific effects. Therefore, the best way to demonstrate the effectiveness of antisense oligonucleotides is by showing a decrease in a specific molecular target, most commonly protein expression, through techniques such as Western blotting. However, it is essential to note that these pilot experiments cannot guarantee that the oligonucleotides used do not have nonsequence-specific effects, and additional controls are needed to ensure this. Additionally, when phosphorothioates are used, it is difficult to determine whether down-regulation of the targeted protein causes the observed biological effect. This distinction is often overlooked in research.

5.3. Proper Controls and Demonstration of Down-Regulation

To address these issues, it is recommended to incorporate individual phosphordiester linkages into the oligonucleotides and protect the 3' and 5' termini with phosphorothioate linkages. Additionally, using chimeric phosphorothioate/phosphodiester backbones can be considered as an alternative approach. It is also important to note that phosphodiester linkages 5' to a purine residue are more resistant to nuclease degradation than those located 5' to a pyrimidine [45].

5.4. Ensuring Targeted Antisense Oligonucleotides with Minimum Off-Target Effects

Keeping the concentration of antisense oligonucleotides low is vital to avoid adverse side effects and ensure specificity, especially when phosphorothioates are used. Oligonucleotides should not be delivered without a carrier in tissue culture, as high concentrations are needed to achieve efficacy without a carrier, leading to nonspecific effects. Instead, commercially available carriers, such as cationic lipids or polyamines, can deliver the oligonucleotides. It is important to optimize the ratio of carrier to oligonucleotide and use the lowest effective carrier concentration to avoid potential toxicity. Furthermore, when using a carrier, it is best to avoid concentrations of phosphorothioate oligonucleotides higher than 4 - 5 μM [13].

5.5. Avoiding High Concentrations of Naked ASOs

Oligonucleotides that contain four consecutive guanosine residues can lead to the formation of G-quartets and tetraplexes through Hoogsteen base-pairing. This ability, depending on the sequence of the oligomer and the placement of the guanosines, can decrease the amount of available single-stranded oligomer and create new, highly charged structures that can have various nonspecific effects on the

sequence [46]. However, this problem can be resolved by replacing one of the guanosine residues with 7-deoxyguanosine, which cannot form Hoogsteen base pairs.

5.6. Avoiding the Use of CpG-Containing Oligonucleotides in Animal Experiments

In animal experiments, potential issues that may arise when using phosphorothioate oligonucleotides, particularly those containing the CpG motif, should be considered. These oligonucleotides have been found to stimulate immune responses, particularly Th1-biased responses, which can lead to tumor rejection. This effect can be controlled by modifying the sequences, flanking the CpG motif, or alkylating the cytosine residue. Palindromic sequences can be immune-stimulating and should be avoided in antisense applications *in vivo*. Reports of antisense-based antitumor effects in animals that have not been controlled for these issues are considered suspect [47].

6. Future of ASOs

Antisense oligonucleotides (ASOs) have shown promising potential in the treatment of many rare diseases. While challenges remain to be addressed, such as off-target effects and toxicity, technological advances are helping to increase specificity and reduce toxicity, making ASO therapy a more effective and safe treatment option for patients. One of the most exciting future perspectives for ASOs is the treatment of neurological disorders. ASOs have a remarkable ability to enter the central nervous system (CNS) after intrathecal delivery and have not shown an immune reaction at therapeutically relevant doses. ASOs are a promising option for treating disorders such as spinal muscular atrophy (SMA) and Huntington's disease. Following guidelines when using antisense oligonucleotides is important to ensure they are used appropriately. Guidelines for using ASOs may change as new oligonucleotides and related molecules are developed. However, following these guidelines and continuing to research how these molecules work will allow the full potential of this technology to be reached. Another important area of focus is the development of chemically modified ASOs that are more stable and have a longer half-life. It is crucial to ensure that the therapeutic payload reaches its target in the body and has the desired effect. Overall, the future of ASO therapy looks bright. With continued research and development, we can expect to see more effective and safe treatments for rare diseases in the near future.

7. Conclusion

Rare diseases often have poor clinical outcomes, are difficult to diagnose, and lack effective treatment options due to their complexity and rarity. Developing new therapeutic agents is crucial for improving these conditions' management, treatment, and symptom alleviation. Gene therapy, particularly ASOs, offers a promising approach to addressing rare diseases. Although ASOs have potential off-target effects, their high customizability and targeted action make them valuable

tools for developing effective treatments. Robust methodologies and precise combinations are essential for maximizing their therapeutic benefits. By utilizing ASOs in gene therapy, researchers can strive to develop more effective treatments for patients with rare diseases.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

References

[1] Doxakis, E. (2020) Therapeutic Antisense Oligonucleotides for Movement Disorders. *Medicinal Research Reviews*, **41**, 2656-2688. https://doi.org/10.1002/med.21706

[2] Kuijper, E.C., Bergsma, A.J., Pijnappel, W.W.M.P. and Aartsma-Rus, A. (2020) Opportunities and Challenges for Antisense Oligonucleotide Therapies. *Journal of Inherited Metabolic Disease*, **44**, 72-87. https://doi.org/10.1002/jimd.12251

[3] Scoles, D.R., Minikel, E.V. and Pulst, S.M. (2019) Antisense Oligonucleotides: A Primer. *Neurology Genetics*, **5**, e323. https://doi.org/10.1212/nxg.0000000000000323

[4] Aymé, S., Kole, A. and Groft, S. (2008) Empowerment of Patients: Lessons from the Rare Diseases Community. *The Lancet*, **371**, 2048-2051. https://doi.org/10.1016/s0140-6736(08)60875-2

[5] Dias, N. and Stein, C.A. (2002) Antisense Oligonucleotides: Basic Concepts and Mechanisms. *Molecular Cancer Therapeutics*, **1**, 347-355.

[6] Lauffer, M.C., van Roon-Mom, W. and Aartsma-Rus, A. (2024) Possibilities and Limitations of Antisense Oligonucleotide Therapies for the Treatment of Monogenic Disorders. *Communications Medicine*, **4**, Article No. 6. https://doi.org/10.1038/s43856-023-00419-1

[7] Roberts, T.C., Langer, R. and Wood, M.J.A. (2020) Advances in Oligonucleotide Drug Delivery. *Nature Reviews Drug Discovery*, **19**, 673-694. https://doi.org/10.1038/s41573-020-0075-7

[8] Zheng, Y.Y., Wu, Y., Begley, T.J. and Sheng, J. (2021) Sulfur Modification in Natural RNA and Therapeutic Oligonucleotides. *RSC Chemical Biology*, **2**, 990-1003. https://doi.org/10.1039/d1cb00038a

[9] Giles, R.V., Spiller, D.G., Clark, R.E. and Tidd, D.M. (1999) Antisense Morpholino Oligonucleotide Analog Induces Missplicing of C-myc mRNA. *Antisense and Nucleic Acid Drug Development*, **9**, 213-220. https://doi.org/10.1089/oli.1.1999.9.213

[10] Gryaznov, S.M., Lloyd, D.H., Chen, J.K., Schultz, R.G., DeDionisio, L.A., Ratmeyer, L., *et al.* (1995) Oligonucleotide N3'-->p5' Phosphoramidates. *Proceedings of the National Academy of Sciences*, **92**, 5798-5802. https://doi.org/10.1073/pnas.92.13.5798

[11] Barresi, V., Musmeci, C., Rinaldi, A. and Condorelli, D.F. (2022) Transcript-Targeted Therapy Based on RNA Interference and Antisense Oligonucleotides: Current Applications and Novel Molecular Targets. *International Journal of Molecular Sciences*, **23**, Article No. 8875. https://doi.org/10.3390/ijms23168875

[12] Holm, A., Hansen, S.N., Klitgaard, H. and Kauppinen, S. (2022) Clinical Advances of RNA Therapeutics for Treatment of Neurological and Neuromuscular Diseases. *RNA Biology*, **19**, 594-608. https://doi.org/10.1080/15476286.2022.2066334

[13] Stein, C.A. (2001) The Experimental Use of Antisense Oligonucleotides: A Guide for the Perplexed. *Journal of Clinical Investigation*, **108**, 641-644. https://doi.org/10.1172/jci13885

[14] Aslesh, T. and Yokota, T. (2020) Development of Antisense Oligonucleotide Gapmers for the Treatment of Huntington's Disease. In: Yokota, T. and Maruyama, R., Eds., *Gapmers*: *Methods and Protocols*, Springer US, 57-67. https://doi.org/10.1007/978-1-0716-0771-8_4

[15] Owoyemi, J.O., Traficante, M.K., Bamgboye, M.A., DiSilvestre, D., Vieira, D.C.O. and Dick, I.E. (2022) Antisense Oligonucleotides as a Treatment Strategy for Timothy Syndrome. *Biophysical Journal*, **121**, 99a-100a. https://doi.org/10.1016/j.bpj.2021.11.2233

[16] Phillips, M., Costales, J., Lee, R., Oliveira, E. and Burns, A. (2015) Antisense Therapy for Cardiovascular Diseases. *Current Pharmaceutical Design*, **21**, 4417-4426. https://doi.org/10.2174/1381612821666150803150402

[17] Benson, M.D., Waddington-Cruz, M., Berk, J.L., Polydefkis, M., Dyck, P.J., Wang, A.K., *et al.* (2018) Inotersen Treatment for Patients with Hereditary Transthyretin Amyloidosis. *New England Journal of Medicine*, **379**, 22-31. https://doi.org/10.1056/nejmoa1716793

[18] Gaudet, D., Alexander, V.J., Baker, B.F., Brisson, D., Tremblay, K., Singleton, W., *et al.* (2015) Antisense Inhibition of Apolipoprotein C-III in Patients with Hypertriglyceridemia. *New England Journal of Medicine*, **373**, 438-447. https://doi.org/10.1056/nejmoa1400283

[19] InSilico Medicine Hong Kong Limited (2022) A Phase 1, Randomized, Double-Blind, Placebo-Controlled, Oral Single and Multiple Ascending Doses, Parallel Group Study to Evaluate the Safety, Tolerability, and Pharmacokinetics of INS018_055 in Healthy Subjects (Clinical Trial Registration No. NCT05154240). https://clinicaltrials.gov/ct2/show/NCT05154240

[20] Mombelli, G. and Pavanello, C. (2013) Novel Therapeutic Strategies for the Homozygous Familial Hypercholesterolemia. *Recent Patents on Cardiovascular Drug Discovery*, **8**, 143-150. https://doi.org/10.2174/15748901112079990001

[21] Kumar, A., Acharya, A., Nandi, D., Sharma, N. and Chitkara, E. (n.d.) Mipomersen: A Novel Therapeutic Drug for the Treatment of Familial Hypercholesterolemia, Hyperlipidaemia, and Hypercholesterolemia.

[22] Miller, T., Cudkowicz, M., Shaw, P.J., Andersen, P.M., Atassi, N., Bucelli, R.C., *et al.* (2020) Phase 1-2 Trial of Antisense Oligonucleotide Tofersen for SOD1 ALS. *New England Journal of Medicine*, **383**, 109-119. https://doi.org/10.1056/nejmoa2003715

[23] Singh, R.N. and Singh, N.N. (2018) Mechanism of Splicing Regulation of Spinal Muscular Atrophy Genes. In: Sattler, R. and Donnelly, C.J., Eds., *RNA Metabolism in Neurodegenerative Diseases*, Springer International Publishing, 31-61. https://doi.org/10.1007/978-3-319-89689-2_2

[24] Knowles, J.K., Helbig, I., Metcalf, C.S., Lubbers, L.S., Isom, L.L., Demarest, S., *et al.* (2022) Precision Medicine for Genetic Epilepsy on the Horizon: Recent Advances, Present Challenges, and Suggestions for Continued Progress. *Epilepsia*, **63**, 2461-2475. https://doi.org/10.1111/epi.17332

[25] Chi, K.N., Higano, C.S., Blumenstein, B., Ferrero, J., Reeves, J., Feyerabend, S., *et al.* (2017) Custirsen in Combination with Docetaxel and Prednisone for Patients with Metastatic Castration-Resistant Prostate Cancer (SYNERGY Trial): A Phase 3, Multicentre, Open-Label, Randomised Trial. *The Lancet Oncology*, **18**, 473-485. https://doi.org/10.1016/s1470-2045(17)30168-7

[26] Sandweiss, A.J., Brandt, V.L. and Zoghbi, H.Y. (2020) Advances in Understanding of Rett Syndrome and MECP2 Duplication Syndrome: Prospects for Future Therapies. *The Lancet Neurology*, **19**, 689-698. https://doi.org/10.1016/s1474-4422(20)30217-9

[27] Cavalieri, S., Pozzi, E., Gatti, R.A. and Brusco, A. (2012) Deep-Intronic ATM Mutation Detected by Genomic Resequencing and Corrected *in Vitro* by Antisense Morpholino Oligonucleotide (AMO). *European Journal of Human Genetics*, **21**, 774-778. https://doi.org/10.1038/ejhg.2012.266

[28] Vega, A.I., Pérez-Cerdá, C., Desviat, L.R., Matthijs, G., Ugarte, M. and Pérez, B. (2009) Functional Analysis of Three Splicing Mutations Identified in the PMM2 Gene: Toward a New Therapy for Congenital Disorder of Glycosylation Type Ia. *Human Mutation*, **30**, 795-803. https://doi.org/10.1002/humu.20960

[29] Tsuboi, Y. (2009) Clinical, Pathological, and Genetic Characteristics of Frontotemporal Dementia and Parkinsonism Linked to Chromosome 17 with Mutations in the MAPT and PGRN. *Brain and Nerve*, **61**, 1285-1291.

[30] Kalbfuss, B., Mabon, S.A. and Misteli, T. (2001) Correction of Alternative Splicing of Tau in Frontotemporal Dementia and Parkinsonism Linked to Chromosome 17. *Journal of Biological Chemistry*, **276**, 42986-42993. https://doi.org/10.1074/jbc.m105113200

[31] Peacey, E., Rodriguez, L., Liu, Y. and Wolfe, M.S. (2012) Targeting a Pre-mRNA Structure with Bipartite Antisense Molecules Modulates Tau Alternative Splicing. *Nucleic Acids Research*, **40**, 9836-9849. https://doi.org/10.1093/nar/gks710

[32] Mukherjee, S. and Maxfield, F.R. (2004) Lipid and Cholesterol Trafficking in NPC. *Biochimica et Biophysica Acta* (*BBA*)—*Molecular and Cell Biology of Lipids*, **1685**, 28-37. https://doi.org/10.1016/j.bbalip.2004.08.009

[33] Friedman, J.M. (1999) Epidemiology of Neurofibromatosis Type 1. *American Journal of Medical Genetics*, **89**, 1-6.

[34] Fernández-Rodríguez, J., Castellsagué, J., Benito, L., Benavente, Y., Capellá, G., Blanco, I., *et al.* (2011) A Mild Neurofibromatosis Type 1 Phenotype Produced by the Combination of the Benign Nature of a Leaky Nf1-Splice Mutation and the Presence of a Complex Mosaicism. *Human Mutation*, **32**, 705-709. https://doi.org/10.1002/humu.21500

[35] Castellanos, E., Rosas, I., Solanes, A., Bielsa, I., Lázaro, C., Carrato, C., *et al.* (2013) Erratum: *In Vitro* Antisense Therapeutics for a Deep Intronic Mutation Causing Neurofibromatosis Type 2. *European Journal of Human Genetics*, **22**, 153-153. https://doi.org/10.1038/ejhg.2013.224

[36] Mancini, C., Vaula, G., Scalzitti, L., Cavalieri, S., Bertini, E., Aiello, C., *et al.* (2012) Megalencephalic Leukoencephalopathy with Subcortical Cysts Type 1 (MLC1) Due to a Homozygous Deep Intronic Splicing Mutation (c.895-226T > G) Abrogated *in Vitro* Using an Antisense Morpholino Oligonucleotide. *Neurogenetics*, **13**, 205-214. https://doi.org/10.1007/s10048-012-0331-z

[37] Regis, S., Corsolini, F., Grossi, S., Tappino, B., Cooper, D.N. and Filocamo, M. (2013) Restoration of the Normal Splicing Pattern of the PLP1 Gene by Means of an Antisense Oligonucleotide Directed against an Exonic Mutation. *PLOS ONE*, **8**, e73633. https://doi.org/10.1371/journal.pone.0073633

[38] Valenzuela, A., Tardiveau, C., Ayuso, M., Buyssens, L., Bars, C., Van Ginneken, C., *et al.* (2021) Safety Testing of an Antisense Oligonucleotide Intended for Pediatric Indications in the Juvenile Göttingen Minipig, Including an Evaluation of the Ontogeny of Key Nucleases. *Pharmaceutics*, **13**, Article No. 1442. https://doi.org/10.3390/pharmaceutics13091442

[39] McDowall, S., Aung-Htut, M., Wilton, S. and Li, D. (2024) Antisense Oligonucleotides and Their Applications in Rare Neurological Diseases. *Frontiers in Neuroscience*, **18**, Article ID: 1414658. https://doi.org/10.3389/fnins.2024.1414658

[40] Burbano, L.E., Li, M., Jancovski, N., Jafar-Nejad, P., Richards, K., Sedo, A., Soriano, A., Rollo, B., Jia, L. and Gazina, E. (2020) Antisense Oligonucleotide Therapy for KCNT1 Encephalopathy.

[41] Siva, K., Covello, G. and Denti, M.A. (2014) Exon-Skipping Antisense Oligonucleotides to Correct Missplicing in Neurogenetic Diseases. *Nucleic Acid Therapeutics*, **24**, 69-86. https://doi.org/10.1089/nat.2013.0461

[42] Jason, T.L.H., Koropatnick, J. and Berg, R.W. (2004) Toxicology of Antisense Therapeutics. *Toxicology and Applied Pharmacology*, **201**, 66-83. https://doi.org/10.1016/j.taap.2004.04.017

[43] Diesbach, P.d. (2000) Identification, Purification and Partial Characterisation of an Oligonucleotide Receptor in Membranes of Hepg2 Cells. *Nucleic Acids Research*, **28**, 868-874. https://doi.org/10.1093/nar/28.4.868

[44] Bennett, C.F., Dean, N.M. and Monia, B.P. (1998) Antisense Oligonucleotide. *Advances in Drug Discovery Techniques*, 173.

[45] Vaerman, J.L., Moureau, P., Deldime, F., Lewalle, P., Lammineur, C., Morschhauser, F., *et al.* (1997) Antisense Oligodeoxyribonucleotides Suppress Hematologic Cell Growth through Stepwise Release of Deoxyribonucleotides. *Blood*, **90**, 331-339. https://doi.org/10.1182/blood.v90.1.331.331_331_339

[46] Benimetskaya, L. (1997) Formation of a G-Tetrad and Higher Order Structures Correlates with Biological Activity of the Rela (NF-κB p65) "Antisense" Oligodeoxynucleotide. *Nucleic Acids Research*, **25**, 2648-2656. https://doi.org/10.1093/nar/25.13.2648

[47] Krieg, A.M. (2004) Antitumor Applications of Stimulating Toll-Like Receptor 9 with CpG Oligodeoxynucleotides. *Current Oncology Reports*, **6**, 88-95. https://doi.org/10.1007/s11912-004-0019-0