Antisense Oligonucleotides: A Unique Treatment Approach

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Synthetic Antisense oligonucleotides (ASOs) are novel and efficient laboratory tools to regulate the expression of specific genes, and have only recently come into clinical use. These are synthetic single-stranded DNA analogs, whose sequence is complementary to a target nucleotide and alter protein synthesis by several mechanisms. We herein provide a primer on the topic for pediatricians, as this group of drugs is likely to see many more drugs for previously incurable diseases.

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Nucleic acids come in two forms: deoxyribonucleic acids (DNA) and ribonucleic acids (RNA). RNA has much structural variety with subtypes like messenger RNA (mRNA, that codes for protein) non-coding RNAs, transfer RNA (tRNA), ribosomal RNA (rRNA), and long-noncoding RNAs (lncRNAs) – DNA is a much more stable molecule [1]. Genetic information from DNA encodes to RNA, *ie*, transcription, which is then translated into proteins. Most of the available drugs, such as small molecules and antibodies target mainly proteins due to their mechanisms of action and chemical properties. In recent years, the use of compounds that can bind messenger RNAs (mRNAs) has gained increasing interest, as inhibition of protein expression can be helpful for controlling the course of inflammatory and neoplastic diseases. The two major therapeutic approaches in this field are the antisense oligo-nucleotides (ASOs) that inhibit mRNA translation, and the oligonucleotides, which function *via* RNA inter-ference (RNAi) pathway [2].

Synthetic antisense oligonucleotides are a novel and efficient laboratory tool to regulate the expression of specific genes. These are synthetic single-stranded DNA analogs; usually 15-30 base pairs in length, whose sequence (3' to 5') is antisense and complementary to the sense sequence of the target nucleotide (mRNA) hence called antisense oligonucleotides [2]. They selectively bind to specific pre-messenger ribonucleic acid (pre-mRNA)/mRNA sequences and alter protein synthesis by several mechanisms. First studied in the late 1970s to inhibit oncogenic viral production [3], further research led to designing of highly modified ASOs with more targeted delivery, tolerability, safety, with a prominent role in treating life-threatening diseases that were previously incurable [4].

MECHANISM OF ACTION

The mechanism of action of ASOs may briefly be summarized in three sequential steps, as follows [5]:

Pre-hybridization phase is the phase in which the ASO enters the cell, distributes within the cell, to achieve sufficient concentrations at the target RNA site. The internalization of the ASO within the cell by carrier protein-mediated endocytosis is a complex process – further, the ASO needs to escape the cellular endosomal pathway to reach the target site, which is a rate-limiting process [6].

Hybridization phase is the phase in which ASO sorts through the cellular nucleic acid sequence space to hybridize to its target RNA site. This is a complex process that involves interactions with proteins, such as Ago2, or other cellular components [5].

Post hybridization phase: After binding to the mRNA site depending on the chemical design of the ASO, a variety of events may be induced that alter the target RNA to achieve the desired pharmacological outcome. There are two main mechanisms: the common mechanism is by induction of endogenous RNAse H activity (ASO-RNase H) that cleaves the mRNA-ASO hetero-duplex which leads to degradation of the target toxic mRNA and leaves the ASO intact. The second mechanism includes binding to the RNA and causing translational inhibition by steric hindrance, exon skipping, exon inclusion, destabilization of pre-mRNA in the nucleus, or targeting the destruction of microsomal RNAs that control the expression of other genes [2,7]. For example, ASOs can bind to mRNA

structures and prevent the 5'-mRNA cap formation or, alternatively, they modify the polyadenylation site to prevent mRNA translation or alter RNA stability. Moreover, ASOs can directly stick to the mRNA and sterically block the 40S and 60S ribosomal subunits from attaching or running along the mRNA transcript during translation. Other ASOs bind on pre-mRNA intron/exon junctions and directly modulate splicing by masking splicing enhancers and repressor sequences, skipping exons, or forcing the inclusion of otherwise alternatively spliced exons. These actions are independent of RNase activity, as with Eteplirsen.

The mechanism of ASO action is shown in ***Fig.* 1**. There are many hurdles to incorporating ASOs in therapeutic use, because of their mechanism of cellular uptake and action (***Box* 1**). Thus, modifications are needed on the native ASOs to overcome these disadvantages, and this has led to various versions for clinical use.

First Generation ASOs

These are obtained by replacing one of the non-bridging oxygen atoms in the phosphate group of nucleotide with either sulfur groups (phosphorothioates), methyl groups (methylphosphonates) or amines (posphoroamidates). Phosphorothioate substitution was the earliest and the most commonly used modification that renders the inter-nucleotide linkage resistant to nuclease degradation, supports endogenous RNAse H activity to degrade the target mRNA, improves the pharmacokinetic characteristics by their binding with plasma proteins which alter the half-life and increases the availability of ASO to the target site. The other type is thiophosphoramidate substitution, though it had many side-effects in animal models and *in vitro* experiments [2,8].

Box I Hurdles to Using Anti-sense Oligonucleotides for Therapeutic Purposes

- Nucleic acids are inherently susceptible to degradation by endogenous nucleases: ASOs in their native forms have a very short half-life, even before they are filtered out through the kidney.
- Unfavorable bio-distribution and pharmacokinetic properties: Synthetic ASOs are large (approximately 30 kD) and highly negatively charged molecules and thus do not cross vascular endothelium, dense extracellular matrix and cell and nuclear membranes in order to reach their intracellular DNA or mRNA targets.
- Off-target effects of ASO may lead to a devastating adverse reaction.
- Synthetic ASOs can be immunogenic.
- Sub-optimal binding affinity for complementary sequences.

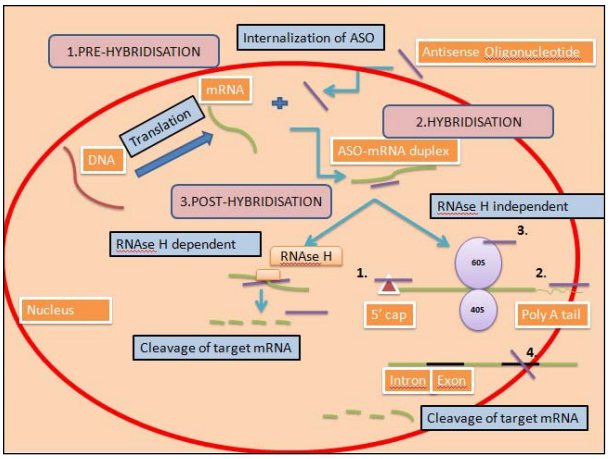

FIG. 1 *RNAse H independent mechanisms to prevent mRNA translation by ASO. Step 1. Binds to 5' cap; Step 2. Binds to poly A tail; Step 3. Stearic hindrance; and Step 4. Modifies exon splicing.*

Second Generation ASOs

Second generation ASOs were developed to overcome the shortcomings of first-generation ASOs. These second-generation antisense agents, contain a Phosphorothioate backbone and replacement of the 2-hydroxyl by many different groups but most commonly by 2'-O-methoxy (OMe), 2'-O-methoxyethyl (MOE), and locked nucleic acid (LNA). 2'-OMe modifications are commonly used in a 'gapmer' design, which is a chimeric oligo nucleotide comprising a DNA sequence core with flanking 2'-MOEnucleotides.2-Methoxyethyl is probably the most common one used in trials. Their mechanism of action is RNAse independent. The advantages of these ASOs are improved nuclease resistance, target-binding affinity, increased thermal stability of complementary hybridization, encourages tighter binding and allowing use of shorter oligonucleotides [2,4,7].

Third Generation ASOs

These are modified ASOs which help in their intracellular uptake and effective delivery to the target. These ASOs are covalently bound to a carrier or ligand, such as lipid particles, liposomes, nanoparticles, and, more recently, the sugar N-acetyl galactosamine to enhance safer delivery to the target site [2,9,10].

PHARMACOKINETICS

The rationale for understanding the pharmacokinetics of ASOs is to correlate effective dose with clearance rates to

optimize effectiveness while reducing potentially harmful side-effects [6]. The route of administration of these drugs is parenteral – intravenous, subcutaneous and intrathecal because oral bioavailability is less than 1% [11].

Absorption: The pharmacokinetic properties of oligonucleotides following parenteral administration are predominantly studied in phosphorothioate oligonucleotides, which after parenteral administration, bind to plasma proteins at >90% and transfer rapidly from blood to tissues with a distribution half-life of 1-2 hour. By 12 hour after dosing, <1% of the administered dose remains in circulation. At the same time, <5% of the administered dose is recovered in urine and feces over the first day, and this broad distribution to tissues causes the rapid disappearance of compound from blood. The highest tissue accumulation has been observed in kidney, liver, spleen, lymph nodes, adipocytes and bone marrow. In marked contrast, those oligonucleotides lacking charge and/or binding to plasma proteins (peptide nucleic acids, morpholinos) are rapidly cleared from plasma, with substantially higher excretion in urine in the first day resulting in lower overall tissue accumulation and ultimate target bioavailability [12].These drugs do not cross the blood-brain barrier and poorly distribute in skeletal muscle, heart, and lung.

Distribution: Although distribution out of plasma to tissue is rapid, the ultimate distribution to the active site within cells following a single dose is maximally realized at 24-48 h. Thus, the onset of action for antisense oligonucleotides is slower than the distribution out of plasma, which is explained due to the intracellular uptake and the kinetics for transport from the cell surface to the nucleus. Once distributed to cells, they are slowly cleared with tissue half-life ranging from 2-4 weeks [11,13].

Metabolism: These drugs are metabolized by endonucleases, which are expressed in most tissues hence liver dysfunction does not appear to affect their action. These drugs are not substrates for cytochrome P450 enzymes, and hence a very low drug-drug interaction is known [11].

Excretion: Excretion is predominantly renal and fecal; biliary uptake is minimal.

CLINICAL USE

Chemically modified 1st and 2nd generation ASOs have generated a new hope in the management of devastating neuromuscular and few other diseases such as Duchenne Muscular Dystrophy (DMD), Spinal Muscular atrophy (SMA), Myotonic dystrophy, familial hypercholesterolemia, Amyotrophic lateral sclerosis, factor IX thrombosis, Huntington chorea and peripheral neuropathies [6,14,15]. Nearly two decades after their advent, the US FDA approved the first ASO for therapeutic use in 1998 (Fomiversan). We have come a long way since then (**Box II**), with approvals coming for two oligonucleotides in 2016 for use in DMD and SMA [16]. These modify the disease make-up and progression by an effect at the gene (mRNA) level, hence provide immense scope for complete cure or at least a better quality of life. A multitude of other drugs is under development or in trials for the treatment for various other diseases, including cancers.

Individual Drugs

Fomiversan: This was the first ASO to be approved by the FDA in 1998, used clinically in CMV retinitis secondary to AIDS [17]. At that time, there was a high unmet need for anti-cytomegalovirus retinitis drugs; however, subsequently, due to the development of high-activity antiretroviral therapy (HAART), the number of CMV cases dramatically decreased [16], and its use has declined.

Pegabtinib: It was approved by the FDA in 2004 to treat Age-related macular degeneration (AMD) of the retina [16,18]. This is caused by the VEGF-stimulated growth of blood vessels (neovascularization)of the choroid of the eye leading to macular blindness. This molecule prevents the binding of VEGF to VEGFR receptors. However, with the advent of cheaper and better alternatives like bevazimumab, its use is on the downswing [16].

Eteplirsen: It was approved by the FDA for DMD in 2016, which was a remarkable step in the future of treatment for this disease. DMD is caused by mutations within the dystrophin gene that disrupt the reading frame or cause premature termination of protein synthesis [19].This was

BOX II Available Anti-sense Oligonucleotides for Clinical Application

Nusinersen:	Spinal muscular atrophy
Eteplirsen:	Duchenne Muscular Dystrophy
Fomiversan:	Cytomegalovirus retinitis secondary to AIDS
Pegabtinib:	Age-related macular degeneration of the retina
Mipomersen:	Familial hypercholesterolemia
Defibrotide:	Severe HVOD following high-dose chemotherapy and autologous BMT
Patisiran:	Transthyretin amyloidosis

HVOD: hepatic veno-occlusive disease; BMT: bone marrow transplantation

also the first approved exon skipping ASO to be used in humans [14]. This molecule is a 30-nucleotide phosphoro-diamidatemorpholino oligomer type third-generation ASO that hybridizes to exon 51 of DMD Pre-mRNA and causes it to be skipped during splicing; this corrects the translational reading frame in certain DMD gene deletions, resulting in the production of shortened but functional dystrophin protein similar to what is found in Becker's muscular dystrophy. It is effective only in DMD caused by exon 51 deletion (13%), which, however, is supported by a limited trial including 12 patients. High costs limit its widespread use [16].

The FDA approval of eteplirsen has been controversial due to the poor generalizability of the trial. Mendell, *et al*. [20] compared the three-year progression of the disease and its effect on ambulation in those receiving Eteplirsen and compared them to historic controls (*n*=13). Ambulatory DMD patients aged between 7-13 years, amenable to exon 51 skipping who were able to walk between 180-440 m on 6-Minute Walk Test and on stable corticosteroids for 24 weeks were randomized into three cohorts (each *n*=4) *viz*, placebo, Eteplirsen 30 mg/kg/week and 50 mg/kg/week. Later all received the drug in an open labeled trial. Six minute walk test and pulmonary function tests were done at baseline, 6, 1 and 24 months. A significant advantage on the walk test and a lower incidence of loss of ambulation (16%) were seen in the eteliprisen group in comparison to matched historic controls (46.2%).

Nusinersen: This is the other ASO approved by FDA in 2016 for spinal muscular atrophy (SMA). SMA is most frequently caused by a homozygous deletion or mutation within the *Survival motor neuron1 (SMN1)* gene located on chromosome 5. Homozygous deletion of *SMN1* exon 7 is confirmatory for the diagnosis of SMA. *SMN1* gene codes for the ubiquitously expressed 'survival motor neuron' (SMN) protein, which is essential for the maintenance of motor neurons. Humans have one more paralogous *SMN1* gene copy, referred to as *SMN2*, which differs from *SMN1* only by a cytosine-to-thymine mutation in exon 7 of the *SMN2* gene,which leads to alternative splicing processes with the consequence that exon 7 is omitted from the majority of *SMN2* transcripts [6,14,21]. Yet a small amount (approximately 10%) of functional SMN protein is expressed *via* the *SMN2* gene. This allows for partial compensation of the lost SMN1 exon 7 by SMN2 synthesis. Clinical phenotype is hence related to the number of *SMN2* copies [21]. Nusinersen is a 2'-OMe phosphorothioate ASO that induces the inclusion of exon 7 in the *SMN1* and *SMN2* mRNA by targeting and blocking an intron 7 internal splice site and producing functional SMN protein [16]. Nusinersen is now indicated in infants with types 1, 2, and 3 SMA. Nusinersen has to be given intrathecally as it does not cross the blood-brain barrier. The mean plasma terminal elimination half-life is 63-87 days, and the mean CNS terminal elimination half-life is 135-177 days. A fixed-dose is recommended because dose-related toxicity has not been demonstrated. The renal route of elimination is applicable for nusinersen and its inactive metabolites [22]. It is supplied as 12 mg/5 mL preservative-free solution and given in a standard dose of 12 mg on days 0, 14, 28, and 63 in two-weekly intervals followed by repeated applications in 4-month intervals [22,23].

Nusinersen is probably the most promising ASO manufactured which could modify the outcome and mortality in infants with SMA. A randomized, double-blind, sham-controlled trial by Finkel, *et al*. [23] in 2017 proved the same. In this trial 122 infants who were less than 7 months of age at the time of screening, having onset of symptoms from less than 6months of age with a confirmed mutation in the *SMN1* gene with two copies of *SMN2* gene were randomized in 2:1 ratio. 81 were to receive the drug and 41 the sham injections. Nusirensen was injected intrathecally 12 mg/ adjusted according to CSF volume on days on days 1, 15, 29, and 64 and maintenance doses on days 183 and 302. The primary endpoints were the motor-milestone response defined according to results on the Hammersmith infant neurological examination and event-free survival which was the time to death or the use of permanent assisted ventilation. Secondary end-points were overall survival and subgroup analyses of event-free survival according to disease duration at screening. Due to a very significant result during the interim analysis which showed a motor milestone response of 41% in test and 0 in control, the trial was prematurely terminated and everyone received the drug. In the final analysis, 51% in the test group showed a motor response. Risk of death or use of permanent assisted ventilation was lower in nusinersen group 47% [hazard ratio (95% CI) 0.53 (0.32-0.89), $P = 0.005$]. Also the likelihood of event-free survival and overall survival were significantly more in the test group – infants with the shortest disease duration prior to drug administered had the highest likelihood of event-free survival [23].

In another multi-center, double-blind, sham-controlled phase 3 trial by Mercuri, *et al*. [24] in 2018, 126 children with SMA who had symptom onset after 6 months of age were randomly assigned in a 2:1 ratio, to undergo intrathecal administration of nusinersen at a dose of 12 mg (nusinersen group) or a sham procedure (control group) on days 1, 29, 85, and 274. The primary endpoint was the least squares mean change from baseline in the Hammersmith functional motor scale-

expanded (HFMSE) score at 15 months of treatment; Secondary endpoints included the percentage of children with a clinically meaningful increase from baseline in the HFMSE score (≥3 points), an outcome that indicates improvement in at least two motor skills. This trial was also prematurely terminated as the pre-specified interim analysis showed a least-squares mean increase (by 4.0 points) from baseline to month 15 in the HFMSE score in the nusinersen group and a least-squares mean decrease in the control group (by-1.9 points) with a significant between-group difference favoring nusinersen (least-squares mean difference in change, 5.9 points; 95% confidence interval, 3.7 to 8.1; $P<0.001$). Results of the final analysis were consistent with results of the interim analysis in which 57% of the children in the nusinersen group as compared with 26% in the control group had a significant increase from baseline to month 15 in the HFMSE score of at least 3 points, and the overall incidence of adverse events was similar in the nusinersen group and the control group (93% and 100%, respectively) [24]. Patients from both the above trials were later enrolled in SHINE, an open-label extension trial. SHINE is structured to evaluate the effects of longer treatment with nusinersen with respect to motor function and quality of life which is still ongoing [25]. The results from the above studies infer that nusinersen could produce positive changes in the SMA patient's clinical course however the results may not be generalizable to all patients with SMA because only patients with types 1, 2, and 3 were included in the trials. Additionally, the long-term benefit and safety is not known at this time. It appears that nusinersen may have more benefit in patients who are younger with less severe disease or less comorbidity.

The most common adverse reactions of nusinersen are (>10%) upper and lower-respiratory tract infections (39%-43%), atelectasis (14%), constipation (30%), headache (50%), back pain (41%), and post-lumbar puncture syndrome (41%). Its high cost and route of administration are its major limitations [22,26].

Mipomersen: Mipomersen is the first FDA-approved systemically-delivered ASO in 2013 for familial hypercholesterolemia [16]. This is a disease caused loss of function mutations in both LDL-receptor genes which results in the reduced liver uptake of plasma LDL cholesterol leading to a very high plasma concentration of low-density lipoprotein. The core protein of the LDL particle is apolipoprotein B [27]. Mipomersen is targeted to the coding region of the apoB mRNA which effectively reduces plasma LDL and cholesterol levels with less deleterious effects on HDL. Side effects are injection site reactions and liver toxicity [28].

Other drugs include Defibrotide for severe hepatic veno-occlusive disease occurring after high dose chemotherapy and autologous bone marrow transplantation [29], and Revusiran, Patisiran and Inotersen for Transthyretin amyloidosis [30-32].

Adverse Drug Reactions

Oligonucleotides are prone to a diverse array of off-target interactions because of their size, negative charge, and potential to be synthetic [4]. Thus, despite a good overall safety profile, a few adverse reactions are encountered due to their off-target effects [33]. However, the number of studies and the sample size included are too small to determine the general side effect profile, the dose relationship and class effect for these drugs.

Binding of nucleic acid to cell surface proteins or to proteins inside cells -oligonucleotides can bind serine/threonine protein kinase PKR or Toll-like receptors (TLRs) and activate the innate immune response/alternate complement pathway. These can also bind to dRNA and DNA by complementary base-pairing. Vasculitis or glomerulonephritis are rare manifestations of immune activation [9]. All ASOs and dsRNAs will be at least partially complementary to DNA or RNA sequences inside cells that are not their intended targets and thus can modify the actions of genes on these mRNA/DNA, which could be harmful [4].

Thrombocytopenia is one of the most common side-effects seen [34]. Two forms of thrombocytopenia are studied. The more common form is milder, transient and dose-dependent wherein bleeding episodes are very rare. In humans, thrombocytopenia has been reported in cancer studies with a number of first-generation ASOs and occasionally with second-generation ASOs such as Mipomersen [35]. Other is rarer and severe form with bleeding episodes [36].

Cost

Despite remarkable progress in the development of ASOs for clinical use, the cost remains a major limitation for widespread use. For Eteplirsen, the costs were estimated at US$ 57,600 (INR 38,59,000) per month [37]. For Nusinersen, the cost of treatment of a patient with SMA amounts to US$ 750,000 (INR 5,02,50,000) for the first year, and half of that every year afterward [38,39].Thus the high costs are a major setback to any healthcare system plus the poor validity of the clinical trials in showing efficacy and adverse effects do not give a definitive risk-benefit advantage.

CONCLUSIONS

The invention of ASOs represents a therapeutic

milestone in those diseases for which we do not have a definitive cure by modifying the disease pathways. ASOs are under development or have already been tested in clinical trials for the treatment of many other diseases like myotonic dystrophy, Huntington chorea, Amyotrophic lateral sclerosis, Hemophilia A, Hereditary neuropathies. There have been some demands from individual patients and patient-support groups in LMICs (including India) to permit use of these drugs through publicly-funded programs. Although these drugs have good safety and tolerability, their high cost, route of administration, localized target (not applicable to all variants of disease), and lack of significant clinical trials describing mechanism of action, target sites, efficacy, and side effects are the major limitations. Hence, further research is required to better elucidate these important aspects, before widespread use would be a possibility.

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