Antisense oligonucleotides: modifications and clinical trials

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There has been an upsurge in the number of clinical trials involving chemically modified oligonucleotide-based drug candidates after the FDA approval of Vitravene, Macugen, and recently, Kynamro. Over the years, different types of backbone, nucleobase and/or sugar-modified oligonucleotides have been synthesized because natural DNA/RNA based oligonucleotides pose some limitations, such as poor binding affinity, low degree of nuclease resistance, affecting their direct use in antisense therapeutics. In this review article, we discuss in detail different modifications of nucleosides/oligonucleotides along with the related clinical trials, which demonstrated their potential as drug candidates for antisense and related nucleic acid based therapeutics.

Introduction

Most of the drugs present in the market interact with proteins; moreover, they often bind to non-target proteins or exert an adverse effect through unknown interactions.1 The dream of modern drug research to develop a therapeutic technology that can act specifically only on the target responsible for the disease has led to the development of drugs that can turn off genes by targeting directly the nucleic acids that code for the proteins. Antisense therapeutics were introduced after Paterson et al.2 in 1977 reported the utility of nucleic acids in modulating gene expression, and shortly after, Zamecnik and Stephenson3 demonstrated the inhibition of viral replication by modified oligonucleotides (ONs).4 In the quest of effective antisense candidates, various chemical modifications of the natural ONs have been studied, such as modifications in the phosphodiester backbone, heterocyclic nucleobase and sugar moiety, which confer high affinity and specificity for their target nucleic acid sequences (Fig. 1).5

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Internucleoside linkage or backbone modified AONs

These are also referred to as the first generation of chemically modified antisense agents. They contain backbone modifications such as 5'-N-carbamate, methylene–methylimine (MMI), amide, triazole, phosphorothioate (PS), phosphorodithioate, thioether, thioformacetal, mercaptoacetamide, methylphosphonate, boranophosphate, N-3'-phosphoramidate.

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(NP), S-methylthiourea, and guanidinium, and they have been designed and synthesized to circumvent the physical and biological limitations of the natural phosphodiester linkage. These backbone modifications can be broadly classified as neutral, anionic or cationic internucleoside linkages (Fig. 2).

Phosphorothioate oligonucleotides (PS-ONs) are the major representatives of this generation and have been used most successfully for gene silencing. The introduction of a PS linkage into ONs confers sufficient resistance to nuclease degradation, leading to higher bioavailability. In addition to nuclease resistance, PS-ONs form regular Watson–Crick base pairs, activate RNase H, carry negative charge for cell delivery, and display attractive pharmacokinetic properties and cellular uptake due to increased binding to plasma proteins and other receptor sites as compared to natural phosphodiesters. However, their profiles for binding affinity to the target oligonucleotide sequences and specificity are less satisfactory. Despite these disadvantages, the FDA approved the first antisense drug Vitravene, a first generation PS-modified AON for the treatment of AIDS-related cytomegalovirus (CMV) retinitis (Fig. 3).

Sugar modified AONs

In recent years, there has been a sudden leap in the synthesis of conformationally constrained nucleoside analogues by modifying the sugar moiety in various ways. These include: (a) synthesis of nucleoside analogues containing an electronegative atom or substituent at the 2′-position of sugar; (b)
synthesis of bicyclic nucleoside analogues having an extra ring fused to the sugar moiety;\(^\text{(e)}\) synthesis of nucleoside analogues of varying sugar ring structures;\(^\text{(f)}\) and synthesis of spiro nucleosides containing a spirocyclic ring at different positions of the sugar ring (Fig. 4).\(^\text{(g)}\) The problems associated with PS-ONs, i.e. poor binding affinity to the target RNA, lack of specificity and low cellular uptake, are to some degree solved by these second generation ONs containing a modified sugar moiety. 2'-O-Methyl (2'-OMe), 2'-O-methoxymethyl (2'-OMOE) and locked nucleic acid (LNA) are the most important members of this class (Fig. 4).

The structural difference between DNA and RNA includes the 2'-substitution on the furanose ring of RNA. Hence, the RNA binding behavior of AONs may be improved by mimicking RNA structures with 2'-modified nucleosides (Fig. 5). Electronegative substituents like fluorine and oxygen influence the furanose sugar 3'-endo conformation\(^\text{14}\) due to the preferred gauche orientation of the 2'-substituent and the ring oxygen (Fig. 5). As a result, RNA and 2'-modified nucleosides are found predominantly in the C3'-endo conformation that is exclusively present in A-type duplexes.\(^\text{15}\) Various reported 2'-substitutions have shown excellent results in antisense therapeutics as they provide high metabolic stability and high affinity to target mRNA; for example, 2'-OMe-, 2'-OMOE- and LNA-containing ONs have entered in human clinical trials.\(^\text{15,16}\)

The FDA in 2004 approved pegaptanib sodium (Macugen), an anti-vascular endothelial growth factor (anti-VEGF) RNA aptamer for the treatment of all types of neovascular age-related macular degeneration.\(^\text{17}\) Macugen consists of 2'-F and 2'-OMe substituted sugar moieties (Fig. 6). Aptamers are single-stranded ONs (DNA/RNA) that form stable three-dimensional structures and are capable of binding with high affinity and specificity to a variety of molecular targets such as proteins and can modulate their functions. Because the targets are in the blood plasma or displayed on the surface of cells, aptamers are likely to be degraded easily by serum nucleases. Therefore, unmodified aptamers have shown half-lives in the blood as short as 2 minutes.\(^\text{18}\) Modifications such as the capping of ONs at the 3'-terminus, often followed by inverting the nucleotide at the 3'-terminus, have shown increased stability against endogenous serum nucleases (Fig. 6).\(^\text{19}\)

### Nucleobase modified AONs

Since the nucleobases provide the prime recognition site for Watson–Crick base pairing via specific hydrogen bonding interactions, the scope of modification of the nucleobase is confined, which can only improve the binding affinity for the complementary ON but not the nuclease resistance.\(^\text{20}\) Although less common than backbone and sugar modifications, chemically modified heterocyclic nucleobases have also found applications as AONs. Carefully designed nucleobase analogues when introduced into ONs can provide information on the importance of specific functional groups in natural bases. Note that even a subtle change can have a dramatic effect because of the change in size, electronic distribution, nucleoside sugar conformation, tautomeric structure or functional group properties. Representative structures of several modified bases, i.e. pyrimidine and purine modification, and universal bases are shown in Fig. 7. The most attractive sites for substitution of the nucleoside bases are those positions that are exposed to solvents in the major groove, i.e. the 4- and 5-positions of pyrimidines and the 6- and 7-positions of purines (Fig. 7). Substitutions at these positions neither interfere with base pairing nor induce steric hindrance and influence the general geometry of the double helix.\(^\text{5,21,22}\)

Natural nucleobases display exquisite selectivity in recognizing complementary bases as given by Watson–Crick rules. A universal base is an analogue that can be substituted for any of the four natural bases in ONs without significantly impairing the duplex stability. In general, universal base analogues use aromatic ring stacking, instead of specific hydrogen bonds, to stabilize a duplex (Fig. 7).\(^\text{22}\)
The stacking interactions between the planar heterocycles of nucleic acids are largely responsible for the stability of DNA and RNA duplexes. Maximizing stacking interactions through chemical modification provides a means of creating duplex helices of greater stability, e.g. tricyclic phenoxazine and G-clamp cytosine derivatives have been shown to enhance stacking. A tricyclic phenoxazine (Fig. 8) serves as a rigid scaffold for the attachment of groups designed to interact with the Hoogsteen binding face of a complementary base paired guanine. Appending an arm with strong hydrogen bond donor, i.e. an aminoethyloxy tether to the phenoxazine, recognizes both the Watson–Crick and the Hoogsteen sites of guanine; hence, it is termed as a G-clamp (Fig. 8).

The G-clamp-containing AON displayed dramatically enhanced stability. The greatly increased affinity and specificity of the base-modified G-clamp was also confirmed by in vivo studies. However, the acyclic derivative lacks the conformational restriction and hence does not demonstrate enhanced affinity. The G-clamp’s affinity for the complementary guanine is due to the appropriate positioning of the strong hydrogen bond donors (Fig. 8).

Other advanced modified AONs

Although AONs made of only sugar modified building blocks are less toxic than PS-AONs and have slightly enhanced affinity towards their complementary RNAs, their efficiency to induce RNase H cleavage of the target RNA is a matter of concern. Since RNase H cleavage is the most desirable mechanism for the antisense effect and 2′-O-alkyl modifications are desirable...
The last 35 years have witnessed an explosive growth in the number of modified ON-related clinical trials. We have collated the data for 76 oligonucleotide drug candidates that have been tested in the clinical trials for treatment of various diseases, and the majority of them have shown promising potential. Please note that we have considered only those ON drugs that have been tested in a minimum of phase I clinical trials or onwards. Most of these chemically modified ONs involve phosphorothioate (PS) chimera and are designed to specific inhibition of gene expression. PMO provide excellent nuclease stability in comparison to that of the unmodified AONs. PMO has demonstrated antisense efficacy in animal models in vivo and in human clinical trials.

Clinical trials of modified oligonucleotides

for nuclease resistance and high binding affinity, a hybrid ON construct incorporating both characteristics has appeared in the form of the ‘gapmer’ antisense oligonucleotide. A gapmer contains a central ‘gap’ of deoxynucleotides sufficient to induce RNase H cleavage flanked by blocks of 2’-O-modified ribonucleotide ‘wings’ that protect the internal block from nuclease degradation, e.g. 2’-OMOE sugar modified nucleosides can be further combined with a phosphorothioate (PS) linkage as in Kynamro, which is the second antisense drug approved by the FDA to reduce low density lipoprotein-cholesterol (LDL-C), apolipoprotein-B, total cholesterol and non-high density lipoprotein-cholesterol in patients with homozygous familial hypercholesterolemia (HoFH) (Fig. 9). Kynamro also represents the first systemic antisense drug and is given as a 200 mg weekly subcutaneous injection as an adjunct therapy to lipid-lowering medications and a controlled diet. Some serious side effects such as liver toxicity have been encountered with Kynamro; hence, it is available with a warning on the package citing the risk of hepatic toxicity. The common adverse reactions to Kynamro include injection site reactions, increased alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels, flu-like symptoms, and abnormal liver function test results. Numerous modified ONs are being tested in multiple clinical trials to explore whether this ‘gapmer’ type chimera has improved therapeutic properties (Table 1).

In order to further enhance target affinity, nuclease resistance, biostability and pharmacokinetics, an advanced third generation of AONs was developed mainly by modifications of the furanose ring of the nucleotide. Peptide nucleic acid (PNA) and phosphorodiamidate morpholino oligomer (PMO) are the most well studied third-generation AONs. Peptide nucleic acid (PNA) is a non-charged nucleotide analogue in which the phosphodiester backbone is replaced by a flexible pseudopeptide polymer N-(2-aminoethyl)glycine and the nucleobases are attached to the backbone via methylene-carbonyl-linkage (Fig. 1). PNA can hybridize with complementary DNA or RNA strands with higher affinity and specificity than natural oligonucleotides. PNA is not a substrate for RNaseH and exerts its antisense effect by forming a sequence-specific duplex with mRNA, causing steric hindrance of translational machinery, leading to protein knockdown.

In phosphorodiamidate morpholino oligomer (PMO), the ribose sugar is replaced by a six-membered morpholino ring, whereas the phosphodiester bond is replaced by a phosphorodiamidate linkage (Fig. 1). Like PNAs, this modification also does not activate RNase H; hence, it acts only as a steric blocker for specific inhibition of gene expression. PMO provide excellent nuclease stability in comparison to that of the unmodified AONs. PMO has demonstrated antisense efficacy in animal models in vivo and in human clinical trials.
corresponding RNA, whereas aptamers directly bind to the protein target (Fig. 10).

From the Table 1, it is quite clear that ISIS pharmaceuticals, which is a pioneer in antisense technology, has contributed about ~20% of all modified ON based drugs that are in clinical trials. Sarepta Therapeutics had six drug candidates in clinical trials involving PMO chemistry. A brief representation of the number of drugs in clinical trials for different assignees is provided in Fig. 11. Please note that assignees having one or two drugs are grouped together as ‘others’ assignees in the graph.

To see the pattern in the number of drug candidates that entered Phase 1 clinical trials over time (2 year intervals), we retrieved the corresponding data for these drug candidates from various sources and prepared a graph to showcase this pattern (Fig. 12). From the graph, it is clear that after the first two drug candidates entered clinical trials in 1997–1998 (both

![Structure of FDA approved antisense drug Kynamro having 2'-OMOE chimera.](image)
<table>
<thead>
<tr>
<th>S. no.</th>
<th>Product</th>
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<th>Disease</th>
<th>Target</th>
<th>Mode of action</th>
<th>Status (phase)</th>
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<td>Phosphodiester with 2′-O-methylated purines and 2′-F-modified pyrimidines</td>
<td>Neovascular age-related macular degeneration (AMD)</td>
<td>Vascular endothelial growth factor (VEGF)</td>
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<td>Oblimersen&lt;sup&gt;37&lt;/sup&gt; (Genasense, Augmerosen, G-3139)</td>
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<td>Profsa Therapeutics &amp; GlaxoSmithKline</td>
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<td>II</td>
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<td>II</td>
<td>Antisense Therapeutics &amp; ISIS Pharma</td>
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<td>AVI-5126 (ref. 71)</td>
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<td>II</td>
<td>Sarepta Therapeutics</td>
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<td>Monarsen52 (EN-101)</td>
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<td>Heptazyme57 (LY-466700)</td>
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<td>Sirna (formerly Ribozyme)</td>
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Table 1 (Cont.)

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<td>NeoPharm Methrogel</td>
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<td>EnGeneX Hobbiden</td>
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<td>AV-4665 (ref. 89)</td>
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Table 1 (Contd.)

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<td>EZN-2968 (ref. 99)</td>
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<td>Lymphoma and solid tumours</td>
<td>Hypoxia-inducible factor-1alpha (HIF-1α)</td>
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<td>Survivin</td>
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<td>ALN-VSP&lt;sup&gt;1,03&lt;/sup&gt;</td>
<td>Modified dsRNA in liposome formulation</td>
<td>Liver cancer and solid tumours</td>
<td>VEGF and kinesin family member 11</td>
<td>RNAi</td>
<td>I</td>
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<td>68</td>
<td>CALAA-01 (ref. 103)</td>
<td>dsRNA in nanoparticle formulation</td>
<td>Solid tumours</td>
<td>M2 subunit of ribonucleotide reductase (RRM2)</td>
<td>RNAi</td>
<td>I</td>
<td>Calando Pharma</td>
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<td>69</td>
<td>OMJP-GCGR&lt;sub&gt;Rx&lt;/sub&gt; (ref. 104)</td>
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<td>Diabetes</td>
<td>Sodium-dependent glucose transporter 2</td>
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<td>ISIS Pharma and Ortho-McNeil-Janssen Pharma</td>
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<td>OGX-225 (ref. 106)</td>
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<td>Prostate and breast cancer</td>
<td>Insulin growth factor binding protein, IGFBP 2 AND 5 C-myb</td>
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<td>OncoGeneX</td>
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<td>LR-3001 (ref. 107) (G-4460)</td>
<td>PS</td>
<td>CML</td>
<td>Signal transducer and activator of transcription3 (STAT-3)</td>
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<td>AIR-645 (ref. 109) (ISIS-369645)</td>
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<td>75</td>
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<td>Polo-like kinase (PLK1)</td>
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<td>Tekmira Pharma</td>
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<td>76</td>
<td>ARC-520 (ref. 111)</td>
<td>2′-OMe, 2′-F, PS and 3′-3′-phosphodiester</td>
<td>HBV</td>
<td>Conserved regions of HBV</td>
<td>RNAi</td>
<td>I</td>
<td>Arrowhead Research</td>
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</table>

<sup>a</sup> 2′-OMOE chimera, 2′-O-methoxyethyl-DNA chimeric oligonucleotides with phosphorothioate linkages; 2′-O-Me chimera, 2′-O-methyl-DNA chimeric oligonucleotide with phosphorothioate linkages; LNA chimera, locked nucleic acid-DNA chimera with phosphorothioate linkages.
drugs in 1998), there was an increase in the number of drug candidates entering clinical trials and a maximum of 34 drug candidates entered between 2005–2008. From then, there was a decrease in the number of drug candidates, and only two drugs entered clinical trials in 2011–2012. A maximum of 18 drug candidates entered clinical trials in 2007–2008, which was followed by 16 drugs in 2005–2006. Out of these, a maximum of 12 drug candidates entered Phase 1 clinical trials in 2005.

Conclusion

The recent approval of Kynamro by the FDA has added a much needed boost to research on antisense based therapeutics, which since 1998 was thought to be directionless and futile. Chemical manipulations of natural oligonucleotides are required as the direct use of these nucleotides as therapeutic agent suffers from some limitations such as low binding affinity to the complementary nucleic acid and poor nuclease stability. Hence, in the search for suitable antisense drug candidates, vast number of modifications have been carried out, e.g. backbone, nucleobase and sugar moiety modification of the natural DNA/RNA, leading to the development of three FDA-approved drugs. Furthermore, with persistent promising clinical trials involving these modified oligonucleotides, it can be anticipated that more new potent antisense drugs may appear in the near future.

Acknowledgements

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113 Data retrieved from http://www.clinicaltrials.gov/ and respective companies websites.