

Antisense Oligonucleotides: problems with use and solutions

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Les oligonucléotides antisens (OAS) montrent un grand potentiel comme outil moléculaire et comme nouvel agent thérapeutique. Cependant, plusieurs problèmes limitent leur utilisation, en particulier leur toxicité, leurs effets indésirables et leur faible pénétration dans les cellules. Chez l'homme, les études cliniques concernant l'utilisation des oligonucléotides phosphorotioates (PS-ODNs) ont montré diverses toxicités hématologiques qui sont principalement dues à des effets non spécifiques. La charge et la polarité des PS-ODNs freinent leur entrée dans les cellules. En outre, le mécanisme principal de leur mode d'action est limité à l'induction du clivage de l'ARNm par la ribonucléase H. Pour surmonter les problèmes de la disponibilité et de la livraison des OAS au niveau de leur site d'action et pour augmenter leur spécificité, des vecteurs sont utilisés.

La seconde génération des OAS offre d'autres mécanismes d'action pour inhiber la production de la protéine cible et constitue donc une alternative à la thérapie par les PS-ODNs. Les modifications qui ont été apportées permettent aux OAS d'inhiber la signalisation en privant la cellule du récepteur ou du médiateur cible. Cette propriété permet d'utiliser les OAS comme outil de recherche fondamentale ou comme agent thérapeutique.

Antisense oligonucleotides show great potential as a molecular biological tool and therapeutic agent but there are some difficulties while using them such as toxicity, non-specific side effects, and low intracellular uptake. In human clinical trials, phosphorothioate oligonucleotides (PS-ODNs), the first generation antisense oligonucleotides show various hematologic toxicities mainly due to non-specific effects. Due to their charge and polarity, uptake by targeted cell is not efficient. Additionally, the main mechanism of action of first generation antisense oligonucleotides is limited to the induction of ribonuclease digestion of mRNA. To overcome the problems experienced with antisense oligonucleotides, drug delivery vectors can help in the specific delivery of the oligonucleotides, their uptake from the circulation, and the prevention non-specific effects. Second generation antisense oligonucleotides offer other mechanisms of action to inhibit the production of proteins thus having a potential an alternative antisense therapy to PS-ODNs. With these modifications, Antisense oligonucleotides can therefore efficiently disrupt signaling by causing a reversible knockout of receptors or intracellular secondary mediators in assays to explore receptor function or as well as a therapeutic agent.

Antisense oligonucleotides

Inhibition by antisense oligonucleotides (oligonucleotides) was first suggested and implemented by Paterson et al.,¹ a little more than 20 years ago by inhibiting single-strand DNA translation in a cell free system. A year later, the first therapeutic possibilities for antisense oligonucleotides were explored as treatment for Rous sarcoma infections.² It was later on discovered that several naturally occurring antisense oligoribonucleotides existed as a control of bacterial plasmid and phage processes by inhibiting mRNA translation by inducing its degradation.^{3,4} From their beginnings, antisense oligonucleotides have progressed to become a useful tool in molecular biology and also as a therapeutic

agent. Vitravene (ISIS2922), which targets the CMV IE-2 gene, was the first antisense oligo based drug approved by the FDA. Other potential oligo therapies are in the phases I to III clinical trials for several diseases such as AIDS, cancer, Crohn's disease, CMV retinitis, hematologic disorders and restenosis.⁵ The drug targets of interest are protooncogenes, viral reproductive proteins, growth factor receptors, other extracellular signaling proteins, transcription factors and various other proteins of the signal transduction machinery. As a molecular biological tool, antisense oligonucleotides can also be used to perform selective knockouts of mRNA functions either *in vivo* or *in vitro* and from the phenotypic outcome a function may be ascribed to the target from the loss of function that occurs. The power of antisense oligonucleotides

resides in their specificity and broadness of use. Antisense oligonucleotides can specifically inhibit a gene transcript and can go so far as to inhibit an allele or even a splice variant, while avoiding other members of the same family. Acquisition of such specificity is one of the main goals pharmacology as this can avoid non-specific side-effects. The broadness of use of antisense oligonucleotides comes about from the fact that they are chains of RNA or DNA, and by altering the base sequence of the oligo one can specifically target any mRNA product from the genome. Oligonucleotides mediate their functions by Watson and Crick ionic base pairing where the oligo single stranded DNA or RNA binds to its complementary strand (the sense strand) of mRNA. In theory, the length of the oligo need be at least 13 base pairs long to uniquely bind to a specific transcript of the human genome, but the most often used length for efficient inhibition is 15-20 base pairs. The selection of the sequence of the oligo used is most often procured by empirical testing of several antisense sequences. The mechanisms of action, once bound, are diverse either by inducing or inhibiting the mRNA processing enzymes (Fig 1). These include blocking transcription by the oligo forming a Hoogsteen triple helix⁶, 5'-cap formation and polyadenylation by inhibition of the enzymes, inhibition of splicing, prevention of mRNA nuclear export, induction of RNases for mRNA degradation, initiation of translation by blocking initiation factor binding and elongation.⁷ All these have been are potential targets, but the most often used mechanism, the one used by all of the antisense drugs that are in clinical trials, is the induction of RNase H cleavage of the mRNA. Harnessing of these other mechanisms will provide interesting avenues for the future of antisense oligonucleotides.

Limitations of Antisense Strategy

Two major problems exist in antisense pharmacology, that of toxicity and that of drug delivery and uptake. Natural oligonucleotides, composed of the ribose or deoxyribose sugar-phosphate backbone and bases, are quickly degraded in the body by nucleases so they are not effective molecules. Nucleotide-5' monophosphates, resulting from oligo degradation, can also negatively affect cell growth and proliferation⁸. Phosphorothioate deoxynucleotides (PS-ODN) are the first generation oligonucleotides and have a sulfur atom replacing the non-bridging oxygen of the sugar phosphate backbone (Figure 2). This gives the molecule chirality but preserves the overall charge and can also activate RNaseH for the degradation of mRNA. PS-ODNs are

also the first analogs used in clinical trials and they have several toxicologic problems that are independent of the sequence and most probably due to their binding to proteins on the cell surface or in the serum. PS-ODNs can activate the complement cascade⁹ and cause hematologic changes such as reduced heart rate, blood pressure and cardiac output. There is also a transient inhibition of the clotting times shown by an increased activated partial thromboplastin time (aPTT).¹⁰ Oligonucleotides with a sequence containing unmethylated CpG motifs also have been shown to activate the immune system by inducing the antibacterial and antiviral response with interferons α and γ and by the enhancement of killing by natural killer cells.¹¹ Observed PS-ODN side effects seen in clinical studies performed on humans include thrombocytopenia, fatigue, fever, rashes, leukopenia, and complement activation.¹² Due to their polyanionic nature, PS-ODNs cannot cross the lipid bilayer because of their charge and polarity. Once in the circulation they can be uptaken by many cell types and not just the cell targeted leading to potential side-effects. Other oligonucleotides analogs with morpholino backbones or methoxyethyl modifications, were developed and are actually used in clinical trials. However, this second generation of oligonucleotides remains toxic^{13,14}.

Due to the encountered non-specific effects, it is important in molecular and clinical research to have controls able to dissect the antisense effects from the non-specific. There is no single ideal control and two or more different controls are usually used.¹⁵ Sense controls are complementary to the antisense oligo and cannot bind to the mRNA. Random controls also known as non-sense controls are those where the bases are scrambled. Reverse controls are those where the antisense sequence is reversed and offer the advantages of having the same base composition, hybridization characteristics and free energy parameters. Finally, mismatched controls are those where several bases of the antisense are changed causing less binding to the mRNA for each base changed.

Delivery vectors

Delivery vectors can take care of both toxicity and drug delivery problems by mediating the entry and their delivery to the target, not just of oligonucleotides but of other drugs. Cells inefficiently take up naked oligonucleotides by adsorptive endocytosis¹⁶ while the some of the more recent second-generation oligonucleotides are not taken up at all without permeabilization of the cell, electroporation or use delivery vectors. The vector

can also protect the drug from degradation and also from rapid clearance from the body. The vector must be of small size to allow intercalation between tissues and to allow intracellular transport, they must be non-toxic and stable in the blood stream, they must retain the drug when in the circulation and must release it at its target before elimination. If the delivery vector is too large, this potentiates clearance of the drug from the body. These are quite challenging tasks but many ideas have been developed such as liposomes, protein or peptide constructs and polymers.¹⁷

Liposomes are small microscopic spheres of one or more concentric, closed phospholipid bilayer enclosing an internal aqueous compartment, like the plasma membrane of cells or of certain organelles. Drugs that are polar, such as first generation and second-generation oligonucleotides can be entrapped in the internal space. Cationic lipids with unsaturated hydrocarbon chains, such as phosphatidyl-ethanolamine (PE) can form a positively charged liposome and are effective at supporting transfection of the oligonucleotides. When oligonucleotides are mixed with lipids, complexes form spontaneously due to electrostatic interactions and form a condensed and tight structure.¹⁸ The liposome cannot fuse directly with the cell membrane and must be endocytosed. Once internalized the liposome causes a disruption of the endosomal membrane, resulting in fusion and expulsion of the contents into the cytoplasm.¹⁹ Liposomes can also be conjugated with viral proteins to aid in their fusion to the endosome, and the resulting complex is known as a virosome. Virosome proteins most often used originate from the influenza virus, the Hemagglutinating virus of Japan (HJV), and the adenovirus. Another type of liposome structure is a fusogenic liposome composed of PE and a titratable amphiphile such as cholesteryl hemisuccinate (CHEMS).²⁰ At a physiologic pH, CHEMS retain a bilayer structure maintaining the internal aqueous compartment, but at a pH of 6, such as in the maturing endosome, a non-bilayer is formed which enhances membrane fusion. Targeting of liposomes can be performed by covalently attaching a targeting ligand that will bind to a molecular receptor or other molecule on the target cell. If an antibody is used, the resulting complex is called an immunoliposome, which will bind to an antigen of interest on the target cell. Cellular receptors can also be used for targeting by using folate or transferrin conjugated to the liposome. It can be readily seen that combinations of a virosome with a targeting ligand will make a very efficient vector, although this would form a bigger complex reducing its overall distribution as well as an increase in clearance. An

advantage to liposomes is that they tend to accumulate at sites of infection, inflammation and tumors making these valuable potential targets for liposome therapeutics^{21,22}. Oligo-liposome complexes were also found in the circulation *in vivo* for up to 24 hours following injection²³ showing that liposomes protect the oligonucleotides from degradation and clearance and promise a long half-life in the body. Disadvantages to liposomes include toxicity as well as decreased activity in the serum, but this may be remedied by newer lipid formulations.

Oligonucleotides can also be linked to proteins and peptides that have the ability to penetrate the cell membrane without the liposome complex. This idea is based on the fact that several mechanisms for entering the cell exist in nature that can be used to the advantage of oligonucleotide research. The oligonucleotides attach by electrostatic interactions to poly-L-Lysines, or other cationic sequence, linked to a carrier molecule that is a ligand for a surface receptor similar to the targeting ligands in liposomes. The peptide complex can protect from nucleases, and particular amino acid sequences can be used such as the fusion sequence of the Human Immunodeficiency Virus (HIV) gp41²⁴ which increases cellular internalization. A nuclear localization sequence (NLS) can be added to the protein sequence to help bring the oligo into the nucleus. Another family of proteins of interest is the Penetratins, such as pAntennapedia, which are cationic proteins that can bind DNA and can mediate penetration through the membrane through non-endocytotic means.²⁵ This would be beneficial so as to avoid the risk of the oligo from being degraded by nucleases from the fusion of lysosome to the endosome during endosomal maturation. A disadvantage of using protein conjugates is the risk of developing an immune reaction to the conjugate and other non-specific effects.

Oligonucleotides can also be covalently linked to hydrophobic molecules such as cholesterol thus increasing their lipophilicity and entry into the cell. Epa et al. linked a cholesterol moiety at the 3' and/or 5' end of a PS-ODN targeting the p75 neurotrophin receptor.²⁶ LDL receptor mediated internalization was the suggested mechanism for uptake of the oligo. The 5', 3'-bischolesteryl oligo downregulated both mRNA and protein more than either the 3' or the 5' monocholesteryl oligonucleotides. Advantages to these oligonucleotides are an increased stability to 3' and 5' exonuclease, an increased solubility in the membranes and an increased stability in the serum most probably by being bound to serum lipoproteins.

A disadvantage to these oligonucleotides is that they are not commercially available.

Another potential delivery vector is composed of polymerized nanoparticles that form a colloid suspension where the oligo or drug is entrapped, encapsulated or adsorbed to the surface. One example is the commercially available NanoGel™, composed of poly(ethylene glycol) and polyethyleneimine monomers, and can be used for oral delivery of antisense drugs.²⁷ Targeting ligands, such as transferrin, can also be conjugated to the polymers for the specific delivery of the drug.

Dendrimers are highly branched polymeric structures that have a core molecule, such as a hydroxyl, with the monomer unit attached to it. From the core monomer unit, other monomer units can be attached branching out to form a complex structure. The ends of the dendrimer can be charged as in the case of the polyamidoamine (PAMAM) dendrimer.²⁸ This dendrimer is positively charged at physiologic pH because of amino groups on its surface, able to complex with oligonucleotides, has a limited cellular toxicity and is stable in the serum. Thus, by altering the monomers and monomer combinations to form polymeric gels or dendrimers, one can obtain the required pharmacokinetics and low toxicity for the safe and efficient delivery of the oligonucleotide or other drug of interest.

There is no generic drug delivery vector, and each vector must be tailored for its specific drug it will contain, its site of action and its release of the drug at its site of action. There are many available drug delivery systems either based on natural or synthetic systems and many show great potential in delivering a molecule or drug to the target of interest either in molecular biology research or in therapeutics.

Second Generation Antisense Oligonucleotides

Second-generation oligonucleotides, such as 2'-alkoxy modified, N3'-P5' Phosphoramidate, morpholino modified or peptide nucleic acid modified oligonucleotides, are also resistant to nucleases but do not induce RNaseH activity. These can take the advantage that many steps in mRNA processing can be inhibited by antisense oligonucleotides. They were developed to try and avoid the toxicity associated with PS-ODNs as well as exploiting other mechanisms to inhibit protein production.

The 2' alkoxy modified oligonucleotides show high binding affinity to target mRNA, nuclease resistance and higher lipophilicity compared to PS-ODNs. Various lengths of carbon chains can be linked to the 2' position of the ribose ring. The shorter

the chain the better the binding affinity but the longer the chain the greater the nuclease resistance.^{29,30} Mechanisms of action for the 2' modified oligonucleotides do not rely on RNase H activation but on translation arrest by blocking 80S ribosome complex formation³¹ as well as with splicing interference.³²

N3'-P5' Phosphoramidate oligonucleotides, containing a 3'-amino instead of a 3'-hydroxyl nucleoside, form stable bonds with complementary mRNA. They are resistant to nucleases, are negatively charged at a neutral pH and are also achiral. They show great potential as they have an observed activity superior to PS-ODNs.³³ Like PS-ODNs they induce a decrease of target mRNA with a subsequent synthesis in target protein but they function in an RNase H dependent fashion.³⁴

Morpholino oligomers are a backbone-modified oligonucleotide that function in an RNase H independent fashion and show promise in having little non-antisense effects. Their mechanism of action is by blocking translation initiation when targeted to the 5' UTR of the mRNA.³⁵ Morpholino oligonucleotides have also shown to be useful in inducing the correction of malfunctioning pre-mRNA by altering its splicing.³⁶

Peptide nucleic acid (PNAs) are also oligonucleotides are modified with polyamide chains. They are only effective at interfering with translation initiation and not elongation and therefore the functional PNA oligonucleotides span the AUG initiation codon of the mRNA.³⁷ They are highly stable and bind to their targets with high affinity. Unfortunately they show poor uptake but can benefit by the association or conjugation to potent delivery vectors such as pAntennapedia.³⁸

Conclusion

Antisense oligonucleotides show a great potential as a molecular biology investigative tool as well as highly selective therapeutic agents. Several problems have been encountered with oligonucleotides as they can produce non-specific effects such as hematologic disturbances or activation of immune system components, they have limited uptake due to their polarity and specific delivery to target tissues is difficult to obtain. Second generation antisense oligonucleotides show promise as an alternative to phosphorothioate oligonucleotides as they can function at other levels than RNase H. Drug delivery systems may be the key in making antisense oligonucleotides better therapeutic agents, as they can produce enhanced uptake, they protect from degradation or prevent non-specific effects and they

can allow specific delivery by conjugating targeting ligands to the vector. With the newly developed solutions, these agents show great promise in the future.

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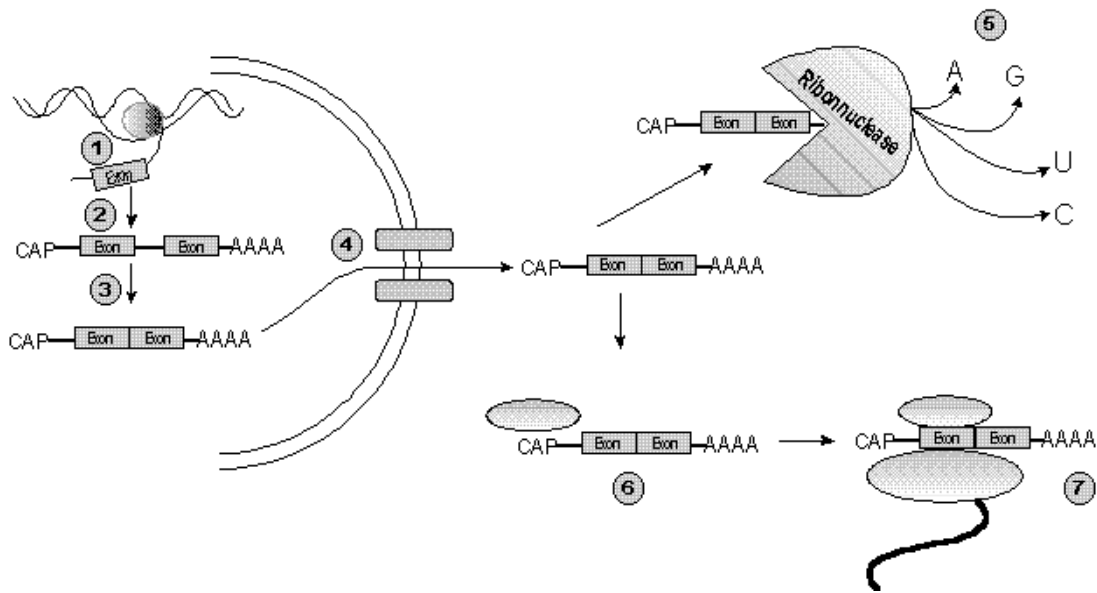


Figure 1: Sites of action of antisense oligonucleotides. Antisense oligonucleotides can inhibit many steps of mRNA processing: transcription (1), 5'-cap formation and polyadenylation (2), splicing (3), mRNA nuclear export (4), induction of RNases for mRNA degradation (5), initiation of translation (6) and elongation (7).

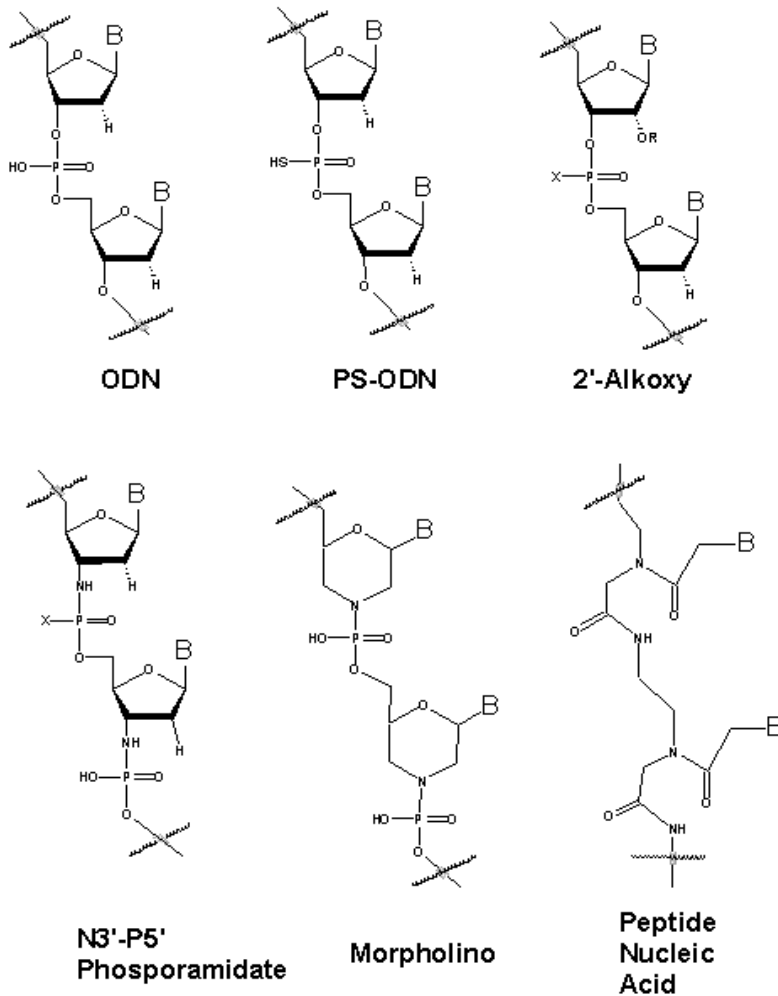


Figure 2: Oligodeoxynucleotides (ODN) and analogs. PS-ODN is a first generation oligo while the other analogs are of the second generation.