NOVEL DRUG DELIVERY SYSTEMS: MAKING SENSE OF ANTISENSE OLIGONUCLEOTIDES

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Breakthroughs in molecular biology and human genome project have opened previously unforeseen possibilities for targeted intervention with gene expression.[1,2] These include approaches such as inhibition of specific genes using antisense oligonucleotide (AON). The allure of antisense technology has been the promise of potent and specific inhibition of any gene of interest. This potential has stimulated a significant amount of research in development of these *"informational drugs"* but problems limit the therapy reaching its potential. One of the greatest obstacles of antisense therapy is the delivery of the oligonucleotide to its target.[3-7] This essay will help the novice to understand a few key principles regarding AON technology and delivery systems used to increase the stability and cellular uptake of AON.

What are AONs?

Antisense technology is a novel drug discovery method. AONs are short (typically 15-20 nucleotides in length), single stranded synthetic stretches of ribo- or deoxyribonucleic acids that recognize and bind specifically to the complementary sequence of a gene or its mRNA and halt a biological event such as transcription, translation or splicing.[9-11] The technology is known as antisense because the oligonucleotides are complementary to the mRNA and has the same sequence as the sense strand of DNA.

How AONs work?

Proteins play a central role in virtually every aspect of human metabolism. Almost all human disease are the result of inappropriate protein production (or disordered protein performance). This is true of both host diseases (such as cancer) and infectious diseases (such as AIDS)[12]. The information required for the human body to produce all proteins is contained in the human genome. Genes are made up of DNA that contains information about when and how much of which protein to produce, depending upon what function is to be performed. The DNA molecule is a "double helix" — a duplex of entwined strands. In each duplex, the bases or nucleotides (Adenine, Thymidine, Guanine, Cytosine) are weakly bound or "paired" by hydrogen bonds to complementary nucleotides on the other strand (A to T, G to C). Such highly specific complementary base pairing is the essence of information transfer from DNA to its intermediary, messenger RNA (mRNA). During transcription of information from DNA into mRNA, the two complementary strands of the DNA partly uncoil. The "sense" strand separates from the "antisense" strand. The "antisense" strand of DNA is used as a template for transcribing enzymes, which assemble mRNA — a process called "transcription." Then, mRNA migrates into the cell where other cellular structures called ribosomes read the encoded information, its mRNA's base sequence, and in so doing, string together amino acids to form a specific protein. This process is called "translation."[12]

Antisense drugs are complementary strands of small segments of mRNA/DNA. Each antisense drug is designed to bind to a specific sequence of nucleotides in its mRNA/DNA or ribosomes to inhibit protein production (Fig. 1). This can happen in one of the following ways –

1. AON may form a triplex with double stranded DNA by Hoogsteen base pairing, thus specifically impairing RNA synthesis (transcription) from that region.[13]
2. AONs may be designed to interfere directly with protein synthesis by binding to complimentary mRNA via Watson-Crick base pairing rules which may result in -

- ➢ Physical blockage of the site preventing the access or binding of various factors like ribosomes, spliceosomes or activation factors or
- ➢ Sensitization of the target mRNA to RNase H leading to degradation of the target RNA or
- ➢ Modification of secondary structure of target RNA which improves the accessibility to ribonucleases that degrade mRNA.[4]

3. AONs may also bind the ribosomal binding site and adjacent regions which may inhibit ribosome binding by mRNA.

Any of the above mentioned process would prevent protein synthesis and turn off a gene's activity.[14] By acting at this early stage in the disease causing process to prevent the production of a disease causing protein, antisense drugs have the potential to provide greater therapeutic benefits than traditional drugs which do not act until the disease causing protein has already been produced. ***It may be said that AONs not only treat but also cure the disease in contrast to conventional drugs that generally offer pallative/symptomatic remedies.***

Choice of AON sequence[15]

There is no sure way to determine *apriori* where on a particular gene is the most active site for an AON. The region surrounding the short codon (AUG) site is the most popular followed by mismatch sites. Recently targeting splicing sites have become increasingly popular in order to inhibit the mRNA processing mechanism as opposed to the messages. The most important point to consider in that the specificity of AON gene inhibition is compromised when homology to other sequences allows the selected AON to bind to non-targeted mRNAs. To reduce this non-specific activity, an AON should target a sequence that is predicted to be unlikely to occur in other mRNAs.

Applications

Of many potential applications of AON two are most prominent: target validation and use as an actual therapeutic agent.

(1) Target validation : It refers to the use of AON in cell culture to determine if down regulating a certain gene target will give desired biological results (i.e. tumor cell line reduction etc.). This information is often used to help develop more classic small molecule drugs.

(2) As an therapeutic agent : AONs have attracted special interest as a new class of chemotherapeutic agents for a number of diseases due to their ability to inhibit gene expression in a sequence specific manner. A number of applications have been suggested for this group of compounds-

- ***As anticancer agents***: Alteration in the normal gene expression profile of a cell are thought to be an early event in oncogene transformation. A large number of oncogenes are transcriptional factors, hence their inhibition by AON would directly lead to prevention of abnormal cell growth.[16]
- ***Anti HIV agents***: AONs could block the production of proteins which are essential for survival of viruses.[17]
- ***CMV retinitis in AIDS patient***: Through blockage of the Cytomegalovirus reproduction. **Vitravene™ (Fomiviresin Na – injectable)** is the first in a class of antisense drugs to be approved by USFDA for marketing. The nucleotide sequence of this drug is **GCGTTTGCTCTTCTTCTTGCG**.[12]
- ***Genital warts:*** Through blockage of human papillomatus reproduction.
- ***Kidney transplant rejection:*** Through blockage of immune cell activities.
- ***Chronic myelogenous leukemia***: Through blockage of cancer cell activities.
- ***Rheumatoid arthritis and other autoimmune diseases:*** Blocks immune cell activities.
- ***For atherosclerosis & Restenosis***: Supression of smooth muscle cell growth.[18]
- ***Crohn's disease and inflammation***: inhibits synthesis of adhesion protein causing inflammatory responses.[14]
- ***Pulmonary fibrosis***: inhibition of TNFa.[19]
- ***Influenza***: inhibits viral PB2 or PA genes for RNA polymerase.[20]
- ***Hepatitis C***: blocks the production of viral proteins.[12]

Initial Euphoria and the Reality

Science named the gene blocking technique-the antisense technology as a **runner up for its "1992 – molecule of the year"**[21]. At that time, the technology appeared to offer a promising way to turn specific genes on or off at will. And that had made it attractive as a potentially powerful tool for uses ranging from fundamental molecular biology to the development of pharmaceuticals. The initial euphoria gave way to a sense of dismissiveness, when researchers encountered difficulties in getting oligos to recognize and bind to specific genes into target tissues. The control oligos that did not even complement the target mRNA seemed to work almost as well as the AONs. Moreover lethal and unexpected side effects were also observed in animal studies. The following obstacles were identified-

1. ***Stability***: The stability of unmodified

oligonucleotides (ONs) ONs is unacceptably low with intracellular half lives of only few minutes[13]. The enzymes that degrade nucleic acid such as phosphodiesterases, DNses, RNses and nucleotidases are found in all tissues.

2. ***Specificity***: Theoretically problems associated with AON specificity should be minimal. Assuming that human genome contains 10^9 coding base pairs and considering that the distribution of the bases is random, a sequence composed of 17 nucleotides should occur only once in these sequences. However AONs were found to bind to other targets and proteins. To maximize specificity AONs should be targeted to unique sequences rather than general binding sites.

3. ***Cellular uptake***: When added directly to the media only 1-2 % of the added AONs become cell associated. This is because these molecules poorly diffuse across the cell membrane owing to their ionic character.[10] Moreover, the oligos inside the cell are trapped in endosomal/ lysosomal compartments and are thus unable to exert their effects in the nucleus or cytoplasm.

The problem of in vivo stability of these molecules has been mainly approached by chemical modification of the AONs through substitution of the natural phosphodiester linkage. The most widely tested class of oligo analogues are the backbone modified derivatives which include methylphosphonate, phosphorothionate, phosphodithionate and peptide nuclei acid analogues.[7, 13,22] While these forms of AONs have been reported to be relatively stable, their in vivo therapeutic applicability is unsatisfactory due to possible side effects and adverse pharmacokinetic properties.[6, 23]

To obtain an efficient antisense effect it is necessary to direct and facilitate the penetration of negatively charged oligos into target cells and for this development of efficient delivery system is necessary for their clinical utilization.[6,18,24,25]

Requirements of a Delivery System[4]

Apart from the requirements that a drug delivery system should possess namely delivery of drug in its therapeutically effective form to desired site of action and other ex vivo requirements like robustness and stability, a delivery system for ON's should additionally possess features that afford-

1. Protection of ONs from nuclease degradation
2. Enhancement of cellular uptake of the oligos
3. Improvement of ON potency
4. Modification of intracellular distribution of ON
5. Increased retention of the oligo nucleotides in cells
6. Potential for slow release depots.

Delivery Systems for AONs

Despite the rapid progress of AONs into clinical trials, investigations are still in progress on development of delivery strategies and routes of administration that will make the technology clinically successful.

1. Liposomes

Liposomes (lipid vesicles) are sealed sacs in the micron or submicron range dispersed in an aqueous environment. The wall of the sacs consists of bilayers composed of suitable amphiphiles. The nature of the bilayer ensures the formation of internal aqueous compartment, which can differ from the outside medium. The presence of two different environment in the carrier, the aqueous "milieu interne" and the membrane makes liposomes a versatile carrier for a broad spectrum of hydrophobic, amphipatic and hydrophilic agents. They are the most intensively studied delivery systems for AON's owing to their ability to protect the entrapped agent from external media and delivery the same directly into the cells; thereby overcoming stability and permeability problems associated with delivery of AONs. Moreover, it is also possible to specifically target liposomes by certain coupling proteins or antibodies on their surface. A number of different types of liposomes have been reported in literature as carriers for AONs.

1. Anionic liposomes[26,27]
2. pH sensitive liposomes[28]
3. Immunoliposomes[29]
4. Fusogenic liposomes[30]
5. Cationic liposomes[31]

Among all these the cationic liposomes (cationic lipids) have provided good clinical results. The transport of AONs by cationic lipids is based on the electrostatic interaction between negative charges of AON and positive charges of lipids. In contrast to all other liposomes cationic lipids do not require any encapsulation step. In this case the oligos are directly mixed with preformed liposomes and since oligos are complexed to liposomes and thus are encapsulated, it is necessary to use backbone modified oligos which

are relatively resistant to nucleases.[31-33]

Fusion of the cationic lipids with negatively charged cell membranes leads to release of the associated molecules into the cytoplasm. AONs might also be release by endocytosis.[34] Cationic liposomes have been found to increase the quantity of delivered AONs both in the cytoplasm and in the nucleus. The most commonly used cationic lipid formulation is Lipofectinâ which is found to increase the potency of an antisense drug atleast 1000 fold.[35]

However use of cationic liposomes is limited by their ampiphilic nature, which may result in cytotoxicity and the need to stabilize the AONs against nucleases. A number of modifications have been reported to overcome these limitations. Cationic liposomes appear very promising as carriers to deliver AONs. With their numerous advantages complemented with in vivo studies, a number of AON may find cationic liposomes as their carriers.

2. Transdermal delivery systems

There are many problems that are often associated with treatment of cells by conventional methods such as intravenous injection. For example cells of melanomas tumors are typically difficult to target by injection technique because they are in the form of relatively thin tissue. Moreover injections can traumatize tissue thereby possibly spreading potentially malignant growth.[16] Topical delivery by electroporation could be an interesting alternative to deliver such highly charged molecules directly to the pathological tissue.

The application of high voltage pulses to skin increases the transdermal transport. The mechanism underlying this phenomenon is hypothetized to be electroporation i.e. creation of aqueous pathways in the lipid bilayers of the stratum corneum. Using a 15-mer phosphorothionate it has been shown that

- AON delivery can be controlled by the electrical parameters of the pulses and AON concentration
- AON remains stable for at least 4 h in the skin
- Local concentration higher that 1 mM can be achieved &
- FITC labeled AON reaches nucleus within 5 min after electroporation.[36]

This method is advantageous as it allows topical treatment of skin lesions having a genetic component such as melanoma tumors. The amount of nucleotide necessary to treat a particular lesion is significantly reduced by localized application of the nucleotide thereby substantially diminishing the cost of the treatment. Optionally electroporation of the skin can be conducted in conjunction with an additional electrical protocol such as iontophoresis. However more in vivo studies needs to be carried out for the clinical utility of the method. Also there are no reports on utilization of transdermal electroporation for systemic delivery of AONs.

3. Transmucosal delivery systems

Most applications of AON for therapeutic purposes are intended for the treatment of chronic diseases such as cancer, AIDS, genetic disorders and cardiovascular diseases where repeated injections are not very convenient for the patients. Therefore, development of alternative routes of administration and/or controlled release drug delivery systems are very valuable for improving patient convenience and compliance. Delivery using a controlled release delivery system via transbuccal route has great potential because buccal epithelium has better permeability than skin and is well suited for retentive systems. Li[37] et al investigated buccal permeation of a model AON ISIS 3082. The permeability of AON through buccal mucosa indicated the potential of transbuccal route for delivery of AON.

4. Controlled Release Biodegradable Microspheres

In order to achieve in vivo efficacy of AONs for target protein with a slow turnover repeated administration of AON is required to provide a sustained pharmacological effect. The release of AON from a sustained release delivery system would provide the controlled delivery of AON at a predetermined rate to the target tissue over an extended period of time whilst protecting the ON from degradation by nucleases present in biological milieu.

Polylactide and polylactide-co-glycolide (PLGA) polymers have been extensively studied for the formation of biodegradable Microspheres to control the release of various drugs. A 20 mer phosphorothionate against the human papilloma virus was the model oligo for the study. The solvent evaporation technique employed was suitable for production of microspheres with encapsulation efficiency of 60-70% and size 20-30 mm. The in vitro release study demonstrated sustained release of ON from PLGA microspheres over a 28 day period. Biodegradable PLGA microspheres offer the potential for the delivery of AONs in vitro and in vivo.[6,11] However, manipulation of polymer properties including chemistry, molecular weight, microsphere

size and ON loading is essential to tailor the rate of release for desired therapeutic application.

5. Novel polymer based delivery systems

A number of novel polymer based delivery systems have been employed for the delivery of AONs.

- ***Protein based polymers***, which are high molecular weight polymer formed by repeating peptide sequences. These polymers prepared using molecular biology methods bind to AONs and release them over an extended period of time.[38]

- ***Poly (amidoamine)s*** (PAA) are synthetic, linear, biodegradable polymers that undergo a change in tertiary conformation in response to a drop in pH from 7.4 to 5.5 the pH of extracellular fluids and the secondary lysosomes respectively. They may be used as systemically administered vehicles for AONs.[39]

- Cationic and hydrophilic ***poly aminoacid*** carriers have been used for AON delivery.[23]

- ***Zyn linkers***[40] are small lipid like amphiphiles, which rapidly bind rapidly to cells and tissues depositing large number of molecules per cell after brief exposure without altering cell viability, immunogenicity or function. Because they bind to the lipid regions of membranes, zyn-linkers facilitate delivery to intracellular targets through normal membrane trafficking processes but do not require the presence of specific cell surface receptors. AONs can be coupled to Zyn linkers and released at controlled rates depending on the type of linker used.

Conclusion

The benefit of oligo carriers in in-vivo application in the present scenario is still unclear and warrants further studies. The real in-vivo potential of liposomes as carriers need further investigations that take into account the choice of oligo target and type of liposome. Finally the ideal combinations concerning both modified oligos and carrier systems are yet to be devised. The success of some liposomal formulations and other delivery systems suggests that future efforts in novel drug delivery systems of AONs should be rewarding. The merging need of synthesizing these oligos and their delivery has led to merger of companies like ISIS pharmaceutical (leader in AON technology) with Elan Pharmaceuticals (a leading drug delivery systems technology firm).[41] Such technological collaborations would make the dream of *"switching of gene's activity on and off at will"* a reality. In this context, the 'magic bullet' designed by Paul Ehlrich to specifically transport drugs in the body might be close at hand in liposome encapsulated oligos.

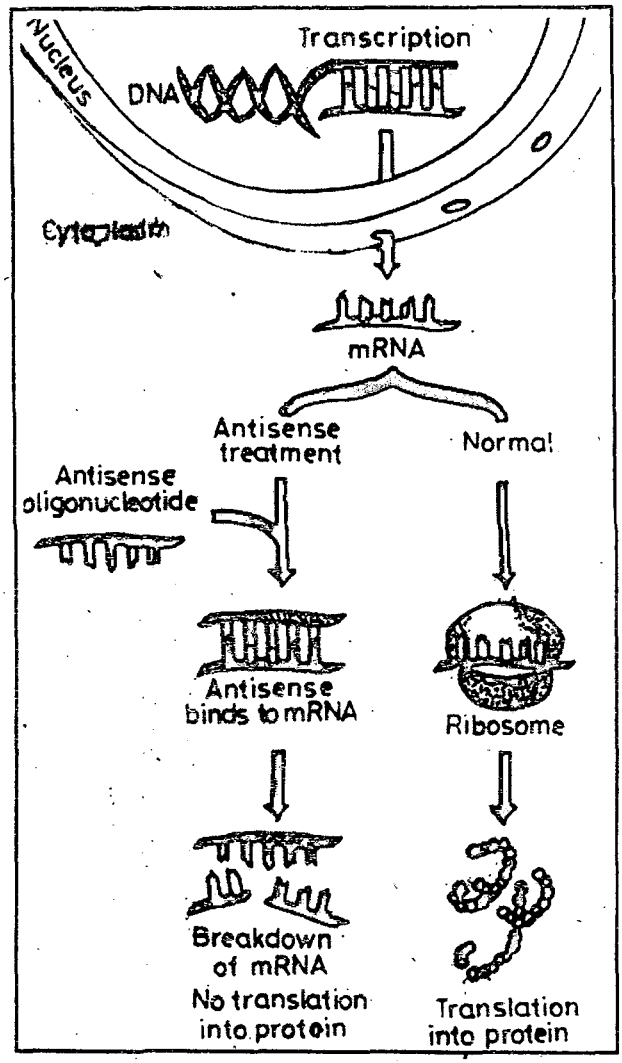

Fig.1 : Mechanism of action of antisense drugs

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