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Review

Nucleic acid drugs: a novel approach

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Nucleic acid base sequence of proteins plays a crucial role in the expression of gene. The gene is responsible for the synthesis of proteins and these proteins, which are synthesized, are responsible for the biological process and also for dreadful diseases as well. Once if the nucleic acid sequence is altered, we would be able to block or transfer the message for protein synthesis, there by preventing the particular protein, which is responsible for the disease. These nucleic acids act as drugs by different mechanisms, they may bind with the synthesized proteins, and they can hybridize to a messenger RNA leading to translation arrest or may induce degradation to target RNA. In this way the nucleic acids act as drug for inhibiting gene expression or protein synthesis. This article examines about the different types of nucleic acid drugs, their mechanisms, advantages, drawbacks and their future prospects.

Key words: Aptamers, ribozymes, antisense oligonucleotides.

INTRODUCTION

The discovery of that genetic information is coded along the length of a polymeric molecule composed of only 4 types of monomeric units is one of the major scientific achievements of this century. This polymeric molecule DNA is chemical basis of heredity and is organized into genes, the fundamental units of genetic information. Genes control the synthesis of various types of RNA most of which are involved in protein synthesis (Daryl, 1993)

The main function of a gene is to encode a message that can be transferred and expressed in order that protein may be synthesized. Three over all processes are involved in protein synthesis. First the genome (Cell's DNA) must be replicated Secondly a specific base sequence (a gene) must be transcribed from DNA to form a messenger RNA (mRNA) molecule having a complementary base sequence. Finally translation of the message by transfer RNA (tRNA) at the ribosome is executed to assemble a protein. Modulation of specific gene expression and noting concomitant changes in cell physiology and function can provide critical insight into the biological functions of particular genes. Specific modulation of gene expression may also be beneficial in inflammatory, neoplastic or viral diseases since it would be desirable to effect only the inflammatory genes, oncogenes or viral genes without harming the normal functions of the cell.

The majority of currently employed pharmacological approaches for modulation of gene function rely upon the interaction of low molecular weight chemical compounds with proteins targets so as to alter the function of the protein. Those chemical compounds are nucleic acids (Vyas and Dixit, 2002).

Nucleic acids may be defined as the macromolecules in which the nucleotides remain linked to each other by phosphodiester bonds between the 3' and 5' positions of the sugars.

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TYPES OF NUCLEIC ACID DRUGS

Nucleic acids are responsible for the direction of metabolism throughout the life of a cell. They direct the synthesis of proteins, control the synthesis of enzymes, and are responsible for the transfer of genetic information from one offspring to another. For the clinician, they are major interest as they are undoubtedly involved in the causation of cancers and other diseases. These nucleic acid drugs can be subdivided in to five classes based upon their target site and mechanism of action. These are Aptamers, Antisense oligonucleotides, Ribozyme nucleic acids, RNA interference and Antigene nucleic acids.

APTAMERS

Aptamers from the latin aptus (meaning to fit) are single stranded or double stranded nucleic acids. These are selected and amplified oligonucleotides that have been isolated from random pools of synthetic oligonucleotides according to their ability to bind with high affinity to biological target molecules (Ellington and Szostak, 1990). Aptamers as therapeutics would most likely bind proteins involved in the regulation and expression of genes dependent upon activity of protein.

The production of aptamers involves synthesis of random pools of oligonucleotides containing approximately 10 different molecular species, each having a different nucleotide sequence (Riordan et al., 1991). These pools are then incubated with target molecules and substances that bind with the highest affinity are isolated by physical or filter binding. The next step involves amplification of the isolated pool by enzymatic procedures, and then binding selection and amplification cycles are repeated until the pool is enriched with only those oligonucleotides that have the greatest affinity. This technique allows for the selection of oligonucleotides that by chance, have the correct three-dimensional structure to bind to the target molecule. In subsequent steps, the high affinity oligonucleotides are evaluated for their ability to inhibit the activity (for example, enzymatic activity) of the target to which they bind.

Aptamers as therapeutic and diagnostic agents

Using this method, many powerful antagonists of such proteins have been found. In order for these antagonists to work in animal models of disease and in humans, it is necessary to modify the aptamers. First of all, sugar modifications of nucleoside triphosphates are necessary to render the resulting aptamers resistant to nucleases found in serum. Changing the 2'OH groups of ribose to 2'F or 2'NH2 groups yields aptamers, which are long lived in blood. The relatively low molecular weight of aptamers (8000-12000) leads to rapid clearance from the blood.

Aptamers can be kept in the circulation from hours to days by conjugating them to higher molecular weight vehicles. When modified, conjugated aptamers are injected into animals, they inhibit physiological functions known to be associated with their target proteins. A new approach to diagnostics is also described. The use of photo-cross-linkable aptamers will allow the covalent attachment of aptamers to their cognate proteins, with very low backgrounds from other proteins in body fluids. Finally, protein staining with any reagent, which distinguishes functional groups of amino acids from those of nucleic acids (and the solid support), will give a direct readout of proteins on the solid support.

Only a few instances of oligonucleotide aptamers displaying biological effects have been reported. Double stranded aptamer which acts on B cells, phosphorothiote which acts on T cells, phosphodiester aptamers which acts on thrombin for anticoagulation are few examples of aptamers (Riordan et al., 1991; Ess et al., 1994).

Advantages when compared to antibodies

Aptamers can be isolated by a simple *in vitro* process for virtually any target, even if it is toxic or has low immunogenicity. They are specific drug-like antagonists of protein function. Being specific interaction partners, they can be integrated into almost any target validation and drug screening program. Aptamers tend to bind grooves and clefts on proteins and can recognize binding pockets in ways similar to that of small molecules. They can be chemically synthesized offering a wide variety of targeted modifications such as fluorescent reporters or affinity tags. They are also stable under various buffer conditions and resistant to harsh treatments such as physical or chemical denaturation with no loss off activity.

ANTISENSE OLIGONUCLEOTIDES

Zamecnik and Stephenson (1978) were the first to propose the use of synthetic oligonucleotides for the therapeutic purposes. Antisense therapy is designed to prevent or at least lower expression of a specific gene. An oligonucleotide that has a sequence that is complementary to the mRNA of the target gene is introduced into the cell. It will bind to the mRNA of the target gene and block translation of the message into protein. It may bind to DNA in the nucleus, blocking transcription or to the transcript during its processing and transport from the nucleus to the cytoplasm: all these interactions would reduce expression of gene.

To inhibit translation, the antisense oligonucleotides must reach the interior of the cell unaltered. That ability depends upon stability of the oligonucleotide toward extra and intracellular enzymes, ability to penetrate through the cell

membrane and once if it has reached the cytoplasm it must bind specifically and with sufficient affinity to the target mRNA to inhibit its translation into the corresponding protein. In order to meet all these requirements it is necessary for normal oligonucleotides to be chemically modified in a suitable manner. A minimum length for antisense oligonucleotides in order to get specific binding is 11 bases, but most being tested are in the 15-25 base range. The most commonly used antisense oligonucleotides (ASONs) are phosphorothioates and methyl phophonates. Methyl phosphonates are uncharged and therefore more lipophilic than native DNA or RNA and may penetrate the cells better. However, solubility may be a problem with methyl phosphonates and the doses of methyl phosphonates are high because they become bound to membranes and less of a dose is available to bind to mRNA. Therefore, phosphorothioates gained favor with several companies (Elbashir et al, 2001; Stein, 2001).

One of the major challenges for antisense approaches is the stabilization of oligonucleotides, as unmodified oligodeoxynucleotides are rapidly degraded in body fluids by nucleases. A vast number of chemically modified nucleotides have been used in antisense experiments. According to their generations they have been categorized in to three types.

First generation antisense-oligonucleotides

Phosphorothioate (PS) oligodeoxynucleotides are the major representatives of first generation DNA analogs that are the best-known and most widely used ASONs. In this class of oligonucleotides, one of the nonbridging oxygen atoms in the phophodiester bond is replaced by sulfur. PS DNA oligonucleotides were first synthesized in the 1960s by Eckstein and colleagues (2000) and were first used as ASONs for the inhibition of HIV replication by Matsukura and coworkers (1987).

The introduction of phosphorothioate linkages into oligonucleotides was primarily intended to enhance their nuclease resistance. PS DNAs have a half-life in human serum of approximately 9–10 h compared to 1 h for unmodified oligodeoxynucleotides (Phillips and Zhang, 2000). In addition to nuclease resistance, PS DNAs form regular Watson–Crick base pairs, activate RNase H, carry negative charges for cell delivery and display attractive pharmacokinetic properties.

The major disadvantage of PS oligodeoxynucleotides is their binding to certain proteins, particularly those that interact with polyanions such as heparin-binding proteins. The reason for this nonspecific interaction is not yet fully understood, but it may cause cellular toxicity (Brown et al., 1994; Levin, 1999).

Second generation antisense-oligonucleotides

The problems associated with phosphorothioate oligo deoxynucleotides are to some degree solved in second generation oligonucleotides containing nucleotides with alkyl modifications at the 2' position of the ribose. 2'-*O*-methyl and 2'-*O*-methoxy-ethyl RNA are the most important members of this class. ASONs made of these building blocks are less toxic than phosphorothioate DNAs and have a slightly enhanced affinity towards their complementary RNAs. Questions regarding its efficiency to induce RNase H cleavage of the target RNA are the matter to concern regarding this second generation oligonucleotides (Crooke et al., 1995).

Third generation antisense-oligonucleotides

In recent years a variety of modified nucleotides have been developed to improve properties such as target affinity, nuclease resistance and pharmacokinetics. The concept of conformational restriction has been used widely to enhance binding affinity and biostability. In analogy to the previous terms ‘first generation’ for phosphorothioate DNA and ‘second generation’ for 2'-*O*-alkyl-RNA, these novel nucleotides will subsequently be subsumed under the term ‘third generation’ antisense agents. Few novel modified nucleotides, which have a great potential as antisense molecules are peptide nucleic acids (PNAs), N3’ P5’ phosphoroamidates (NPs), 2-Deoxy-2’-fluoro-β-d- arabino nucleic acid (FANA), and locked nucleic acid (LNA) (Nielsen, 1999; Gryaznov and Chen, 1994; Damha et al., 1998; Koshkin et al., 1998).

RIBOZYMES

Catalytic RNAs, or ribozymes, are RNAs, which catalytically cleave covalent bonds in a target RNA. The catalytic site is the result of the conformation adopted by the RNA-RNA complex in the presence of divalent cations. In the early 1980s, Cech and coworkers discovered the self-splicing activity of the group I intron of *Tetrahymena thermophilia* (Cech, 1981) and coined the term ‘ribozymes’ to describe these RNA enzymes. Shortly thereafter, Altman and colleagues discovered the active role of the RNA component of RNase P in the process of tRNA maturation (Guerrier, 1983). This was the first characterization of a true RNA enzyme that catalyses the reaction of a free substrate, i.e. possesses catalytic activity *in trans*. A variety of ribozymes, catalyzing intramolecular splicing or cleavage reactions, have subsequently been found in lower eukaryotes, viruses and some bacteria.

Although natural ribozymes can be divided into several broad classes based upon structure and mechanism, two structural classes are the basis for the design of most trans acting ribozymes. (i) Hammer head ribozymes are found in the self-cleaving domain of plus-strand satellite RNAs from tobacco ringspot virus and (ii) Hairpin ribozymes are found in the self-cleaving domain of minus strand satellite RNAs

from tobacco ringspot virus. A hammerhead ribozyme was isolated from viroid RNA and its dissection into enzyme and substrate strands (Uhlenbeck, 1987; Haseloff and Gerlach, 1988) transformed this *cis*-cleaving molecule into a target-specific *trans*-cleaving enzyme with a great potential for applications in biological systems. This minimized hammerhead ribozyme is less than 40 nucleotides long and consists of two substrate binding arms and a catalytic domain.

For the development of a therapeutic hammerhead ribozyme similar problems have to be solved as described for ASONs. Some steps, however, are more challenging due to the catalytic nature of ribozymes. Firstly, suitable target sites have to be identified, secondly the oligoribonucleotides have to be stabilized against nucleolytic degradation and thirdly the ribozymes have to be delivered into the target cells. Stabilization of ribozymes is even more difficult than protection of ASOns, as the introduction of modified nucleotides very often leads to conformational changes that abolish catalytic activity (Beigelman et al., 1995).

RNA INTERFERENCE

Only recently, research in the antisense field increased in impact by the discovery of RNA interference (RNAi). This naturally occurring phenomenon as a potent sequence-specific mechanism for post-transcriptional gene silencing was first described for the nematode worm *Caenorhabditis elegans* (Fire et al., 1998). RNA interference is initiated by long double-stranded RNA molecules, which are processed into 21–23 nucleotides long RNAs by the Dicer enzyme. This RNase III protein is thought to act as a dimer that cleaves both strands of dsRNAs and leaves two-nucleotide, 3' overhanging ends. These small interfering RNAs (siRNAs) are then incorporated into the RNA-induced silencing complex (RISC), a protein·RNA complex, and guide a nuclease, which degrades the target RNA.

The 21 nucleotide-long siRNA duplexes with 3' overhangs can specifically suppress gene expression in mammalian cells (Yu et al., 2002). So it is thought to provide a significantly higher potency compared to traditional antisense approaches. Not only short double-stranded RNA molecules but also short hairpin RNAs (shRNAs), i.e. fold-back stem-loop structures that give rise to siRNA after intracellular processing, can induce RNA interference.

An alternative approach to prolong siRNA-mediated inhibition of gene expression is the introduction of modified nucleotides into chemically synthesized RNA, despite the fact that even unmodified short double-stranded RNA revealed an unexpectedly high stability in cell culture and *in vivo*. For certain applications, however, further enhancement of the siRNA stability might be desirable (Amarzguioui et al., 2003).

Taken together, first promising *in vivo* experiments with siRNA have already been performed and further therapeutically important genes are expected to be targeted soon. No toxic reactions after siRNA application have been observed in the studies performed to date, but great care has to be taken to rule out severe side -effects of long-term induction of RNAi before trials can be started to treat human diseases. Because silencing of gene expression by siRNAs is similar to traditional antisense technology, researchers will be able to benefit from the lessons learned for more than a decade such as the requirement to use proper controls to proof a specific knock-down of gene expression and a careful analysis of possible unspecific effects mediated by the immune system.

ANTIGENE NUCLEIC ACID COMPOUNDS

Nucleic acids targeted to genomic DNA have been termed antigene nucleic acids. Antigene nucleic acids are designed to bind to single stranded or double stranded DNA. Binding of single stranded DNA may occur at a replication or transcription bubble, and thereby interfere with either of these processes, although gene specificity would be expected only when the antigene compound interferes with transcription processes. Alternatively the antigene compound may bind the major groove of double stranded DNA and form a triple helix or triplex. Triplex formation may then prevent the interaction of various protein factors required for transcription or it may physically block the initiation or elongation of the transcription complex.

Each of the nucleic acid bases possesses hydrogen bond donor and acceptor groups, which enable it to engage specific interaction with various ligands. Parts of these groups are involved in hydrogen bonding interactions within Watson-Crick base pairs of double helical DNA. But several recognition sites still remain in both major and minor grooves. Crystal structures of nucleic acid components have revealed that nucleic acid bases themselves can use some of these hydrogen-bonding possibilities. For example thymine can form two hydrogen bonds with the N (7) and NH2 (6) groups of adenine and/or with the NH2 (6) and N (1) groups (Roy, 1993; Hanvey et al., 1992).

A triplex of bases can be obtained where one adenine is hydrogen bonded to two thymines. Similarly, cytosine, once protonated, can bind to guanine in a Watson-Crick G.C. base pair. These base triplets are isomorphous. Consequently, a homopurine-homopyramidine sequence of duplex DNA could be recognized by a homopyrimidine oligonucleotide forming a local triple helix. Additionally, oligonucleotides containing G and T residues will form triplexes with DNA target sites. The orientation of the binding of the G and T triplex forming oligonucleotides is dependent upon the exact sequence of the target site.

DISCUSSION

Though the nucleic acids play a vital role in preventing dreadful diseases, they must overcome several measures before they can be widely applied as therapeutics. Researchers are concern about the stability of polynucleotides in biological systems, optimizing the affinity and efficacy of the drug without reducing its selectivity, targeting and delivering across cell membranes.

Since phosphodiester nucleic acids are extremely sensitive to nucleases in serum and cells, they undergo degradation. Chemical modification may affect the affinity or activity of the nucleic acid drugs. This can be overcome by introduction of hairpin forming sequence which stabilize the transcript towards exonucleases, provided the hairpain does not adversely affect the affinity of the drugs.

Subcellular distribution of nucleic acid drugs is an important aspect of their activity. Oligonucleotides, when directly introduced into the cytoplasm of living cells, accumulate within the nucleous. If the target is in the nucleus this will be beneficial and the biological properties of antigene oligonucleotides and aptamer compounds will be improved. However, as some mRNAs are localized in the cell RNA targeting drugs such as ribozymes should localize with their substrates if biological effects are to be expected.

CONCLUSION

After a long period of ups and downs, antisense technologies have gained increasing attention in recent years. Advances in recombinant DNA technology and synthetic chemistry have led to novel nucleic acid drugs that inhibit gene expression and protein function as evidenced by various studies. Major improvements have been achieved by the development of modified nucleotides that provide high target affinity, enhanced biostability and low toxicity. By developing a suitable drug delivery system, these drugs can emerge as novel therapeutics in the area of antiviral and anticancer in near future.

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