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Review article

Oligonucleotide conjugates – Candidates for gene silencing therapeutics

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ABSTRACT

The potential therapeutic and diagnostic applications of oligonucleotides (ONs) have attracted great attention in recent years. The capability of ONs to selectively inhibit target genes through antisense and RNA interference mechanisms, without causing un-intended sideeffects has led them to be investigated for various biomedical applications, especially for the treatment of viral diseases and cancer. In recent years, many researchers have focused on enhancing the stability and target specificity of ONs by encapsulating/complexing them with polymers or lipid chains to formulate nanoparticles/nanocom plexes/micelles. Also, chemical modification of nucleic acids has emerged as an alternative to impart stability to ONs against nucleases and other degrading enzymes and proteins found in blood. In addition to chemically modifying the nucleic acids directly, another strategy that has emerged, involves conjugating polymers/peptide/aptamers/antibodies/proteins, preferably to the sense strand (3′end) of siRNAs. Conjugation to the siRNA not only enhances the stability and targeting specificity of the siRNA, but also allows for the development of self-administering siRNA formulations, with a much smaller size than what is usually observed for nanoparticle (~200 nm). This review concentrates mainly on approaches and studies involving ON-conjugates for biomedical applications.

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1. Introduction

Oligonucleotides (ONs) show great potential for therapeutic use due to their ability to bind complementary endogenous messenger RNA (mRNA) leading to silencing of specific genes via several possible mechanisms. There are a number of different types of regulatory ONs, including single stranded antisense RNA (asRNA) of 13–25 nucleotides in length, double stranded small interfering RNA (siRNA) of 20–25 base pairs in length, small nuclear RNA (snRNA) of approximately 150 nucleotides in length, micro RNA (miRNA) of 22 nucleotides in length with short hairpin loops and mRNA of approximately 1500–2000 nucleotides. All of these types of

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oligonucleotide are found in nature and play complex roles in the regulation of gene expression [1].

There has been much discussion about the possibility of using these regulatory mechanisms to treat a wide range of diseases, but despite many years of research two antisense drugs: Fomivirsen (brand name Vitravene) and Mipomersen (brand name Kynamro) have so far been approved, while others are in ongoing clinical trials [2–4]. The reasons for this are largely due to issues with delivery of RNA, which make it unsuitable for therapeutic administration – it is a large, anionic molecule which has poor bioavailability and is highly susceptible to degradation by endogenous nucleases [5]. The size and charge mean that RNA cannot cross the plasma membrane, which it must do to reach its site of action in the cytoplasm or nucleus [5]. Therefore, much research has focussed on finding a suitable delivery system for RNA molecules, which simultaneously protects from nucleases, targets to the target tissue, increases uptake through the plasma membrane and facilitates intracellular trafficking.

1.1. Mechanisms of gene knockdown

Regulatory ON molecules bind to their complementary mRNA or pre-mRNA targets via base pairing, but there are several mechanisms by which gene knockdown can occur, depending on the type of regulator and the site of action. Ribonuclease H (RNase H) is a class of enzymes which degrades the RNA moiety in the RNA/DNA duplexes in mammalian cells, and this pathway may be exploited by non-natural ONs to knock down selective genes [6]. The RNase H pathway is most commonly associated with asRNA, wherein RNase H specifically cleaves the 3′-O-P bond of RNA in the DNA/RNA duplex to produce 3′hydroxyl and 5′phosphate terminated products [7]. The majority of ON drugs currently in clinical trials, including Fomivirsen and Mipomersen make use of the RNase H pathway, and are usually chemically modified to prevent degradation *in vivo* and have increased bioavailability [1].

Another possible mechanism that involves degradation of the mRNA is the RNA interference (RNAi) pathway, which is activated by siRNA and miRNA. siRNAs are short 21–23 base pair double stranded ONs, which are processed from long dsRNA in the cytoplasm by an endoribonuclease enzyme called Dicer. The processed siRNAs then act catalytically by binding to proteins, which make up the RNA-induced silencing complex (RISC) containing the Argonaute 2 (Ago2) enzyme. The activation of RISC is induced upon unwinding of the sense/antisense strand and thermodynamic selection of antisense strand. The activated RISC with the antisense strand binds to the complementary mRNAs with high sequence similarity, targeting the mRNA for degradation, thereby inhibiting the protein translation [5]. In contrast, miRNA has a hairpin structure and does not bear full complementarity with the target strand, binding only 6–8 nucleotides in the 3′ untranslated region of the mRNA [8]. Unlike siRNAs, miRNA are processed from dsRNA in the nucleus by an endoribonuclease enzyme (Drosha) and transported to the cytoplasm by the nuclear exportin-5 miRNA [9]. Once in the cytoplasm miRNA undergoes cleavage by Dicer to form siRNAs, activating the RISC complex, but gene silencing is thought to occur via blocking of translation or by sequestering the mRNA into P-bodies where other degradation enzymes act upon it [8]. Since miRNA is not sequence specific, one sequence may regulate many genes. For this reason, there has been less interest in using miRNA for therapeutic uses.

Gene silencing may also be achieved at the mRNA level by non-degrading mechanisms. Splice switching oligonucleotides (SSOs) are short, synthetic, antisense, modified oligonucleotides that bind to splice junctions on pre-mRNA, thereby blocking the RNA-RNA base pairing or RNA-protein binding that occurs between the splicing machinery and pre-mRNA [10]. This results in an alternative splice mRNA product, which in turn leads to a different protein sequence being translated [11]. This type of RNA-regulating ON has the potential to treat diseases caused by incorrect mRNA splicing, and has shown particular promise in the treatment of Duchenne Muscular Dystrophy (DMD) [11].

1.2. Chemical modifications

As mentioned previously, a major barrier to the use of ONs as therapeutic agents is the difficulty in delivering them to the site of action [12]. Naked ONs have a short half-life *in vivo* due to attack by nuclease enzymes, renal excretion, and accumulation in the liver and kidneys [12]. These issues of bio-distribution have partially been addressed by chemical modifications on the ONs, and in addition these modifications are widely used to improve uptake. The backbone of the ON chain is often changed to a phosphorothioate (PS) in which the oxygen anion is replaced by sulfur and increases nuclease resistance as well as decreasing renal clearance due to increased binding to serum proteins [13]. PS-ONs were one of the first class of chemically modified ONs to be developed, but they suffer from low bioavailability and off-target effects [14]. Substitution at the 2′ position of the nucleoside sugar by methyl (OMe) or methoxyethyl (MOE) groups increases binding affinity to the target mRNA, as well as decreasing serum protein binding [15], and these modifications are used in several antisense drugs currently in clinical trials [4]. However, these 2′-O substitutions on antisense oligonucleotides inhibit RNase H activity on the target strand, and therefore their use must be limited in order to retain the gene silencing effect [16]. This is often achieved by using gapmers, in which unmodified nucleosides are flanked by regions of 2′-O-substituted bases [16]. These types of chemical modifications are also used in siRNA to improve nuclease resistance, in addition to 5′-O-methyl substitution of the sense strand, which prevents this strand from binding to RISC [17].

Other chemical modifications involve more complex changes to the ON backbone. Locked nucleic acids (LNAs) include a methylene bridge between the 2′-O and the 4′-C of the sugar, which result in much higher binding affinity and enzyme resistance [18]. There are several LNA drug candidates in clinical trials, including Miravirsen which suppresses production of a miRNA (miR-122) involved in the life cycle of the hepatitis C virus [19]. LNAs may also be used in siRNA to confer higher stability and functionality [20]. Peptide nucleic acids (PNAs) consist of a neutral, peptide-like backbone instead of ribose sugars, and similarly phosphorodiamidate morpholino oligomers (PMOs) substitute the ribose sugar for a morpholino ring. These ONs possess high resistance to nucleases and low binding to serum proteins due to their neutral backbones [21]. However, their failure to activate RNase H means that they are used either as gapmers or in functions which do not require mRNA degradation, such as splice switching ONs. Several PMOs are being evaluated at different phases of clinical trials, for example AVI-4126, AVI-4065, AVI-4557. These PMOs have been evaluated pre-clinically and/or as first-in human trial, as therapeutic interventions for the treatment of restenosis, Hepatitis C virus or downregulation of cytochrome P450, respectively [22,23]. The most recent, GRN163L, a phosphothioamidate oligonucleotide conjugated to a lipid was shown to inhibit telomerase, limiting the lifespan of human pancreatic cells [24]. Table 1 lists studies highlighting a variety of conjugate linkages used to conjugate an oligonucleotide (PNA/ASO/PMO) to peptide ligands/polymers/small molecules for various biological applications, specifically focusing on gene silencing. Another review published recently focuses on nucleic acid bioconjugates, specifically focusing on the cancer therapy and detection [25].