'HOW TO UNDERSTAND IT' – ANTISENSE OLIGONUCLEOTIDES AND OTHER GENETIC THERAPIES MADE SIMPLE

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Word count: 1918

Abstract

Many genetic, neurological diseases result from the dysfunction of single proteins. Genetic therapies aim to modify these disease-associated proteins by targeting the RNA and DNA precursors. This review provides a brief overview of the main types of genetic therapies with a focus on antisense oligonucleotides (ASOs) and RNA interference (RNAi). The different mechanisms of action of ASOs and RNAi are highlighted using examples of new genetic therapies for spinal muscular atrophy, Duchenne muscular dystrophy and familial amyloid polyneuropathy.

Introduction

Dysfunction of single proteins cause many genetic, neurological diseases. To date treatments for such monogenic conditions have been limited and have traditionally involved small molecule drugs that modulate downstream pathways (e.g. corticosteroids in Duchenne muscular dystrophy). A recently-emerging, more efficient strategy is to modify disease proteins by targeting RNA and DNA precursors. Genetic therapies such as antisense oligonucleotide (ASOs) and RNA interference (RNAi) do exactly this. Through canonical Watson-Crick base pairing (Figure 1A), these drugs bind individual RNAs to modulate gene expression and thus protein availability [1]. To understand the different mechanisms of action of genetic therapies it is important to have a basic knowledge of how chromosomal (genomic) DNA acts as a template for protein production via RNA intermediates (Figure 1B).

Types of gene therapy

It is helpful to divide genetic therapies into three main categories A) short synthetic nucleotides, e.g. ASOs and RNAi, B) virus-mediated gene therapy, and C) genomic DNA editing/engineering. ASOs and RNAi-based therapies are both synthetic strands of nucleotides that bind target RNAs through Watson-Crick base pairing. However, they differ in their composition and modes of action; ASOs are single-stranded DNA or RNA sequences, whereas RNAi is synthesised and delivered as a small double-stranded RNA complex (Figure 2). ASOs function in a variety of ways depending on the biochemistry of the ASO and the target RNA sequence to which it binds (Figure 2A), whereas RNAi therapies function solely to downregulate their target transcript (i.e. the mRNA) and protein. RNAi is an innate cellular process in which foreign, double-stranded RNA molecules are identified and used as templates for the specific cleavage of complementary RNA. Having evolved as a defence mechanism against RNA-based viruses, RNAi can be used to restrict gene expression by administration of double-stranded RNA molecules. Viral gene therapy exploits the ability of viruses to invade selected tissues and utilise the host cell machinery to transcribe and translate the virus-delivered gene of interest. Gene editing, or genome engineering, the most successful approach of which utilises the bacterial CRISPR/Cas9 system,

involves the removal of sections of malfunctioning (mutated) genomic DNA and replacement with a 'normal' sequence.

Current challenges to ASO and RNAi therapies

The ability of exogenously delivered ASOs and RNAi-based therapies to modulate protein expression has been known for many years, however, their translation into clinical use has been hampered by their susceptibility to nuclease degradation, inability to cross the cell membrane due to their strong negative charge, and their activation of the innate immune system. Advances over the last two decades in the delivery and chemical structure of ASOs and RNAi have helped to overcome some of these hurdles [2].

Modifying the chemical structure of ASOs

An ASO consists of a short, single-strand of DNA or RNA, composed of phosphate backbone-connected sugar rings that are attached to one of four bases (Figure 1A). Modifications to both the backbone and sugar rings can alter the ASO resistance to nuclease degradation, binding to plasma proteins to maintain stable serum concentrations, RNA complementarity, and ability to activate the innate immune system. For example, one of the earliest modifications was the replacement of the phosphate backbone with a phosphothiorate (PS) linker. This relatively simple change protects against nuclease-mediated degradation and is able to trigger RNase H-mediated degradation of target RNA, but unfortunately also potently triggers the innate immune system [1]. Phosphorodiamidate morpholinos (e.g. Eteplirsen) and 2'-O-methyl ASOs (e.g Nusinersen) are characterised by modifications to the ribose sugar ring which render the ASO resistant to nuclease degradation, confer reduced toxicity and enhanced target specificity.

Mechanisms of genetic therapies

1) RNA degradation

ASOs can mediate target RNA degradation by recruiting the enzyme RNase H (Figure 2A). In a normal dividing cell, DNA replication is dependent on the enzyme DNA polymerase, the action of which

requires a small endogenous RNA primer (i.e. a short strand of nucleotides complementary to a segment of DNA) to trigger DNA replication. Once DNA replication has taken place, the enzyme RNase H recognises the DNA/RNA complex and degrades the now redundant RNA primer. DNA-based ASOs of this type act in the nucleus where they bind to their target RNA, which mimics the DNA/RNA complex recognised by RNase H resulting in mRNA degradation. The ability of an ASO to recruit RNase H is dependent on its chemical structure [1].

2) Blocking protein translation

ASOs that do not recruit RNase H (e.g. morpholino ASOs) may still restrict protein translation by binding their target RNA and preventing ribosomal attachment (Figure 2A).

3) Modification of RNA splicing

Genes include a promoter region (to which the enzyme RNA polymerase binds and begins transcription) and coding segments (exons) interspaced by non-coding regions (introns) (Figure 1B). DNA transcription produces a complementary RNA molecule called pre-mRNA that contains both introns and exons. Specific nucleotide sequences within and surrounding the exon/intron boundaries then recruit splicing factors that facilitate the removal of the intronic sequences (i.e. splicing) producing mature mRNA molecules containing only the required exons ready for ribosomal translation. ASOs that are modified so as not to recruit RNase H can be designed to bind to these regulatory sequences and alter pre-mRNA splicing (Figure 2A).

RNAi mechanism of action: RNA degradation

Unlike ASOs, RNAi-based therapies (e.g. siRNAs) work by hijacking the endogenous RNAi pathway in order to target specific RNAs for degradation to reduce protein levels. When double-stranded RNA enters the cell, it is recognised and cleaved into shorter fragments by a protein called Dicer (Figure 2B), and subsequently loaded into a multiprotein conglomerate called the RNA-induced silencing complex (RISC). RISC is present in most eukaryotic cells, and facilitates the separation of the two RNA

strands. The passenger strand is degraded, while the guide strand remains associated with RISC to act as a template for RISC-mediated cleavage of complementary RNA, reducing protein translation [2].

ASOs in spinal muscular atrophy (SMA)

SMA is an autosomal recessive disease caused by low levels of survival motor neuron (SMN) protein via deletions or inactivating mutations in the *SMN1* gene. Humans possess a second, almost identical gene called *SMN2* that differs by only a single nucleotide at the beginning of exon 7 weakening the splice site signal. This results in exon 7 exclusion from the majority of *SMN2* transcripts and the production of a truncated, unstable protein (called SMNΔ7). Nevertheless, a small amount of functional SMN protein is produced from *SMN2*, the amount of which inversely correlates with SMA severity. ASO therapy in SMA has focused on correcting the *SMN2* splicing defect to produce more functional SMN protein [3]. The recently licenced treatment for SMA, Nusinersen, is an ASO that binds to a regulatory sequence in intron 7 (called 'intronic splicing silencer N1' or ISSN1) that normally suppresses exon 7 splicing. ASOs targeting this sequence potently stimulate *SMN2* exon 7 inclusion resulting in increased SMN protein levels (Figure 3A).

ASOs in Duchenne muscular dystrophy (DMD).

DMD is a severe, childhood-onset disease mostly resulting from deletions within the dystrophin gene, *DMD*. These deletions in the 79-exon *DMD* frequently disrupt the reading frame (Figure 1A), which generates a downstream premature stop codon and causes a complete lack of dystrophin (the premature stop codon triggers degradation of the transcript by a process termed nonsense-mediated decay). Becker muscular dystrophy (BMD) is a milder disease caused by dystrophin truncations (due to 'in frame' deletions) rather than absence. The skipping of exons to correct reading frame disruptions caused by nonsense, DMD-linked mutations (accounting for 83% of mutations in DMD) has been identified as a viable strategy for generating truncated, but functional, dystrophin protein [4]. This can in theory reduce DMD severity and produce a milder phenotype akin to BMD. To date,

ASO therapy has been focused on skipping *dystrophin* exon 51, although only 13% of DMD patients have mutations that would be amenable to this therapy. Eteplirsen, a morpholino-based ASO, approved by the US Food and Drug Administration (FDA) for treating DMD in 2016, binds to the exon/intron splice site at the beginning of exon 51 resulting in it being skipped and thus absent in dystrophin mRNA (Figure 3B).

RNAi- and ASO-mediated RNA degradation in familial amyloid polyneuropathy (FAP)

Patisiran (RNAi) and Inotersen (ASO), targeting transthyretin mRNA for degradation have completed phase III clinical trials in FAP: results are awaited. FAP is an autosomal dominant disease caused by missense mutations in the transthyretin gene (*TTR*), which render transthyretin amyloidogenic, resulting in the deposition of TTR amyloid in the peripheral and autonomic nerves, heart and vitreous humour. TTR is a relatively redundant protein that transports vitamin A and a small amount of thyroxine. Complete absence of TTR is relatively harmless, but can predispose to vitamin A deficiency. For this reason, FAP is an attractive disease for genetic therapy as allele-specific ASOs/RNAi are not required. The overall aim of these therapies is thus to deplete total levels of TTR (healthy and mutant forms) to restrict amyloid deposition (Figure 3C, [5]).

Additional methods for optimising ASO/RNAi delivery

To further facilitate ASO/RNAi delivery to target cells, nanoparticles have been developed that envelope the negatively charged ASO or RNAi allowing it to traverse the cell membrane [2]. Cell-penetrating peptides, which are small peptides typically less than 30 amino acids, can be linked to ASOs to aid traversal of the cell membrane of target tissues. The blood-brain barrier poses a significant hurdle to CNS delivery, meaning that early generation ASOs designed for neurological diseases such as SMA have required intrathecal or intraventricular delivery; however, CNS-targeting strategies are providing promising pre-clinical results.

Viral gene therapy

One of the main impediments to successful gene therapy is an effective, non-toxic means of delivering a transgene to its target tissue. Much research has therefore focused on developing effective viral vectors that are able to target specific tissues [6]. Examples include lentivirus (similar to the HIV retrovirus), adenovirus, and adeno-associated virus (AAV). Lentivirus infects cells and integrates segments of viral DNA into the host cell chromosomal DNA, which can result in stable long-term expression, but unfortunately has the potential to disrupt endogenous gene function, hence there are clinical safety concerns associated with this virus. In contrast, adenovirus and AAV deliver genetic material to the target cell, but do not integrate DNA into the host genome. Due to greater targeting of neuronal tissues and extended periods of gene expression, AAV has become the most promising vector for viral-mediated gene therapy in neurological disease. Research into viral gene therapy is now translating into benefits for patients with the recent demonstration that a single intravenous infusion of AAV encoding the SMN1 gene results in longer survival, and superior motor function in infants with SMA compared to historical controls [7].

AAV viral vectors are limited by the total amount of DNA that can be incorporated into the viral capsid, typically between 4.5 and 5.5 kb. In the case of Duchenne MD, full length dystrophin mRNA is 14kb and cannot be delivered in its entirety using AAV virus

Genome editing

Gene editing allows the chromosomal DNA of a cell to be altered and for an underlying mutation to be permanently corrected, which has obvious benefits for genetic diseases. Various methods of genome engineering have been developed (e.g. zinc finger nucleases and TALENs), however, the CRISPR/Cas9 system has rapidly emerged as the most versatile and simple to use. CRISPR/Cas9 is an integral part of the bacterial defence system that has evolved to identify and degrade foreign segments of DNA inserted by invading viruses. Guided to specific nucleotide sequences by small RNAs, Cas9 is an endocuclease encoded by a CRISPR associated (CAS) gene (hence the name) that cuts both DNA strands. The region between two specific cuts can be replaced using template DNA with

homology to the Cas-targeted region. Harnessing the CRISPR/Cas9 system in mammalian cells provides a tool for precision genome engineering that has great potential for future clinical application [8]; however, several limitations including off-target genetic modifications and sub-optimal template DNA integration will first have to be fully resolved.

Summary

The recent emergence of ASO therapies for SMA, DMD and FAP is the result of decades of research. With phase II trials of ASOs ongoing in Huntington's disease (NCT02519036), amyotrophic lateral sclerosis (NCT02623699), and acute non-arteritic anterior ischaemic optic neuropathy (NCT02341560), the number of licenced ASO therapies is likely to expand [2]. The cost of Nusinersen to treat a child with SMA is approximately £500000 for the first year, requires intrathecal delivery and repeated treatments. How we fund and ration these expensive drugs and accommodate the social and health care requirements of a new patient population is an emerging issue [9].

Key points

1. ASOs are short, single-stranded sequences of DNA or RNA designed to target specific RNAs to modify gene expression.

2. ASOs exert their therapeutic effects in a variety of ways depending on their chemical structure. Mechanisms include promoting RNA degradation, preventing protein translation, and modifying RNA splicing.

3. RNAi-dependent therapies employ double-stranded RNA to mediate degradation of complementary RNA.

4. Viruses are used to deliver genetic material to cells to upregulate gene expression.

5. CRISPR/cas9 is a bacterial protein that can be utilised to engineer chromosomal DNA.

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Figure 1. From genes to proteins. A. Genomic DNA has a double helix structure that depends upon two antiparallel chains of repeating nucleotides, which are composed of a phosphate group, a sugar called deoxyribose, and one of four nitrogenous bases (adenine, A; cytosine, C; guanine, G; thymine, T). The storage of heritable biological information is mediated by Watson-Crick pairing of complementary bases (A to T and C to G) through hydrogen bonding. Amino acids are coded in mRNA by sequences of three bases, referred to as codons. Transcripts can be read in three different frames, but the start codon dictates which frame is chosen. Note that mRNA contains the base uracil (U) in place of thymine, and that individual amino acids are represented here by the three letter code. The addition or deletion of bases that are not multiples of three will result in a frame shift (e.g. frame 1 to frame 2 or 3) whereby all downstream codons are 'out of sync' (deletion not depicted in frame 2 or 3). This eventually leads to a premature stop codon (e.g. frame 3), which, if it occurs sufficiently early in the transcript, results in its degradation by a process known as nonsense-mediated decay. **B.** Genes are stretches of DNA that contain information for the production of proteins. DNA is transcribed into a complementary molecule called pre-messenger RNA (pre-mRNA), which contains both protein coding (exons) and non-coding (introns) regions, sandwiched between 5' and 3' untranslated regions (UTR), which are integral to gene regulation. Post-transcriptional modifications to pre-mRNA such as 5' capping, removal of introns (splicing), and polyadenylation, result in the production of mature mRNA, which serves as the template for ribosome-dependent protein production.

Figure 2. ASOs versus RNAi. A. ASOs impact gene expression through three principal mechanisms: 1) RNase H-mediated mRNA degradation, 2) steric block of ribosome binding, and 3) splicing modulation. **B.** Entry of double-stranded RNA into cells results in RISC-mediated cleavage of mRNA through activation of the RNAi pathway (see main text for details on these mechanisms).

Figure 3. Examples of ASO and RNAi-based therapies for neurological diseases. A. Nusinersen is a splice-modifying ASO that targets the ISS-N1 sequence within exon 7 of the *SMN2* gene. This masks negative splicing factors, ultimately resulting in greater production of the SMN protein, which is lacking in patients with spinal muscular atrophy. **B.** The ASO Etiplersen mediates skipping of exon 51 in the dystrophin gene, *DMD*, and is therefore only of use to 13% of DMD patients. For example, a deletion across exons 49-50 could disrupt the reading frame and thereby create an out-of-frame premature stop codon in exon 51, resulting in the complete absence of dystrophin. Etiplersen-mediated skipping of exon 51 bypasses the premature stop and restores the reading frame to produce truncated, but functional, dystrophin protein. **C.** Patisiran is a RNAi therapy that selectively targets *transthyretin* (*TTR*) mRNA for RISC-mediated degradation via the RNAi pathway. Both healthy and mutated transthyretin transcripts are degraded, resulting in reduced deposition of amyloidogenic TTR protein in familial amyloid polyneuropathy. An ASO targeting *TTR* mRNA for degradation, Inotersen, has also completed phase III clinical trials in FAP. E, exon; I, intron. Figure adapted from [10].