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OLIGONUCLEOTIDE THERAPEUTICS: DRUG DISCOVERY THROUGH TIGHTLY COUPLED CHEMISTRY AND BIOLOGY

Oligonucleotide-based therapeutics are set to transform the landscape of treatable human disease. Many traditional pharmacological treatments use small molecules to directly modulate the activity of proteins involved with disease. Yet only about 25% of disease-related proteins have been drugged to date, and the vast space of the human genome provides a variety of biochemical mechanisms to target for treating disease.[1]

"The range of modalities available to us in drug discovery continues to evolve and provide us with ever more opportunity to explore the relationship

In this conceptual illustration, a strand of synthetic nucleic acid (silver) binds to a complementary strand of naturally occurring genetic material (blue).

Credit: ktsdesign/Corporate+

between disease, pathway, and the most appropriate modality for therapeutic intervention," Dave Madge, vice president of WuXi AppTec's discovery services team, said in a webinar in late 2022.[2]

Nucleic acid therapies are increasingly prominent as a treatment modality. They influence cell function before protein production, interacting with genetic material to alter gene expression. They can also provide instructions for functional proteins or initiate degradation.[3]

Oligonucleotide-based therapeutics were originally used for rare diseases, including a treatment tailored for one girl.[4] The US Food and Drug Administration has now approved at least 15 oligo-based therapeutics to treat macular degeneration, Duchenne's muscular dystrophy, and a genetic factor that increases risk for heart disease, among other diseases. Other oligonucleotide therapeutics are in clinical trials to treat heart disease, neurological disorders, and various eye disorders.[3]

While creating oligonucleotide therapeutics is theoretically as straightforward as a sequence of chemical building blocks, designing effective treatments for clinical applications involves understanding both the chemistry and biology of nucleic acids.

OLIGONUCLEOTIDE INTERVENTIONS

Oligonucleotide therapeutics disrupt processes within the central dogma of biology: genetic information in DNA encodes RNA, which encodes protein, the biomolecule primarily responsible for cellular function. Most oligonucleotide therapeutics have a sequence complementary, or antisense, to the target

TABLE 1. TYPES OF OLIGONUCLEOTIDE THERAPEUTICS

Oligonucleotide therapeutic class	Nucleic acid composition	Method of action
Antisense gapmers	Single-stranded DNA with RNA-like flanking regions	Bind to RNA and induce its degradation
Antisense steric blockers	Single-stranded DNA or RNA	Prevent assembly of RNA-binding factors
Antisense splice site modulators	Single-stranded DNA or RNA	Include or exclude exons to restore functional protein expression
Agomirs	Single-stranded RNA	miRNA mimics
Antagomirs	Single-stranded RNA	miRNA inhibitors
siRNA	Double-stranded RNA	Decrease expression of a specific gene
Aptamers	20–100 bases, single stranded DNA or RNA	Secondary structure allows binding to target proteins to alter activity

Source: "A Perspective on Oligonucleotide Therapy: Approaches to Patient Customization," Thakur Shikha et al.

genetic information. Many interact with RNA to trigger its degradation, alter processing such as splicing events, or otherwise impact protein production. Other oligonucleotide therapeutics target noncoding RNAs, such as microRNAs (miRNAs), to influence gene expression.

Another class of approved therapeutic oligonucleotides is small interfering RNA (siRNA). This double-stranded RNA recruits protein machinery, called the RNA-induced silencing complex, to destroy a target sequence. Once surrounded by the recruited proteins, the duplex unwinds, leaving a single strand of siRNA available to bind to target RNA. The target joins the protein complex, where it is cut and rendered useless for gene expression.

Table 1 contains a variety of oligonucleotide therapeutics and their mechanism of action. The variation in nucleic acid therapeutics enables researchers to match a therapeutic mechanism to the molecular biology of a disease, Madge said in the webinar.[2]

CHEMICAL MODIFICATIONS FOR STABILITY AND SELECTIVITY

Oligotherapeutic design starts by choosing a nucleic acid target based on its presumed physiological significance. Next, scientists convert the target sequence into its complement. They then use software tools to screen the complementary sequence for factors that minimize toxicity and maximize efficacy.

Considerations during screening include:

- sequence similarities to other RNA molecules, which could create off-target activity
- overall number of the nucleotides guanine and cytosine, which impact binding strength and likelihood of therapeutic oligos binding to one another
- similarity to sequences containing common single nucleotide variants, which could reduce therapeutic efficacy in patients with these altered sequences.

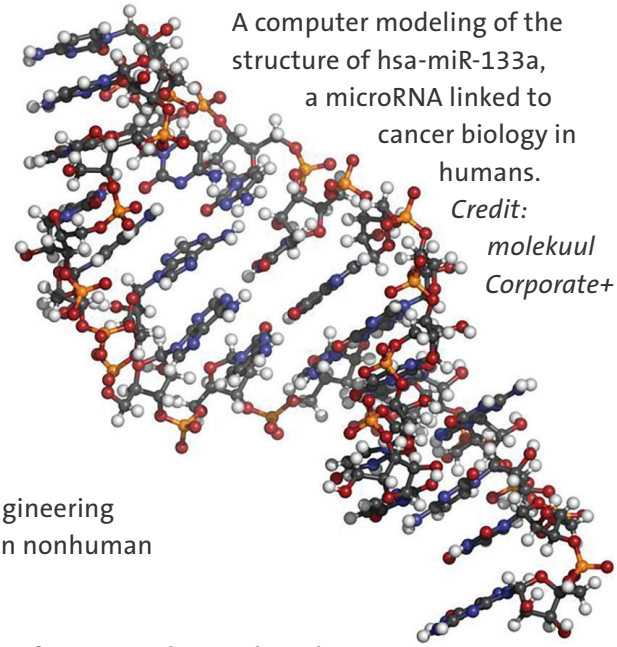
Next, researchers identify appropriate chemical modifications for the target sequence. Nucleotide building blocks contain three parts: a sugar, a phosphate group, and a nucleobase. Any of these components can be modified chemically to promote the potency, stability, safety, and bioavailability of the complete oligonucleotide. Modification also enables targeting of a specific tissue or cell type.[5] For example, appending a sugar group called triantennary *N*-acetylgalactosamine, or GalNAc, to the end of a nucleic acid strand facilitates delivery to liver cells via binding to the asialoglycoprotein receptor.

Without modified nucleotides, oligo therapeutics would be ineffective.[6] Unmodified nucleic acids are quickly broken down by enzymes located outside cells. Their large size and overall negative charge also restrict the crossing of cell membranes.

BIOLOGICAL TESTING

After design and synthesis, the next step in oligonucleotide drug discovery is

biological testing. Both in vitro and in vivo tests require additional effort to gain information most relevant to future clinical testing. A suite of cell-based assays provides initial information about function and selectivity. Standard preclinical tests in animal models (such as transgenic mice) involve different types of genetic engineering to replicate human biology in nonhuman organisms.



A computer modeling of the structure of hsa-miR-133a, a microRNA linked to cancer biology in humans. *Credit: molekuul Corporate+*

Cell-based assays involve transferring an oligonucleotide into cells and monitoring them for a lack of expression of the targeted gene, also called a knockdown effect. Gene expression can be monitored through nucleic acid measurements, including direct sequencing or reverse transcriptase quantitative polymerase chain reaction (qPCR). Protein levels can also be measured to provide information about gene expression.

For these tests, researchers prefer to use primary cells extracted directly from tissue rather than continuous cell lines established for research. Primary cells retain the genetic and biomolecular information of tissue more closely than cells containing mutations for immortality.[7] This means the response of primary cells to an oligonucleotide therapeutic is more related to that of a whole organism.

Despite their utility, primary cells are challenging to work with. They are difficult to culture, struggle to efficiently take in oligonucleotides, and have a limited life span. Carefully designed high-throughput assays can provide information about knockdown efficacy when the signal is inherently low, such as in primary cultures of mouse neurons.[8]

For in vivo tests in animal models such as mice, there are several options to re-create human biology. Hydrodynamic injection mice, or HDI mice, receive genetic information via a fluid injection, which results in temporary gene expression in their liver. For instance, mice injected with the genome of the hepatitis B virus can be used as models to test oligonucleotide therapeutics for a hepatitis B vaccine.[9]

A mouse can also receive human genes by packaging the genetic information within an adeno-associated virus (AAV). This viral vector effectively targets specific tissues, thus enabling gene delivery to tissues other than those in the

liver. The AAV vector's limited packaging capacity controls the size of the genetic information that can be delivered, however.

Transgenic mice can be genetically engineered to express a gene that is the target of an oligotherapeutic. Expression of the introduced gene is stable, so this model is a reliable test system for how an oligotherapeutic interacts with its target.

Liver-humanized mice contain human cells in their liver.[10] Like transgenic mice, they are a source of information about on-target effects. Because liver-humanized mice contain the full functionality of human biology in their human cells, they can also provide information about off-target effects of an oligonucleotide therapeutic. Although transgenic and humanized mouse models provide unmatched benefits for the quality of information gained from in vivo testing, these models are expensive—in terms of both time and cost—to produce.[2]

IDENTIFYING OFF-TARGET SITES

Oligonucleotides bind to their target gene through well-established sequence-specific pairings. But these therapeutics can also bind to genes with sequences similar to the target and create the potential for toxic, off-target side effects. Computer-based screening using a database of genomic and RNA sequences can identify candidates for potential off-target binding sites.[11]

Sequencing RNA from treated cells can provide cell-wide information about off-target effects. This experimental technique, called RNA-Seq, involves extracting RNA from treated cells, isolating specific forms, and converting the isolated RNA to complementary DNA (cDNA). This conversion captures the sequence information of RNA in a molecule that has increased stability relative to RNA. The cDNA is then amplified and sequenced. Plots of relative levels of gene expression throughout the cell can reveal patterns that indicate off-target effects.

COMBINING ANALYTICAL TECHNIQUES FOR QUALITY CONTROL

Oligonucleotide therapeutics have several characteristics that complicate their analysis for pharmacokinetics studies.[2] Oligonucleotide therapeutics are relatively large molecules, often 1–30 kDa; they are polyanionic, may incorporate diverse modifications, and may have nonspecific binding to proteins or other nucleic acids.

A combination of analytical techniques is needed to identify these molecules by charge, mass, binding in a hybridization assay, or imaging. Effective method selection accounts for an oligo's chemical structure and sample type, as well as for the necessary sensitivity and development stage of a given study.

Liquid chromatography/mass spectrometry (LC-MS) methods are used to accurately quantify and discriminate metabolites from full-length

oligonucleotides, and this approach is typically applied when measuring concentrations in specific target organs and to support in vitro metabolic, pharmacokinetic, and biodistribution studies. Hybridization-based enzyme-linked immunosorbent assay (ELISA) and hybridization-based LC-fluorescence methods have better sensitivity and can be used to track lower concentrations in vivo over time for pharmacokinetic studies. Reverse transcriptase-quantiative PCR (RT-qPCR) methods can provide ultra-high sensitivity for oligonucleotide quantitation, but, like hybridization-based ELISA assays, this method is unable to distinguish full-length oligonucleotides from their close metabolites.

Oligonucleotide therapeutics have a growing role in the pharmaceutical landscape. Structure and sequence are key to their stability and efficacy. Discovering, designing, and analyzing oligonucleotide therapeutics involve connections between chemical structures and biological function at every step.

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