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New reASOs to pursue the therapeutic potential of synthetic nucleic acids for neurological diseases

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The use of short, synthetic nucleic acids as potential therapeutics was proposed more than three decades ago. However, improvements in the design and synthesis of nucleic acids have recently piqued the interest of researchers and physicians searching for alternative approaches for treating diseases that do not conform to traditional drug discovery programs. The advent of novel modified nucleic acids with better efficacy, stability and cell penetrance combined with advancements in various delivery systems and methods, including intrathecal (IT) administration to allow direct entry to the central nervous system, provide sufficient rationale to cultivate the therapeutic capabilities of synthetic nucleic acids for neurological diseases.

In this issue of *JAMA Neurology*, Dr. David Corey presents the current state of synthetic nucleic acids as therapeutic agents for several neurological diseases stemming from different types of genetic mutations¹. Also in the review, Dr. Corey highlights details in the advancement of oligonucleotide therapy for two neurological diseases, Friedreich's ataxia (FRDA) and spinal muscular atrophy (SMA), which are in discovery and clinical stages of development, respectively.

Therapeutic intervention using nucleic acids has also been pioneered as an antisense oligonucleotide (ASO) approach. Typically, ASOs control expression of their targets by binding mutated transcripts and eliminating them from cells via RNase H or RNA interference mechanisms or by modulating their splicing^{2,3}. The initial interest in targeting repeat expansion diseases was focused on CAG coding expansions (also referred to as polyglutamine expansion diseases), such as Huntington's disease or spinocerebellar ataxias type 1 or 3, and toxic RNA diseases, such as myotonic dystrophy type 1 (DM1). The nucleic acids were designed to target the mutated transcript encoding the expanded CAG or CUG repeats for degradation or induce exon skipping to reduce expression of the toxic allele⁴. Two ASOs mentioned in Dr. Corey's review for SMA (Nusinersen) and Duchenne's

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muscular dystrophy (DMD) (Eteplirsen/AVI-4658) also act by redirecting splicing of the mutated gene, which results in expression of the corrected protein. Both of these candidates are in various stages of clinical trials^{1,5}.

Recent findings published by Dr. Corey's group demonstrate a novel use for synthetic nucleic acids in restoring Frataxin (FXN) levels in cell line models derived from patients with FRDA⁶. FRDA is an autosomal recessive neurodegenerative disease caused by decreased expression of the nuclear encoded mitochondrial protein FXN. Transcriptional downregulation of *FXN* expression is caused by large GAA trinucleotide repeat expansions within intron one of the *FXN* gene. Several hundreds of the polypurine-polypyrimidine repeats inhibit initiation and progression of the transcription machinery. Recent studies identified persistent R-loops within the expanded GAA region in *FXN* as critical instigators of transcriptional silencing⁷. R-loops are stable structures formed by interactions between the genomic DNA template and a nascent transcript. While short and metastable R-loops composed of a few base pairs are constantly being formed and resolved during RNA synthesis, long and persistent DNA-RNA hybrids interfere with normal transcription and replication, ultimately affecting genome stability⁸.

Dr. Corey and colleagues aimed to eliminate the formation of these R-loops by using anti-GAA-specific synthetic nucleic acids to compete with the formation of RNA-DNA hybrids. Increased FXN transcript and protein levels were achieved in FRDA patient cell lines following treatment with either duplex RNAs or single-stranded locked nucleic acid (LNA) oligonucleotides. The mechanism of increased expression does not rely merely on activating transcription at the *FXN* locus, nor is the RNAi pathway required. Instead, data from either approach indicate that the ability of the engineered nucleic acids to specifically bind the mutant FXN pre-mRNA and block its interaction with the genomic locus is sufficient to restore FXN mRNA and protein levels⁶.

Formation of R-loops is facilitated frequently by high GC content as well as the repetitive nature of the transcribed sequence. In fact, R-loops have been identified in at least three loci associated with repeat expansion diseases: *FXN* (GAA repeats, FRDA), *FMR1* (CGG repeats, fragile X syndrome), *C9orf72* (CCCCGG repeats, ALS/FTD)^{7,9}. Additionally, expanded CTG repeats, as observed in DM1, form R-loops in model systems¹⁰. In all cases, formation of stable DNA-RNA hybrids is accompanied by epigenetic changes and defects in transcription, as well as instability of the repeating DNAs. Thus, in addition to relieving transcription inhibition, elimination of detrimental R-loops might also result in stabilization of the expanded repeat sequences and prevention of somatic instability¹¹. Altogether, the use of synthetic nucleic acids to target R-loops in a sequence-specific manner likely has much broader implications, beyond FRDA.

Off-target effects remain a major obstacle in the development of synthetic nucleic acids as therapeutic agents¹. In the case of FRDA, it appears that a minimum of two conditions are necessary for the formation of R-loops at the GAA tract that help mitigate potential off-target effects: 1) the sequence must be transcribed and 2) must meet a certain length threshold for stable R-loop formation. Thus, the numerous short GAA loci found across the

human genome are unlikely to be potential off-target sites for oligonucleotides targeting R-loop structures.

The nucleic acid targeting approach described by the Corey group is unique in that it takes into account structural properties of the nucleic acids, for example the propensity to form R-loops, rather than solely the sequence, which in the case of tandem repeats would not assure appropriate levels of specificity. The concept of chemically targeting a particular GAA repeat structure was previously proposed by targeting the triple helical conformation that these repeats can adopt. While effective, the use of highly specific DNA binding polyamides did not achieve levels of reactivation of *FXN* expression as high as those observed following treatment with the synthetic oligonucleotides^{6,12}.

Adding a layer of complexity to the design of therapeutic strategies for FRDA is the epigenetic component of the *FXN* transcriptional defect. Treatment of FRDA cells with synthetic oligonucleotides restored histone H3 and H4 acetylation at the *FXN* locus, signifying a correction of the local chromatin environment to a transcriptionally permissive state⁶. Similarly, the use of histone deacetylase inhibitors (HDACi) also results in increased histone acetylation at the *FXN* gene in FRDA samples and increased *FXN* expression^{13,14}.

Strikingly, the magnitude of the increase in *FXN* mRNA and protein levels after treatment with GAA-specific duplex siRNAs or LNAs is appreciably greater than treatment with HDACi^{6,13,14}, suggesting that targeting the underlying molecular process rather than consequent histone modification changes is more efficacious. Taken together, these results imply that the RNA polymerase impediment and R-loop formation are the primary events leading to transcription deficiency in FRDA. However, considering the beneficial effect on *FXN* expression elicited by both types of treatments (chemical or epigenetic modulators and synthetic nucleic acids), a combined therapy approach will be a promising avenue to explore.

One of the greatest advantages of the approach described by Corey is the extensive knowledge base already established in the field. As stated in the review, there are more than 30 years of studies using ASOs and other therapeutic nucleic acids. Extensive data related to dosing, toxicity, and biodistribution, to name a few, have already been collected and can be used to pave the way for moving R-loop specific nucleic acids much faster to the clinic.

So, what are the next steps? A successful example described in the Corey review (SMN) went through a battery of analyses in various human cell line and animal models. In the case of the GAA repeat/R-loop targeting nucleic acids, the efficacy was demonstrated in one human fibroblast cell line. Importantly, gymnotic delivery of the therapeutic oligonucleotides has to be optimized to replace the variable transfection approach, which is incompatible with therapeutic applications. Confirmatory studies in several FRDA patient-derived cell lines, perhaps containing varying numbers of expanded GAAs, followed by analyses in neuronal and cardiac cell models of FRDA need to be conducted. Detailed off-target analyses using whole genome sequencing approaches will justify moving from proof-of-concept work to animal models of FRDA. Although current FRDA transgenic and knock-in mouse models contain a minimal number of GAA repeats (~200 units) and very mild and

late onset phenotype, increased expression of Fxn should be clearly detected in different tissues, including the effect on dorsal root ganglia upon IT delivery¹⁵. Additionally, the effect of long-term targeting of the R-loops on somatic instability of the GAAs needs to be determined in both cell culture and mouse models. In summary, given the recent progress in discovery and in several aspects of clinical development of ASOs, this alternative treatment option for neurological diseases with unmet therapeutic needs is met with optimism and enthusiasm by the scientific community.

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