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Morpholino-driven gene editing: a new horizon for disease treatment and prevention

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Abstract

The development of genetic and molecular biology tools permitting the connection of specific genes to their functions has accelerated our understanding of molecular pathways underlying health and disease. The resulting gains in knowledge have propelled gene targeting to the forefront of promising therapeutic strategies. Here we discuss the uniquely powerful and adaptable approach of morpholino-driven modification of normal and mutant gene expression as a pathway to health.

Keywords

vivo-morpholino; oligonucleotide; gene silencing; ATP-sensitive potassium channel; skeletal muscle

Modern medicine is rapidly evolving based on the development of multiple technologies that have permitted pinpointing specific molecular determinants of disease pathophysiology. Identification of such targets offers unprecedented potential for development of novel therapeutics, yet traditional pharmacologic approaches often lack the temporal, spacial and molecular specificity needed to achieve ideal outcomes. In this context, genetic silencing and editing techniques have emerged as powerful, specific, and highly adaptable strategies with the capacity for treating a wide variety of diseases. This approach may be used to interfere with disease processes promoted by the actions of normal “wild-type” molecular components, as well as to diminish the consequences of harmful mutations and disease modifying polymorphisms, or to battle gene expression by infectious organisms. Although

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Conflicts of Interest

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Author Contributions

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gene knockdown or editing techniques have been successful in research applications for decades, having served as an important method for advancing our understanding of normal development and disease pathophysiology, central challenges to widespread clinical translation due to issues of targeting, systemic delivery in adults, stability, and cell-penetration, are just recently being overcome.

Here we discuss the use of morpholinos and their adaptations, particularly Vivo-morpholinos - an approach with particular advantages that shows promise for bringing custom gene knockdown and editing into mainstream medicine.

Structure, mechanism of action and toxicity of morpholino oligonucleotides

Invented in 1989, morpholino oligonucleotides are “uncharged stereoregular nucleic acid analogs” (1). These are molecules similar to RNA or DNA in that they comprise the same nitrogenous bases, although bound to morpholine rings instead of ribose or deoxyribose rings, and linked through uncharged phosphorodiamidate groups instead of anionic phosphates. They are typically 18–30 bases in length and bind to targeted RNA sequences by base-pairing. However, unlike siRNA, these phosphorodiamidate morpholino oligomers (PMOs or Morpholinos) do not result in degradation of their target RNA since their backbone structure is foreign and not recognized by cellular nucleases. Rather Morpholinos act by “steric blocking”: binding to a target sub-sequence within the RNA thereby getting in the way of molecules that might otherwise interact with the RNA. As a result, various biological processes may be modulated or prevented from occurring: pre-mRNA splicing, mRNA translation, miRNA maturation and activity, ribosomal frameshifting, and/or ribozyme activity [(2–4), Fig. 1]. The uncharged nature of the phosphorodiamidate groups avoids adverse interactions with cellular proteins and the molecules do not appear to activate the innate immune response (4). Morpholinos, injected or electroporated into embryos at various stages, have been invaluable in defining the role of specific mRNA transcripts in development (4).

In vivo delivery of Morpholinos

In the majority of cases, Morpholinos require assistance (microinjections, electroporation, or association with delivery molecules) for their efficient intracellular entry. In order to improve intracellular delivery of Morpholinos, covalent conjugation to arginine-rich cell penetrating peptides to form peptide-linked phosphorodiamidate Morpholino oligomers (PPMOs, Sarepta Therapeutics, Inc., Cambridge, MA), or to octaguanidinium dendrimer scaffolds to form Vivo-morpholinos (Gene Tools, LLC, Philomath, OR), was developed (2–4). These compounds may be delivered intravenously, intraperitoneally or locally to various tissues, and show rapid and efficient entry to cells at relatively low doses making them powerful tools for gene editing “therapy” *in vivo*.

***In vivo* gene silencing/editing with Morpholinos**

Unmodified Morpholinos (PMOs) demonstrate promising therapeutic potential against Duchenne muscular dystrophy. Duchenne muscular dystrophy is a severe X-chromosome-linked genetic disease caused by mutations in the gene *DMD* that encodes dystrophin, a protein involved in maintaining the integrity of skeletal and cardiac myocyte membranes. Mutations in *DMD* alter the structure or function of dystrophin or prevent any functional dystrophin from being produced. Dystrophin deficient muscles are subject to membrane destabilization, muscle deterioration and loss. PMOs have been designed to cause translational skipping of exons. Fortuitously, these unmodified Morpholinos can cross the pathologically leaky cellular membrane of dystrophin deficient myocytes, resulting in restoration of a functional, though abbreviated, dystrophin protein that improves muscle and cardiac function [(2–4), Table 1]. This technology is currently under evaluation in clinical and pre-clinical trials.

However, delivery through “competent” cell membranes in other disease states requires the modified morpholinos: PPMOs or/and Vivo-morpholinos. PPMOs have been used in multiple studies in animal models against genetic, viral and bacterial diseases [(3, 4), Table 1]. They are being developed as pharmaceuticals (Sarepta Therapeutics, Inc) but have not yet reached clinical trial stage. A limitation of PPMOs may be dose-dependent renal toxicity above a threshold concentration (4).

Vivo-morpholinos appear to be the most effective approach for *in vivo* delivery, tissue distribution and antisense effect of current gene knock-down/editing agents as therapeutics or to create disease models as demonstrated by numerous animal studies [(3, 4), Table 1]. Vivo-morpholinos consistently result in at least 50% gene knockdown (3, 4), although the efficiency of gene modification in brain and vasculature following systemic delivery is limited. However, like PPMOs, no Vivo-morpholino has yet reached the clinical trial stage. Because of their lipophilic properties and chemical and biological stability, Vivo-morpholinos irreversibly penetrate cell membranes and provide a prolonged effect on the target gene of 17 weeks or more (3, 4). As such, Vivo-morpholino targeting of *Dmd* in mdx mice (an animal model of Duchenne muscular dystrophy) demonstrated substantial amelioration of pathology during long term administration (2, 4). Since multiple mutations can cause Duchenne muscular dystrophy, a cocktail of Vivo-morpholino oligomers has been developed that leads to multiple exon-skipping and could thus serve as a more universal and effective approach to treatment than unmodified Morpholinos (2, 3).

The only significant reported toxicity from systemic Vivo-morpholino administration occurred following intravenous delivery of oligomers containing sequences capable of 3'-5' hybridization. These particular Vivo-morpholinos exhibited dendrimer clustering causing increased blood viscosity and clot formation leading to death (Table 1 – PMID 24505100 and 24806225). This toxicity may be overcome by careful design and testing of oligomer sequences with low self-hybridization potential or alternatively by vehicle (saline)-based masking of charges on the dendrimers. In addition, guanidinium has significant toxic effects at higher doses, however these appear to be minimal at the Vivo-morpholino concentrations

sufficient for substantial gene knock-down (3, 4). Further studies directed at short- and long-term toxicities are needed for Vivo-morpholino studies to be translated to human use.

Morpholinos can also be used to alter expression of normal genes to generate a desired therapeutic effect. A recent study from our laboratory illustrates the potential clinical range and power of Vivo-morpholino technology in this regard (5). The goal of this project was to modify the energy efficiency of skeletal muscles to promote thermogenesis and increased caloric consumption as a strategy to combat obesity. Recently, we discovered that muscle energy efficiency is regulated by sarcolemmal ATP-sensitive potassium (K_{ATP}) channels, formed through association of Kir6.2 pore-forming and SUR2A regulatory sulfonylurea receptor subunits. Specifically, we found that potassium efflux *via* sarcolemmal K_{ATP} channels was activated by low-intensity muscle activity and opposed depolarizing currents thus shortening action potential duration and limiting calcium influx. The resulting decrease in calcium-dependent functions translates into less energy used for contractions, calcium re-sequestration, and heat production. Conversely, loss of normal Kir6.2 function augments ATP turnover and elicits an extra energetic cost of muscle performance. Pharmacologic blockade of skeletal muscle K_{ATP} channels to increase cellular energy expenditure as a treatment for obesity would be a logical clinical translation of these findings. Unfortunately, no K_{ATP} channel antagonist is selective for skeletal muscle K_{ATP} channels which share the same isoform composition as cardiac K_{ATP} channels and local administration of a K_{ATP} channel blocker would need to be repeated frequently and risk systemic leak with potentially detrimental consequences on cardiac energetic efficiency and stress resistance. To overcome these limitations, we employed intramuscular injections of anti-Kir6.2 Vivo-morpholino (5). We found that three once weekly local intramuscular injections of anti-Kir6.2 Vivo-morpholino into bilateral limb muscles of mice caused the effective knock-down of K_{ATP} channels accompanied by altered membrane electrical response and augmented force generation only in targeted muscles while K_{ATP} channel expression and function in adjacent muscles and heart remained unaffected, presumably due to the rapid intracellular transit of the Vivo-morpholino. Even in response to low-intensity activities, treated mice increased their muscle and body temperature as well as oxygen consumption significantly more than controls, although with only a mild decrement in maximal exercise tolerance. This study serves as a proof-of-principle that Vivo-morpholino technology can be used successfully to target normal components of molecular pathways in a local manner to achieve a desired clinical result, in this case modification of energy efficiency in select skeletal muscles.

Conclusion

Morpholino-based oligomers are effective, stable and highly adaptable anti-sense DNA analogs for gene silencing and editing. The development of cell-penetrating modifications has greatly facilitated their applicability for *in vivo* use by permitting systemic and/or precise local delivery in adult subjects. Their exceptional duration of biologic effect further enhances their utility. These unique properties make them very attractive foundations for the development of therapeutic agents and animal disease models through targeting the expression of normal and mutant genes. Further definition of potential toxicities, as well as safe and effective methods of application, will be essential for clinical translation.

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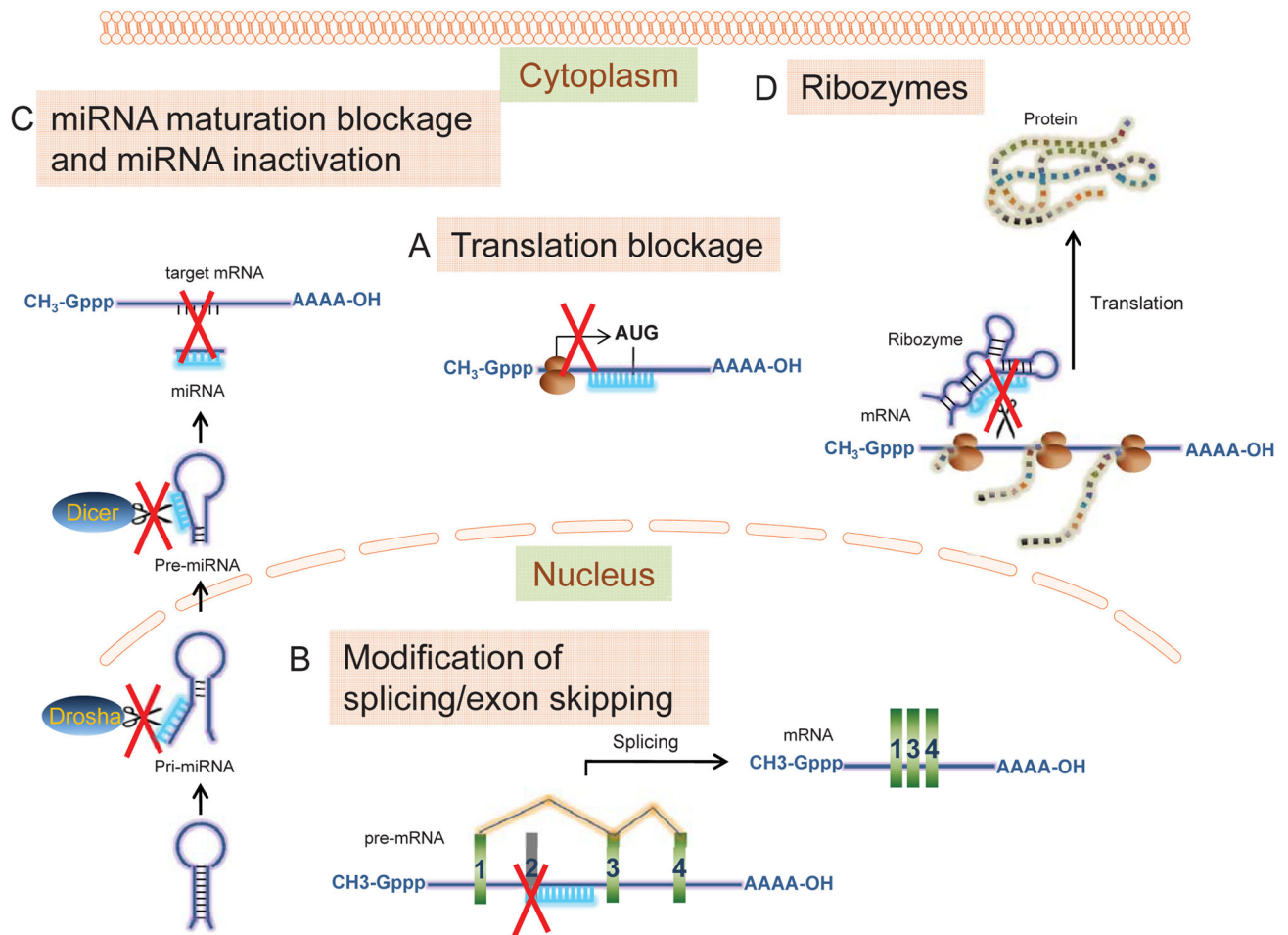


Figure 1. Mechanisms of action of anti-sense morpholino oligomers

A) Translational blockage. PMOs block the translation initiation complex binding sites on mRNA and prevent translation from occurring. **B) Modification of splicing/exon skipping.** PMOs block splice sites on pre-mRNA, prevent recognition of these sites by the spliceosome that in turn causes exon skipping. **C) miRNA maturation blockage and miRNA inactivation.** PMOs may block maturation enzyme cleavage sites (i.e. Drosha, Dicer) on pri- or pre-miRNA to prevent its maturation. PMOs may complementarily bind to mature miRNA and prevent it from binding to target mRNA. **D) Ribozymes.** PMOs may bind to enzymatically active RNAs (ribozymes) blocking their active sites and preventing them from cleaving their target mRNAs.

Table 1

Disease/process	Organism	Type of PMO modification	Target gene/tissue	Mechanism	PMID (Year)
Disease models					
Myotonic dystrophy type 1	Mouse	Vivo	PKM2/skeletal muscle	Modification of splicing	23901116 (2013)
Muscle hypertrophy	Mouse	Vivo	Myostatin/skeletal muscle	Exon skipping	20924365 (2011)
Hyperbilirubinemia in hypertension	Mouse	Vivo	UGT1A1/liver	Translation blockage	20668235 (2010)
Hypothalamic-pituitary-adrenal axis suppression	Rat	Vivo	CRH/hypothalamus	Translation blockage	21979757 (2012)
Systemic hyperphenylalaninemia	Mouse	Vivo	Pah/liver, kidneys	Exon skipping	25226162 (2014)
Intermediate spinal muscular atrophy	Mouse	Unmodified, Vivo	SMN2/skeletal muscle, spinal cord	Modification of splicing	23339722 (2013) 26264577 (2015)
Muscular dystrophies					
Duchene muscular dystrophy	Dog	Unmodified	Dystrophin/skeletal muscle	Exon skipping	19288467 (2009)
Duchene muscular dystrophy	Mouse	CPP	Dystrophin/skeletal muscle, heart	Exon skipping	18806224 (2008) 18545222 (2008) 18784278 (2008) 19692354 (2009) 20700113 (2010) 19815563 (2010) 21505427 (2011) 22683468 (2012)
Duchene muscular dystrophy	Mouse	Vivo	Human dystrophin transgene/skeletal muscle	Exon skipping	21611204 (2011)
Duchene muscular dystrophy	Mouse	Unmodified	Dystrophin/skeletal muscle	Exon skipping	16444267 (2006) 16285002 (2006) 19469709 (2009) 21102560 (2010)
Duchene muscular dystrophy	Mouse	Vivo	Dystrophin/skeletal muscle	Multiple exon skipping	22869723 (2012) 25647512 (2014)
Duchene muscular dystrophy	Dog	Vivo	Dystrophin/skeletal muscle	Multiple exon skipping	22888777 (2012)
Duchene muscular dystrophy	Human	Unmodified	Dystrophin/skeletal muscle	Exon skipping	19713152 (2009) 21784508 (2011) 23907995 (2013) 26086759 (2015)
Facioscapulohumeral muscular dystrophy	Mouse	Vivo	PITX1/skeletal muscle	Translation blockage	24232919 (2014)
Duchene muscular dystrophy	Mouse	CPP	Dystrophin, myostatin/skeletal muscle	Exon skipping	24942628 (2014) 25959011 (2015)
Spinal muscular atrophy	Mouse	Unmodified, Vivo	SMN2/skeletal muscle	Modification of splicing	24781211 (2014) 24636820 (2014)

Disease/process	Organism	Type of PMO modification	Target gene/tissue	Mechanism	PMID (Year)
Cancer					
Cancer/breast cancer	Mouse	Vivo	STAT3/tumor	Modification of splicing	22006329 (2011)
Acute lymphoblastic leukemia	Mouse	Vivo	Gfi1/tumor	Translation blockage	23410974 (2013)
Squamous cell carcinoma	Mouse	Vivo	IL-10/immune cells	Translation blockage	25990221 (2015)
Nijmegen Breakage Syndrome	Mouse	Vivo	Nibrin/tumor	Modification of splicing	26265251 (2015)
Colon cancer	Mouse	Vivo	Fli-1/tumor	Modification of splicing	25534570 (2015)
Eye pathology					
Corneal angiogenesis	Mouse	Unmodified, Vivo	Fli-1/corneal	Modification of splicing	22199251 (2012) 22438952 (2012)
Corneal angiogenesis	Mouse	Vivo	KDR/corneal	Modification of splicing	22997228 (2013)
Heart diseases					
Dilated cardiomyopathy (DCM)	Mouse	Vivo	TTN/heart	Exon skipping	25759365 (2015)
Modulation of physiological processes					
Energy efficiency	Mouse	Vivo	K _{ATP} channel/skeletal muscle	Translation blockage	25648265 (2015)
Physical activity	Mouse	Vivo	DRD1, Vmat2, Glut4/skeletal muscle, brain	Translation blockage	23630592 (2013)
Physical activity	Mouse	Vivo	Anxa6, Casq1/skeletal muscle	Translation blockage	24505100 (2014)
Alcohol seeking	Mouse	Vivo	Orexin/hypothalamus	Translation blockage	24806225 (2014) 25329297 (2014)
Other genetic disorders					
Pompe disease	Mouse	CPP	Gys1/skeletal muscle	Exon skipping	25350581 (2014)
X-linked agammaglobulinemia	Mouse	CPP	BTK/B lymphocytes	Exon skipping	25105368 (2014)
Huntington's disease	Mouse	Unmodified	HTT/brain	Translation blockage	25035419 (2014)

Abbreviations: PKM2 - pyruvate kinase; UGT1A1 - UDP glucuronosyltransferase 1 family polypeptide A1; CRH - corticotropin-releasing hormone; Pah - phenylalanine hydroxylase; PITX1 - paired-like homeodomain transcription factor 1; STAT3 - signal transducer and activator of transcription 3; Gfi1 - growth factor independence 1; IL-10 - interleukin 10; Fli-1 - vascular endothelial growth factor receptor 1; KDR - vascular endothelial growth factor receptor 2; TTN - titin; KATP channel - ATP-sensitive potassium channel; DRD1 - dopamine receptor D1; Vmat2 - vesicular monoamine transporter 2; Glut4 - glucose transporter type 4; Anxa6 - annexin 6; Casq1 - calsequestrin 1; Gys1 - muscle specific glycogen synthase; BTK - Bruton's tyrosine kinase; HTT - huntingtin;