Journal of Drug Discovery, Development and Delivery

Open Access

Austin Publishing Group

Special Article - Antisense Drug Research and Development

Guide for Morpholino Users: Toward Therapeutics

Moulton JD*
Gene Tools, LLC, USA

***Corresponding author:** Moulton JD, Gene Tools, LLC, 1001 Summerton Way, Philomath, Oregon 97370, USA

Received: January 28, 2016; **Accepted:** April 29, 2016; **Published:** May 03, 2016

Abstract

Morpholino oligos are uncharged molecules for blocking sites on RNA. They are specific, soluble, non-toxic, stable, and effective antisense reagents suitable for development as therapeutics and currently in clinical trials. They are very versatile, targeting a wide range of RNA targets for outcomes such as blocking translation, modifying splicing of pre-mRNA, inhibiting miRNA maturation and activity, as well as less common biological targets and diagnostic applications. Solutions have been developed for delivery into a range of cultured cells, embryos and adult animals; with development of a non-toxic and effective system for systemic delivery, Morpholinos have potential for broad therapeutic development targeting pathogens and genetic disorders.

Keywords: Splicing; Duchenne muscular dystrophy; Phosphorodiamidate morpholino oligos; Internal ribosome entry site; Nonsense-mediated decay

Morpholinos: Research Applications, Therapeutic Promise

Morpholino oligos bind to complementary sequences of RNA and get in the way of processes. Morpholino oligos are commonly used to prevent a particular protein from being made in an organism or cell culture. Morpholinos are not the only tool used for this: a protein's synthesis can be inhibited by altering DNA to make a null mutant (called a gene knockout) or by interrupting processes on RNA (called a gene knockdown). Some DNA alterations cause production of a protein to decrease without stopping all production; confusingly, these are also called gene knockdowns. DNA alterations are permanent, while knockdowns of RNA are either transient, generally last several days after dosing with antisense (such as Morpholinos), or are long-term, depending on continued production of knockdown RNA in cells (such as shRNA transcribed in cells from a plasmid).

Once introduced into cells, Morpholinos freely diffuse between the cytosol and nuclear compartments and bind complementary sequences of RNA. Morpholinos have been used as gene knockdown reagents in cell cultures and in animals, bacteria, protists, plants and fungi. The RNA-blocking property of Morpholinos has also been used to cause a range of different outcomes beyond simple gene knockdowns. Morpholinos have different effects depending on the sort of target they bind. Morpholinos can:

- Block ribosome assembly and stop translation of a protein from an mRNA;
- Bind splice junctions and deny access to the small nuclear Ribonuclear Proteins (snRNPs) that mark the junctions for spliceosomes, altering splicing;
- Bind to precursors of miRNA, inhibiting the maturation of the miRNA;
- Bind to mature miRNA, inhibiting the activity of the miRNA;
- Bind to miRNA recognition elements on mRNA, relieving the transcript from miRNA regulation;
- Block regulatory proteins from binding to RNA, shifting alternative splicing;
- Block association of RNAs with cytoskeletal motor protein complexes, preventing RNA translocation;
- Inhibit poly-A tailing of pre-mRNA;
- Trigger frame shifts at slippery sequences;
- Invade RNA secondary structure, changing its conformation;
- Serve as guide sequences for RNase-P activity; or
- Block activity of a ribozyme.

Morpholinos have been broadly used in the developmental biology community to knock down genes in embryos of organisms such as zebrafish (Danio rerio), African clawed frogs (Xenopus sp.), chicks (Gallus gallus), sea urchins (e.g. Strongylocentrotus sp.), sea squirts (Ciona sp.), and many more. The oligos are usually microinjected though fine glass needles into early embryos at the one-to-few cell stage. Many kinds of antisense have toxic effects during development of an embryo. Because Morpholinos have little interaction with protein they are unusually non-toxic antisense, sufficiently non-toxic to make them the first choice of most developmental biologists for transient gene knockdowns. In contrast, injection of oligos containing phosphorothioate intersubunit linkages often kills embryos [1]. Morpholinos are highly specific antisense, having less interaction with unintended RNAs than antisense which employs protein activity; this is because a Morpholino must be complementary to a longer sequence of RNA than antisense using catalytic activity (e.g. RNAi, phosphorothioate RNA, etc.) [2]. Less specific antisense causes changes in gene expression during development of the embryo and can cause developmental defects (teratogenesis) [3].

If you want to use an antisense oligo for a therapeutic, non-toxicity and specificity are excellent characteristics with which to

Moulton. © All rights reserved

Citation: Moulton JD. Guide for Morpholino Users: Toward Therapeutics. J Drug Discov Develop and Deliv. 2016; 3(2): 1023.

start. Morpholinos are also reasonably water soluble, with most oligos staying in solution for many months to years at 1mM concentration in room temperature water; this gives them a great advantage over another kind of potential therapeutic antisense, the Peptide Nucleic Acids (PNA) [4]. Clinical trials conducted by Sarepta Theraeputics Inc. (formerly AVI BioPharma Inc.) have shown excellent safety of the oligos in humans (Table 1).

Efficacy of Morpholino oligos in humans has been shown in clinical trials for Duchenne muscular dystrophy. The splice-modifying Morpholino eteplirsen has partially restored function to the dystrophin protein, enough to show significant clinical benefit on a six-minute walk test [5] *versus* the untreated control group.

Introduction to the Molecules

Morpholino oligos are uncharged analogs of nucleic acids. Typical Morpholino oligos are from 18-30 subunits long, with a nucleic acid base on each subunit. They are used to alter gene expression by binding to complementary targets on RNA molecules and blocking processes. The interactions of Morpholinos with proteins are very weak so they are not cleaved by nucleases and have little effect on cells apart from their RNA binding activity. The structure, design and characteristics of Morpholino oligos have been reviewed elsewhere [6].

Morpholino oligos are referred to by a variety of names and acronyms. We usually call them Morpholinos, but in the literature they are called Phosphorodiamidate Morpholino Oligos (PMO), Morpholino Oligos (MO), Morpholino Antisense Oligos (MASO) and Neu-genes. Variations include delivery-enabled Vivo-Morpholinos, photocleavable Photo-Morpholinos, and cell-penetrating Peptide-conjugated Phosphorodiamidate Morpholino Oligos (PPMO and pip-PMO).

Mechanism of Action

Antisense oligos are molecules that bind to RNA by base pairing and change gene expression. The region of RNA complementary to an antisense oligo is called a target. The sequence of an mRNA is called the sense sequence.

Some antisense oligos rely on the activity of other molecules such as proteins for their effects on gene expression. An example is phosphorothioate DNA, which interacts with both target RNA and RNase-H; the RNase-H recognizes the antisense-DNA pair and cleaves the RNA. Another form of antisense requiring activity of an enzyme is RNAi (including siRNA and shRNA), which uses the protein activity in RISC to cleave its complementary target RNA or to inhibit the translation of some partially complementary mRNAs.

Morpholino antisense acts differently. Morpholinos do not require the activity of a protein in order to affect gene expression, nor do they degrade their target RNA. Instead, a Morpholino is like masking tape for an RNA target sequence: it binds to its RNA target and sticks there, getting in the way of other molecules that might otherwise bind to or move past the target site. In some locations on RNA, binding a Morpholino oligo can change gene expression. Because a Morpholino can alter gene expression just by sticking in the appropriate place and getting in the way of other molecules, it is considered a form of steric blocking antisense. To emphasize that it does not need enzymatic activity to affect gene expression, it is referred to as RNase-H independent antisense. Some other oligo types, such as 2'-O-methyl phosphorothioate, peptide nucleic acid, and locked nucleic acid oligos, are also steric blocking, RNase-H independent antisense.

Benefits of Morpholino Structure

Lack of electrostatic charge

We hypothesize that the lack of protein interaction with Morpholinos is due to their lack of electrostatic charge. Nucleic acids interact with proteins through relatively weak hydrogen bonding and stronger charge-charge interactions. Proteins that have evolved to bind nucleic acids stabilize binding by attraction between cationic amino acids of the protein and the anionic charge on the backbone phosphates of the nucleic acid, along with weaker hydrogen-bonding interactions. Morpholinos arriving at the active site of a nucleic-acid binding protein will not be held by the charge-charge interactions because the Morpholino backbone has no charge. This leaves hydrogen bonding, which is insufficient force to hold a Morpholino in the catalytic site of a nuclease or bind it into a Toll-like receptor.

Morpholinos do not bind to serum, which fits with our hypothesis that the Morpholino's lack of electrostatic charge prevents protein binding. Morpholino-serum interaction was assessed by surface plasmon resonance spectroscopy and no significant binding was detected (Hong Moulton, Pers. Comm). As a result, unmodified Morpholino oligos have a short serum half-life relative to other oligo types. While the exposure time of cells to Morpholino in the blood is relatively short, the oligos are free in solution and available for cell interactions as they are not associated with serum protein.

Stability

The complete resistance of Morpholinos to nucleases has been shown in a study exposing the oligos to a range of nucleases and proteases for up to five hours in cell-free enzyme systems [7] and by exposure to liver lysates [8]. No degradation of oligos was detected in these tests. Later work exposed Morpholino-peptide conjugates to serum and living cells. After varying incubation times, the Morpholinos were extracted and assessed by MALDI-TOF spectrometry. While intermediate and complete degradation products of the peptide component were detected, no degradation of the Morpholino component was detected [9].

Lack of innate immune response

Most other oligo types trigger innate immune responses by binding to Toll-Like Receptors (TLR). For example, double-stranded RNA triggers antiviral responses, including interferon production, by binding to TLR. When Morpholinos were tested for interferon induction and B cell activation, none was detected (A. Kreig, Pers. Comm. to J. Summerton) [2]. Since then, no reports of innate immune stimulation by Morpholinos have since appeared in over 15 years of commercial production as research reagents.

Non-toxicity

A Morpholino oligo's lack of protein binding removes a mode of toxicity that has been a problem for some other antisense types. In particular, oligos bound by a phosphorothioate intersubunit linkage interact with proteins though their backbone sulfurs. The stickiness of phosphorothioates to proteins results in toxic side effects [10] that are lacking in the uncharged Morpholinos.

The common use of Morpholinos in developmental systems such as embryos of zebrafish, African clawed frogs, chicks and sea urchins demonstrates their non-toxicity. While siRNA and phosphorothioate oligos have been widely available, it is Morpholinos that have been favored by developmental biologists because they are less toxic and teratogenic than other knockdown agents. The many thousands of papers in developmental biology that report experiments with Morpholinos (cataloged in the publication database at pubs.gene-tools.com) attest to the relative advantage of these oligos over other knockdown reagents.

While the backbone of a Morpholino is very nontoxic, there is some risk of toxicity with any new sequence of Morpholino oligo. This can be due to the oligo finding an unexpected partially-or-fully complementary site on a region of an unexpected RNA where the oligo can alter a biological process, such as in the 5’-UTR or at a splice junction. The unexpected interactions of runx3 oligos with histone proteins was explored in sea urchin; unexpected knockdown of the histone proteins interfered with mitosis [11]. Sequence toxicity can usually be avoided by searching genome and transcriptome sequence databases for mostly-complementary targets, using software tools like BLAST. In development of a therapeutic Morpholino, it would be prudent to test a candidate oligo against primary cells as well as using bioinformatic methods. During first-in-human trials of a new sequence, additional safety could be provided by making a Morpholino complementary to the putative drug Morpholino; this complementary oligo could be administered as an antidote should unexpected sequence toxicity arise, as has been shown for steric-blocking phosphorothioate drugs [12].

Applications: What Can You Do With A Morpholino?

A Morpholino’s function is simply to base-pair with a complementary base sequence. What happens next depends on where the target sequence lies within RNA (we’ll assume the Morpholino binds tightly enough to its target). If the target lies in the upstream Untranslated Region (5’-UTR) of an mRNA, cap-dependent translation is blocked and the protein encoded on that mRNA is not made. If the target lies across enough intron sequence at the splice junction of an mRNA, the pattern of splicing will be altered and the mRNA produced will be different from the mRNA in an untreated cell. Other sites on RNA can be blocked, including miRNAs, miRNA targets, active sites on ncRNA, translocation protein-binding sequences, ribozyme active sites and more. The only difference between a Morpholino designed to block translation and a Morpholino designed to block splicing or other sites is the sequence of the bases in the oligo; other modifications like fluorescent tags or delivery moieties might be added, but what the Morpholino does once it is in the cell is a function of the oligo’s base sequence.

Block ribosome assembly by blocking arrival of initiation complex

The common sort of mRNA translation in animal cells is cap-dependent. This means that the small subunit of the ribosome and various initiation factors bind to the 5’-cap of the mRNA. After rearrangements involving some initiation factors leaving and others joining, the small ribosomal subunit (as part of an initiation complex) travels along the mRNA downstream (toward the 3’ end) until it encounters a start codon with the appropriate sequence neighborhood (e.g. Kozak sequence) to start translation. The initiation complex pauses and loses initiation factors, the large subunit of the ribosome docks with the small subunit and the first tRNA is bound to its codon. Translation proceeds downstream on the mRNA until a stop codon triggers dissociation of the ribosomal subunits and release of the new polypeptide.

If the mature ribosome (with both large and small subunits) encounters a Morpholino oligo bound in the protein-coding region of an mRNA, the ribosome pushes past the Morpholino, melting the oligo off of the RNA. Translation proceeds as if the Morpholino were never there (unless this happens at a “slippery sequence” and triggers a frameshift [13]).

If, however, the initiation complex (with just the small ribosomal subunit) encounters a Morpholino oligo bound to the 5’-UTR or the start of the coding region, the Morpholino halts the initiation complex’s progress toward the start codon. A reasonable way to think of a translation-blocking Morpholino is that it is a ribosome assembly inhibitor; without getting past the Morpholino, the small subunit can’t reach the mature ribosome’s assembly site at the start codon.

Target region: Because a Morpholino can block an initiation complex but not a mature ribosome, the target region for a translation-blocking Morpholino extends from the 5’-cap to the start codon. The oligo can extend into the coding region, but one end must bind to or overlap the start codon. This requirement was shown using a hepatitis B leader sequence in a cell-free translation system. Oligo targets were chosen in the region at and flanking the start codon. Morpholinos that covered the stop codon or bound upstream of the start codon blocked translation, while if the oligo bound coding sequence and a few bases were left uncovered downstream of the start, the oligo lost translation-blocking activity [14].

There is usually plenty of 5’-UTR sequence to find a few good Morpholino targets. The inability of a Morpholino to block the mature ribosome in the coding region turns out to be an advantage for making oligos that are specific for their targets within a transcriptome. Once a target is chosen in the 5’-UTR through start-of-coding, if there is a nearly-identical target in the coding region of a different RNA, even if the Morpholino associates with that coding-region target it is unlikely to affect translation of the corresponding protein (unless it binds a slippery sequence and triggers a frameshift, an unlikely outcome).

Sometimes an Internal Ribosome Entry Site (IRES) is in the 5’ sequence of an mRNA. An initiation complex can form at an IRES and proceed from that site downstream to a start codon, where with the appropriate flanking sequence (e.g. Kozak sequence) a mature ribosome can form. If a Morpholino is bound to sequence 5’ of the IRES, it will not interrupt translation from the IRES. However, if the Morpholino is bound between the IRES and the start, or across the start codon, the oligo can halt the progression of the initiation complex and prevent the formation of a mature ribosome on that mRNA. Because of this, the first translation-blocking Morpholino designed against a new sequence is usually designed as close to the start codon as possible, decreasing the likelihood that IRES between the oligo and the start codon will allow translation to proceed unblocked.

Some transcripts can be translated from several possible start sites. The discussion here assumes that there are no IRES in the two-start-site transcript. A Morpholino bound between two start sites blocks translation from the downstream start site. If the oligo target is spaced a dozen bases or so downstream of the upstream start codon, the oligo should not interfere with translation from the upstream start site. However, if the oligo is bound upstream of both start sites, then the oligo should halt translation from both of the splice sites

Splice modifying

Morpholinos can be designed to alter splicing of pre-mRNA. There are several classes of target sites that can cause changes in splicing. These include the snRNP binding sites in the introns near splice junctions (splice donors and splice acceptors) and the binding sites of splice-regulatory proteins. Most splice-modifying oligos use splice-junction targets. This is probably because database support for locating splice junction sequence is very good; in particular the Ensembl database's Exon display is convenient for splice junction oligo design <www.ensembl.org>. Various prediction algorithms for splice-regulatory targets often disagree and it is common practice to run several algorithms and target their consensus sites, which might or might not successfully alter splicing. While regulatory-site oligos can produce strong splice alteration and have been exploited in pharmaceutical-Morpholino design [15], the greater likelihood that several oligos must be attempted to find a good splice-regulatory-targeted oligo compared with the more reliable targeting of splice-junction oligos means that for now those targeting splice junctions are the dominant oligos used for research.

Blocking splice junctions: Either the upstream (5') end of an intron (the splice donor site) or the downstream (3') end of an intron (the splice acceptor site) can be targeted with a Morpholino. Typically at least 15 bases of intronic sequence are targeted, with the oligo target either abutting or overlapping the exonic sequence. Splice-targeted oligos can be designated by the transcript name and the exon and intron numbers that make up their splice junction: for instance, an e4i4 oligo targets the splice donor site between exon 4 and intron 4. Targeting the very first (e1i1) splice site in a transcript typically results in inclusion of intron 1 in the mature transcript; similarly, targeting the very last (i[*n*-1]e[*n*]) splice junction in an n-exon transcript typically results in inclusion of the last intron (intron *n*-1). Targeting any other splice site (collectively, the internal splice sites) is most likely to result in splicing out the exon abutting the splice junction, thus excluding that exon from the mature mRNA. For example, targeting the i2e3 junction usually results in a mature mRNA in which exon 2 is directly ligated to exon 4, with exon 3 missing. This exclusion of an exon from a mature mRNA is sometimes called "exon skipping".

Range of possible outcomes: skip, 2x skip, intron inclusion, and cryptic site: The expected outcomes described above will not always occur. Sometimes, when an internal exon is targeted, instead of the adjacent exon being excised from that RNA the adjacent intron is included. Sometimes a cryptic splice site is activated, directing splicing to an unexpected location; a cryptic splice site in an exon results in a partial exon excision, while a cryptic splice site in an intron results in a partial intron inclusion. If a cryptic splice site is activated by blocking a splice junction with a Morpholino, a typical outcome is for several kinds of transcripts to be made, some with the expected splice outcome for the oligo target and some with the product of the cryptic site activation. Another possible but relatively rare outcome is for a single splice-junction targeted oligo to cause several exons to be skipped.

Triggering nonsense-mediated decay by frameshift: The Nonsense-Mediated Decay (NMD) system detects mRNAs with premature termination codons and degrades them. After splicing, proteins are left on mRNAs at each exon-exon junction. These proteins are stripped off as a ribosome passes. If an mRNA is translated and one or more exon-exon marker proteins are left bound to the transcript, this triggers nucleolytic decay of the mRNA by the NMD system. Splice-targeted Morpholinos can be designed to cause NMD. When an exon is skipped, this might frameshift the sequence downstream and bring a premature termination codon in-frame. Because the ribosome dissociates from the mRNA at a termination codon, any exon-exon marker proteins remaining downstream of the termination codon can cause activation of NMD. To cause a frameshift, an oligo is targeted to an exon that has a number of bases that cannot be evenly divided by three. This alters the reading frame of exons downstream of the skipped exon, altering the sequence of amino acids translated and likely bringing new termination codon(s) in-frame. Skipping an exon with a number of bases that is evenly divided by three (sometimes called a cassette exon) will not frameshift downstream sequence.

Designing for NMD: When knockdown of protein activity is the experimental goal of a splice-modifying experiment, the most upstream exon is sought with several desirable properties: (1) the base count is not evenly divisible by three so skipping the exon will cause a frameshift, and (2) there is a splice junction with a good Morpholino target site that will make a soluble and optimal-affinity oligo without stable secondary structure. This transcript will go through transcription before NMD is activated, but the early frameshift should ensure that most of the amino acid sequence produced does not have the same activity as the original (wild-spliced) protein. Note that if an important activity of the protein is encoded upstream of the skipped exon, some of that protein moiety will be produced before each new transcript is decayed by NMD.

Current clinical trials of splice-targeted morpholinos for Duchenne MD: Morpholino oligos are undergoing clinical trials for Duchenne Muscular Dystrophy (DMD), a condition caused by mutations in the DMD gene encoding dystrophin protein. This is an especially large gene with many spliceoforms with up to 79 exons. People with DMD typically have deletions, duplications, nonsense mutations or splice mutations so that functional dystrophin protein is not produced. The DMD gene is on the X chromosome, so usually it is boys who are affected; the incidence is about 1 in 3500 male births. Symptoms appear through childhood, leading to loss of ambulation (usually by early teens) and eventually early death by heart or respiratory failure. Splice-targeted Morpholinos are being developed to treat DMD, starting with an oligo designated eteplirsen which is designed to skip exon 51 of the full-length DMD transcript.

The mutations causing DMD are often frameshift mutations. In many populations, the most common whole-exon deletion is deletion of exon 50. Deletion of exon 50 frameshifts downstream sequence. The experimental drug eteplirsen, a Morpholino oligo in Phase 3 clinical

trials, triggers exclusion of exon 51 from the dystrophin transcript. Skipping exon 51 restores the reading frame, producing a transcript missing exons 50 and 51 but otherwise encoding the dystrophin protein. Production of such a protein, missing the peptide domains encoded on exons 50 and 51 but otherwise intact, has improved the clinical outcomes of eteplirsen-treated boys when compared to natural history data from untreated DMD boys in terms of their performance in a six-minute walk test [5]. Many other frameshifting mutations can potentially be treated with the appropriate splice-targeted oligos, with trials commenced for oligo targeting exons 45 and 53 (Table 1). Skipping non-frameshifting exons may be useful in treating nonsense mutations; skipping a cassette exon which contains a stop codon can in principle restore near-full-length translation of dystrophin (with the peptide domain encoded by the missing and skipped exons missing from the protein).

MiRNA inhibition

The maturation and activity of a micro-RNA can be inhibited using Morphoino oligos. Activity can be blocked by directly targeting the miRNA guide strand or by binding a Morpholino across the MicroRNA Response Element (MRE) of an mRNA. Maturation is targeted by binding a Morpholino to the precursor miRNA, preventing one or both of the two double-stranded cleavage events needed for production of the mature double-stranded RNA form of the miRNA [16].

Blocking the guide strand: The guide strand of a miRNA is typically about 21 bases in length. A Morpholino complementary to the guide strand might bind either to the miRNA precursor or to the guide strand associated with the Ribonucleic Acid Induced Silencing Complex (RISC). It is not known if one or both of these mechanisms cause the miRNA repression observed when a Morpholino complementary to a guide strand (and some flanking precursor bases) is introduced into cells.

Blocking the pre/pri-miRNA hairpin: A Morpholino oligo can bind the precursor of a miRNA and prevent the double-stranded nucleolytic cleavage of enzymes such as Drosha and Dicer, inhibiting the maturation of a miRNA. This has been shown by northern blot, confirming the accumulation of the precursor miRNA when nucleolytic processing is inhibited with a Morpholino [16]. If an oligo is desired which binds to many members of a miRNA family, an oligo can be targeted across the guide strand and some flanking sequence (miRNA guide strands average about 21 bases, while Morpholinos are usually synthesized as 25-mers); because the guide sequence is mostly conserved within a miRNA family, this oligo should bind most or all members. In contrast, the loop region of an miRNA and the stem on the far side of the guide strand are under less selective pressure and are far less conserved, so if an oligo is desired which targets primarily one member of an miRNA family then the oligo can be designed complementary to the loop or complementary to the stem but mostly avoiding the guide strand sequence. Of course, it is not always possible to make a good oligo for a particular target type such as a guide-only target or a loop only target because of unfavorable motifs that can appear in Morpholinos and decrease their activity (e.g. tetra-G sequences, stable self-complementarities, etc.).

MiRNA target protection: A Microrna Response Element (MRE) is the site where a miRNA binds to an mRNA and represses translation, typically found in the 3'-UTR of an mRNA. If a Morpholino is bound across an MRE, the Morpholino can release the mRNA from regulation by the miRNA [17]. If translation of the mRNA is being repressed by the miRNA, blocking their interaction results in increased translation of the mRNA.

Blocking other non-coding RNA

Non-coding RNA (ncRNA) can alter gene expression by several described mechanisms, including interaction with mRNA by base pairing [18]. Morpholinos have been used to study the functions of long non-coding RNA by targeting the oligos to conserved sequences and splice junctions [19] and by preventing viral ncRNA from interacting with a transcript [20].

ncRNA has been found to be essential for DNA replication. Known as Y RNA in vertebrate systems and stem-bulge RNA in Caenorhabditis elegans, the roles of these ncRNAs have been defined by using antisense Morpholinos [21, 22].

Small nucleolar RNA (snoRNA) guide modification of ribosomal RNA (rRNA) and small nuclear RNA and are implicated in human disease. Morpholinos that inhibit splicing and maturation of snoRNAs reduced the snoRNA-guided methylation of rRNA [23].

Translocation inhibition

By masking sites on RNA where motor protein adapters bind, a Morpholino can prevent the directed translocation of the RNA. Directed translocation is needed in Xenopus oocytes for successful maturation of Xenopus laevis embryos. Annealing a Morpholino to the site on RNA where an E1r-type protein would otherwise bind prevented translocation of the mRNAs bearing that binding site to the vegetal pole of the oocyte [24]. A Morpholino that disrupted a localization element stem-loop needed for IoLR5 RNA translocation was shown to prevent that translocation in a snail embryo [25]. A Morpholino targeting a dorsal localization element of the zebrafish sqt50 RNA prevented directed localization of that RNA [26].

RNase P guiding

RNase P is a ribozyme that uses complementary base pairing to direct its catalytic activity to a substrate RNA. The Altman lab has developed a technique for replacing the base-pairing recognition sequence of the ribozyme with a separate Morpholino oligo serving as a guide strand. Using this technique they have targeted mRNAs in Plasmodium protists [27] as well as in bacteria [28].

Unusual targets: viral cyclization, ribozyme activity, slippery sequences, poly-A signals, repeats

The flexibility of Morpholinos for targeting other RNA activities has been demonstrated by many other instances that have been reported in just a few papers each. A Morpholino can block the cyclization sequence of an influenzavirus or a flavivirus, inhibiting their replication [29]. The active site of a Schistosome Sm1 ribozyme can be blocked by a Morpholino [30]. Some sequences on mRNAs termed "slippery sequences" are more prone to spontaneous translational frameshifts; binding a Morpholino at a slippery sequence can increase the probability of a frameshift event [13]. The inhibition of RNA polyadenylation either through the binding of a Morpholino to the signal sequence or the cleavage site led to down regulation of a potential therapeutic target [31]. Morpholinos targeted to pathologic

repeated elements in RNA corrected the defects by preventing deleterious protein-RNA interactions [32].

Diagnostic applications

***In situ* hybridization:** A Morpholino oligo can be used as an antisense probe for in situ hybridization. A Morpholino with a carboxyfluorescein fluorescent tag can be recognized by an anti-fluorescein antibody, allowing signal amplification using antibody-conjugated enzymatic activity [33].

Molecular beacons: Morpholinos have been employed as molecular beacons by attaching a quencher at one end and a fluorochrome at the other end of the oligo. Oligo sequences were designed so that the ends were complementary and a stem-loop formed, bringing the fluor and quencher in close proximity. When the oligo bound a target RNA, the fluor and quencher were pulled away from each other, increasing the fluorescent signal [34].

Surface-bound probes: Morpholinos can be attached to solid surfaces as sequence-specific capture probes [35]. A particularly interesting application of surface-hybridized Morpholinos is their use in electronic detection of nucleic acid sequences. When an uncharged Morpholino attached to a surface binds to a complementary negatively-charged nucleic acid, this changes the dielectric constant of the surface and can be detected as a change in the capacitance at the surface [36].

Pretargeting for nuclear medicine theragnostics

Pretargeting is a strategy for delivery of radioisotopes to tumors with minimal collateral damage to healthy tissues. This involves using a Morpholino oligo conjugated to an antibody targeting a tumor marker and a second, complementary Morpholino conjugated to a radioisotope.

When a radioisotope is attached to an antibody, the isotope-bearing antibody can bind to a cancer marker on a tumor and concentrate radioactivity at that location, either for signal generation for cancer diagnostics or to deliver damaging radioactivity to the tumor as a therapeutic strategy. However, because antibodies are very large molecules they diffuse relatively slowly and are slowly passed though the kidney. This can lead to undesirable radiation doses delivered to healthy tissues.

A Morpholino oligo conjugated to an antibody can remain in the blood and tissues for an extended time without causing damage. During this time some antibodies can bind to tumors while others are excreted and then concentration in blood and non-cancerous tissue drops very low. If a complementary Morpholino conjugated with a radioisotope is administered, the radioactive Morpholino conjugate binds to its complement on the antibody with high affinity but then remaining radioactive Morpholino conjugate is rapidly excreted through the kidneys. This relatively rapid clearance of the Morpholino-radioisotope conjugate decreases the radiation dose delivered to non-cancerous tissue compared to the dose that would be delivered if the radioisotope was conjugated directly to the antibody. This reduced radiation dose may benefit both therapeutic and diagnostic applications of radioisotopes in humans [37].

Pretargeting for aggregating selected fluid-mosaic components

When the CD20 marker is concentrated in one area of the cell membrane, the cell undergoes apoptosis. Apoptosis can be artificially stimulated by binding CD20 with an antibody conjugated to a Morpholino and then adding a polymeric scaffold bearing many copies of a Morpholino sequence complementary to the Morpholino on the antibody. The scaffold binds first to one antibody, then to more antibodies as they diffuse nearby through the fluid mosaic of the cell membrane. The polymeric scaffold gathers more and more of the antibody-Morpholino conjugates, artificially concentrating the CD20 molecule in one region until apoptosis is triggered [38].

Assays and Controls (How Do You Know It Works?)

The activity of a Morpholino can sometimes be assessed by phenotypic change of the treated cells or organism, but it is always desirable to correlate the phenotypic outcome with a molecular assay. Different molecular assays are used depending on what sort of target strategy is used for the Morpholino.

Assessing the activity of translation blocking

When a Morpholino translation-blocking oligo is used, it is targeted to a sequence at or upstream of the start codon and prevents the initiation complex from reaching the start codon. When a Morpholino binds to the mRNA this binding can stabilize, destabilize or not affect the stability of the mRNA to nucleases. Since the Morpholino itself doesn't cleave the mRNA, this range of possible effects on RNA stability means that the lifetime and concentration of the mRNA can be increased, decreased or unaffected by translation-blocking Morpholino treatment. Because of this unpredictability, assessing the abundance of the transcript targeted by a translation-blocking Morpholino won't provide much information about oligo activity.

Assessing the amount of the target mRNA's protein product can reliably show the activity of a Morpholino. The concentration of the targeted mRNA's protein product is usually assessed by an immunochemical method. Western blots are preferred as they give mass information and can help confirm specificity of an antibody, but an ELISA assay can also be developed for a particular protein (generally after initial assessment of antibody specificity by Western blot).

Timing considerations are critical for analyzing the outcome of a translation-blocking experiment. When a translation-blocking oligo is put into a cell, there is usually some preexisting protein present. The translation-blocker stops production of new protein from the blocked mRNA, but immediately assaying for the protein product of the blocked mRNA would not show significant decrease in protein concentration. Only after some time passes will the protein level drop though the typical degradation of the protein. If you know the turnover rate of the protein of interest, this can help in determining how long you should wait between dosing and measurement of the protein concentration. Many proteins which are rapidly turned over, such as most transcription factors, can be assessed 24 to 48 hours after dosing and by then the protein signal will have nearly disappeared. If an mRNA encoding a fairly stable protein is targeted, it might be

necessary to re-dose with Morpholino oligo periodically to maintain suppression of the synthesis of the stable protein long enough for substantial decrease in the protein signal.

Another strategy for assessing the efficacy of a translation blocking Morpholino is to combine the Morpholino target sequence with a reporter gene (e.g. GFP) on an expression plasmid. If the reporter signal can be decreased by Morpholino treatment, this suggests that the Morpholino can also decrease expression of the endogenous mRNA target. However, the transcription rate of the reporter gene and the endogenous gene are likely different and this can affect the efficacy of the knockdown. Morpholinos are most effective at knocking down transcripts that are moderately expressed. A highly expressed endogenous transcript, such as actin, can rapidly bind to the free oligo and soon deplete the pool of available Morpholinos. Similarly, a reporter plasmid under control of a strong promotor, such as a cytomegalovirus promotor, can produce transcript enough to deplete the free Morpholino pool rapidly. If a reporter plasmid strategy is to be used, it is prudent to design the plasmid with a fairly weak promotor so that the Morpholino knockdown can persist long enough to detect clearly the decrease in reporter protein signal. As with translation blocking of endogenous proteins, in some experiments it takes time for the reporter protein levels to decrease enough to detect the signal; to avoid this deliver the Morpholino and the reporter plasmid simultaneously into cells or organisms, so that the signal from the reporter is repressed before it has time to accumulate.

Assessing the activity of splice modification

To assess activity of a splice modifying oligo at the molecular level, Reverse Transcriptase PCR is used to amplify sequences where the oligo is expected to cause changes and the PCR product masses are measured by gel electrophoresis. To detect exclusion of a single exon from a mature mRNA, primers are typically designed to the flanking exons. Primers should be set far enough back from the splice junction so that if the exon is excluded, the PCR product will still be long enough that it can take up enough dye to be visible on the electrophoretic gel; 100 bases is an easily-visible length. If an oligo is designed to bind mostly-intronic sequence at either of the splice junctions of exon 5, start by considering primers in exons 4 and 6. If exons 4 and 6 are too short to design primers that will produce a ~100-base product when exon 5 is missing, move one exon farther away and reassess the primer design (for instance try exon 3 and 6, then if that is too short try 3 and 7, etc.). Once you have a set of primers designed, run the PCR assay of treated and untreated cells (or embryos etc.) and compare the masses of the PCR products from treated and untreated samples on an electrophoretic gel, loading the wells with the same amount of RNA. Include the PCR product from a housekeeping gene amplified from treated and untreated samples so that the signal from the targeted transcript can be normalized to the housekeeping gene to control for differences in loading.

While most splice-modifying Morpholinos cause single exons to be excluded from the mature mRNA, there are a range of other outcomes which can occur. These other possible outcomes include intron inclusion, double exon skipping, partial intron inclusion (intronic cryptic splice site activation), and partial exon excision (exonic cryptic splice site activation). If an oligo produces an unexpected outcome, using more PCR primers to test these possibilities can often help determine the actual outcome of using the splice-modifying oligo (e.g. intronic primers, distant primers). Another useful technique to help understand unexpected results is to sequence PCR products, which can reveal activation of a cryptic splice site too close to the normal splice site to resolve easily on an electrophoretic gel.

If a splice modification activates the Nonsense-Mediated Decay (NMD) system, a PCR product might not appear at the expected mass after oligo treatment. Many splice-modifying oligos are intentionally designed to trigger NMD as a strategy for achieving a stronger knockdown of protein activity. Frameshift due to exon exclusion can bring a premature termination codon in-frame and cause rapid decay of the splice-modified transcript. In this case, activity of the splice modifying oligo can only be seen as dimming of the PCR band at the mass produced by the transcript not treated with a Morpholino. Comparing the intensity of the PCR product mass of untreated and treated samples is more reliable if you can also compare the intensities of the housekeeping gene's PCR product, so while the housekeeping gene band might be less useful for experiments where you see a clear mass-shifted signal it becomes very important for experiments where NMD has occurred.

Assessing inhibition of miRNA maturation

Binding a Morpholino into the stem-loop precursor of a miRNA can inhibit the maturation of the miRNA because once the Morpholino has invaded the RNA stem-loop it is no longer the double-stranded RNA substrate for nucleases which cleave miRNA precursors to produce the mature forms. The interrupted maturation of a miRNA can be assessed with a northern blot, where the immature band is less mobile (longer) than that of the mature miRNA duplex [16].

Assessing inhibition of miRNA activity

The activity of a miRNA can be assessed by constructing an expression plasmid that produces a transcript encoding a reporter gene with a miRNA response element in the 3'-UTR positioned to repress translation of the transcript. Cells containing this construct produce little of the reporter protein unless the activity of the appropriate miRNA is repressed [39]. A simpler approach is to measure expression of a protein from an mRNA known to have its translation repressed by the miRNA of interest; delivering a Morpholino to block a guide miRNA directly, to block maturation of the guide miRNA or to block the miRNA response element should all result in an increase in the expression of the mRNA's protein product.

Testing mRNA specificity: mispaired oligos and the two non-overlapping oligo control experiment

Morpholino oligos can cause effects by interaction with partially-complementary RNAs [11]. To determine whether this is a factor for a given Morpholino sequence, it is important to run a specificity control experiment.

In the early 1990s, the typical specificity control was to use a Morpholino with mispairs introduced into the sequence. In embryos, if the mispaired oligo did not produce the phenotype associated with the target RNA's fully-complementary Morpholino (the targeting oligo), this was taken as an indication that the targeting oligo was

specifically interacting with its RNA target. I will argue later that this was a conceptually weak control, but Morpholino users were first driven from this control by practical problems. The earliest mispaired controls commercially offered were four-mispair oligos, but these often produced the phenotype of their targeting oligo. Two steps improved this situation: first, the controls were shifted to five mispairs; second, it was emphasized that the minimum dose (or culture medium concentration) should be determined where the targeting oligo causes its phenotype, then the mispair control oligo should be used at that same dose (or culture medium concentration). Still, Morpholino users sometimes reported the phenotype of the targeting oligo was appearing with the five mispair oligo, while others observed occurrence of entirely different phenotypes (likely due to interaction of the five mispair oligos with untargeted RNAs).

A knockdown specificity experiment is a test of the hypotheses that the observed outcome of administering a knockdown oligo is due to the interaction of the targeting oligo against its complementary RNA target and is not due to interaction with an unexpected RNA. The mispair experiment does not directly address this question.

Another solution to testing Morpholino specificity was proposed in a meeting of the zebrafish model community and has since become a widely-used specificity control strategy; this is the two-nonoverlapping-oligo experiment. Two Morpholinos designed against different targets on the same RNA are used in separate experiments. If the same phenotype is produced by either of the oligos, this directly supports the hypothesis that the phenotypic outcome is due to knocking down the targeted RNA and not due to interaction with an unexpected RNA. We could expect to see the two oligos produce the same phenotype from an off-target interaction only if the off-target RNA (1) contained two sequences nearly-identical to each of the targeted RNA's oligo target sequences and (2) was located in regions of the RNA where binding a Morpholino alters gene expression. It is unlikely that both of these conditions would be met, though there is increased risk between recently duplicated genes.

Another characteristic of Morpholinos that can be used to strengthen the two-nonoverlapping-oligo specificity control experiment is dose synergy. When two translation-blocking Morpholinos target the same mRNA, their combination has greater-than-additive efficacy. This presents another opportunity to confirm specificity of a knockdown [40].

Knocking down zebrafish p53 to suppress p53 response phenotype: Zebrafish embryos can develop successfully without functional p53. Loss of certain proteins causes some cells to undergo apoptosis, a response to protein loss mediated by p53. Inhibiting the production of p53 prevents phenotypes associated with the p53 response to loss of some proteins. In zebrafish, a p53 translation-blocking oligo should be coinjected with each new targeting Morpholino to determine whether the resulting phenotype is due only to loss of the targeted protein or is due to the p53-mediated response to the loss of that protein [41].

Delivery

Unmodified Morpholino oligos do not readily diffuse through the plasma membranes of most cells. This presents an experimental hurdle for achieving good antisense activity, because the RNA targets for translation-blocking or splice-modifying oligos are within the cytosol and nuclear compartments of cells. Techniques have been developed to deliver Morpholinos into cells in cell cultures, animal embryos, adult animals, plants, protists, fungi and prokaryotes. Most Morpholino work has been done in animal embryos, especially in systems where the Morpholino can be microinjected into the egg or very early zygote and so is present in the daughter cells of the injected cell.

Microinjection: Microinjection is the injection of material across a membrane, directly into a cell or into a yolk. When unmodified Morpholinos targeting either splicing or translation are microinjected into cells, both translation blocking (active in the cytosol) and splice-modifying (active in the nucleus) oligos have activity, demonstrating that the oligos will pass the nuclear membrane. Observations of fluorescently-tagged Morpholinos in cultured cells confirm the distribution of oligos through both cytosol and nucleus [42].

Zebrafish are especially amenable to Morpholino microinjection because their cells are interpermeable to Morpholinos and other large molecules during very early development. Dye studies with fluoresceinated dextran and with Lucifer yellow have shown that injection into a single cell of a zebrafish embryo at the 1, 2, 4, or 8 cell stage results in distribution of the dye thoughout the embryo, with some interpermeability persisting through the 32 cell stage [43-45]. This permeability extends to exposure of the embryonic cells to compounds dissolved in the yolk, so Morpholinos can be injected into the early yolk and then enter cells until interpermeability is lost.

Xenopus, the African clawed frog, does not share the period of interpermeability characteristic of zebrafish. Injection into one cell of the two-celled Xenopus zygote results in Morpholino activity in half of the frog, allowing experimental design using convenient contralateral controls [46].

Other organisms that are microinjected with Morpholinos into eggs or early embryos include sea urchins and sea stars, ascidians (such as Ciona sp.), various teleost fish, mice, and occasionally nematodes (such as Caenorhabditis elegans). The only reported use of Morpholinos in plants involved microinjection into oocytes, requiring an innovative laser-activated microinjection system to overcome turgor pressure [47].

Electroporation: Electroporation allows delivery into organized tissues especially in late embryonic systems. Electroporation has been the delivery method of choice in embryonic chicken, especially for delivery into the neural tube [48]. Electroporation has also been used with embryos of zebrafish [49] and Xenopus sp [50]. Such delivery into tissues usually involves injection followed by electroporation to permeabilize the cells near the injection site. Electroporation has been reported in various cultured cells. Some protists, notably Giardia sp., are typically dosed with electroporation [51]. Delivery to protoplasts of the fungi Mucor mucedo and Mucor circinelloides was reported using electroporation [52].

Bare versus delivery-enabled oligos: Vivo-Morpholinos, peptide conjugates: Morpholino oligos do not readily cross most plasma membranes. This has presented the greatest remaining challenge to the development of Morpholino oligos as therapeutic

drugs. Studies using techniques like microinjection have shown the efficacy of Morpholinos if they are in the cytosol & nuclear compartments, but unmodified Morpholinos injected into the blood of healthy mice have little systemic effect.

In order to improve the potential therapeutic value of Morpholinos, chemical moieties were developed which, when covalently conjugated with the oligos, would improve uptake of the oligos by cells in tissues. Early studies with natural cell-penetrating peptides such as HIV Tat showed the promise of this approach [53]. Soon more effective delivery moieties were found, notably the peptoid $(RXR)_4$, which is comprised of arginine and aminohexanoic acid [54]. Cell penetrating peptides and peptoids are collectively abbreviated as CPP. Morpholino-CPP conjugates have been used for many studies in virology [29]. Muscle-targeting CPP moieties have been used in mouse models of muscular dystrophy [55]. A Morpholino-CPP conjugate targeting c-myc, designed to prevent scarring after cardiac interventions, has been in clinical trial (clinicaltrials.gov ID: NCT00451256)

Vivo-Morpholinos are a widely-used and commercially available delivery moiety consisting of an eight-branched dendrimer carrying a guanidinium moiety at each branch tip. Like the guanidinium of an arginine in a cell-penetrating peptide, the guanidinium of the Vivo-Morpholino's dendrimer helps the oligo conjugate reach the cytosol/nuclear compartment [56].

The mechanism of cell entry has been studied for the arginine-rich CPP-Morpholino conjugates. At lower concentrations the delivery is energy-dependent, temperature-dependent and decreased by endocytosis inhibitors [57] though at higher concentrations there is some temperature- and energy-independent delivery and so it is thought to involve a combination of endosomal escape and direct plasma membrane penetration.

Delivery-enhanced Morpholinos in use up to the beginning of 2016 involve some toxicity conferred by the delivery moiety. In particular the arginines of the arginine-rich cell-penetrating peptides and peptoids confer both enhanced delivery and increased toxicity as their number per Morpholino is increased. The dose of a Vivo-Morpholino must be adjusted to the window between onset of useful antisense activity and unacceptable toxicity. For mice undergoing tail-vein injection, a dose of 12.5 mg/mg lies within this window for many Vivo-Morpholino sequences.

Delivery into the brain: Morpholinos can be introduced into the cerebrospinal fluid, typically by intracerebroventricular infusion [58] or i.c.v. injection [59] in mice, where the unmodified oligos were reported to have antisense activity. Osmotic pumps have been used to infuse Morpholinos into the brain over time [60]. Most recent studies have used Vivo-Morpholino oligos when dosing the brain with Morpholinos [61].

Local injection: Delivery-enabled Morpholinos (Vivo-Morpholinos, CPP conjugates) can be injected into specific tissues [62] or can target the viscera more generally by intraperitoneal injection [63]. Local injections can provide higher concentrations in small regions, concentrations that cannot be reached by systemic delivery due to dose-limiting toxicity.

Cell bathing: Whether in culture or *in vivo*, most cells do not readily take up unmodified Morpholino oligos from the extracellular solution. There are examples of useful levels of unassisted delivery of unmodified Morpholino into the cytosol & nuclear compartment of cells after bathing in an oligo solution, but these are unusual cases; for example, the previously mentioned infusions into mouse brains [58], uptake by explants of the embryonic mouse pancreas around stage E11 [64] and entry of unmodified Morpholinos into skeletal muscle cells after injections into the blood of mdx mice, a mouse model of Duchenne muscular dystrophy [65]. These are cases where uptake is facilitated by slow turnover of extracellular fluid, by unusual permeability of a limited cell type, or by the pathology of a disease. Delivery to most cell cultures by cell bathing requires exposure to high extracellular concentrations of Morpholino oligo for a long period.

Endo-Porter assistance (endosomal release): Endo-Porter is a peptide that was developed to facilitate the delivery of Morpholinos into cultured cells. Endo-Porter is an endosomal release agent; it is uncharged at neutral pH but becomes cationic when protonated in the low pH of the late endosome. Endo-Porter releases the contents of the acidic endosome into the cytosol; this has been shown for Morpholinos [66] as well as oxygen-responsive fluorescent probes [67]. Endo-Porter is poorly soluble in water but is soluble in DMSO. Cultured cells are bathed with fresh medium containing the cargo to be delivered (e.g. Morpholinos) and Endo-Porter solution, usually as a formulation in DMSO, is added to the culture medium. When the Endo-Porter enters the aqueous medium it aggregates into particles; the culture should be swirled immediately to disperse the Endo-Porter and limit the size of the aggregates. Endo-Porter particles will settle slowly to the bottom of the culture container, bringing them into contact with adherent cells. If suspension cultures are to be delivered with Endo-Porter, the cultures should be gently and continuously swirled (as on a slow orbital shaker) to keep the particles in suspension [68].

Vivo-MO in cell cultures: Vivo-Morpholino, with their octa-guanidinium delivery moieties, offer convenient delivery to most cultured cells. The delivery moiety brings the Morpholinos though membranes so the process of delivery in cultured cells is very simple: pipet the Vivo-Morpholino solution into the cultures. Vivo-Morpholinos are typically formulated at 0.5mM in phosphate-buffered saline. To reach a concentration of 10 microMolar in a culture, 1 part Vivo-Morpholino solution is added to 49 parts of medium, or 20 microliters Vivo-Morpholino into 980 microliters of medium for 1ml final solution.

Vivo-MO bath-immersion of zebrafish embryos: Zebrafish embryos have been incubated in a solution containing Vivo-Morpholinos to successfully knock down a target gene [69]. While this technique has not been widely employed, it might offer a low-cost alternative to purchasing a microinjection apparatus for labs doing limited embryo experimentation.

Other Lipofection Agents: While the cationic lipids in lipofection agents can form electrostatic complexes with natural nucleic acids, they cannot electrostatically complex with Morpholino oligos. Still, some groups have reported achieving Morpholino delivery with lipofection reagents alone [70] or by annealing a Morpholino with a partially-complementary DNA oligo and delivering the heteroduplex

Table 1: Clinical trials using Morpholino oligos active or recruiting at the start of 2016.

Study	Sponsor
Exploratory Study of NS-065/NCNP-01 in DMD Condition: Duchenne Muscular Dystrophy Drug: NS-065/NCNP-01 Phase: 1	Sponsor: National Center of Neurology and Psychiatry, Japan Collaborator: Nippon Shinyaku Co Ltd. ClinicalTrials.gov ID: NCT02081625 Active, not recruiting
Dose-Titration and Open-label Extension Study of SRP-4045 in Advanced Stage Duchenne Muscular Dystrophy (DMD) Patients Condition: Duchenne Muscular Dystrophy Drug: SRP-4045 Phase: 1 & 2	Sponsor: Sarepta Therapeutics ClinicalTrials.gov ID: NCT02530905 Active, not recruiting
Safety Study of Eteplirsen to Treat Advanced Stage Duchenne Muscular Dystrophy Condition: Duchenne Muscular Dystrophy Drug: eteplirsen (AVI-4658) Phase: 2	Sponsor: Sarepta Therapeutics ClinicalTrials.gov ID: NCT02286947 Active, not recruiting
Efficacy, Safety, and Tolerability Rollover Study of Eteplirsen in Subjects With Duchenne Muscular Dystrophy Condition: Duchenne Muscular Dystrophy Drug: eteplirsen (AVI-4658) Phase: 2	Sponsor: Sarepta Therapeutics ClinicalTrials.gov ID: NCT01540409 Active, not recruiting
Safety Study of Eteplirsen to Treat Early Stage Duchenne Muscular Dystrophy Condition: Duchenne Muscular Dystrophy Drug: eteplirsen (AVI-4658)	Sponsor: Sarepta Therapeutics ClinicalTrials.gov ID: NCT02420379 Recruiting
Phase I/II Study of SRP-4053 in DMD Patients Condition: Duchenne Muscular Dystrophy Drug: SRP-4053 Phase: 1 & 2	Sponsor: Sarepta Therapeutics ClinicalTrials.gov ID: NCT02310906 Recruiting
Confirmatory Study of Eteplirsen in DMD Patients Condition: Duchenne Muscular Dystrophy Drug: eteplirsen (AVI-4658) Phase: 3	Sponsor: Sarepta Therapeutics ClinicalTrials.gov ID: NCT02255552 Recruiting

with a lipofection reagent [30]. In the 1990s Morpholinos were available in the Special Delivery system, which combined a Morpholino bound to a partially-complementary DNA oligo with a separate vial of Ethoxylated Polyethyleneimine (EPEI). The anionic DNA and cationic EPEI formed electrostatic complexes which could enter cultured cells, carrying along Morpholino oligos associated with the DNA oligos by base pairing [71].

In vivo therapeutic application: Duchenne muscular dystrophy

Eteplirsen is a Morpholino oligo that causes exclusion of exon 51 of the human dystrophin transcript. In clinical trials continuing for over four years, eteplirsen has been administered to boys with frameshift mutations causing Duchenne muscular dystrophy whose dystrophin reading frame can be restored by skipping exon 51. The slight restoration of dystrophin expression measured in these trials is thought to depend on pathology of the disease, the leakiness of the dystrophic muscle to large molecules. Though their 6-minute walking distances are declining slowly, the boys have retained more walking ability than a natural-history group to which they are being compared; [5] maintaining a sham-dosed placebo-control group for this progressive, fatal disease over a period of years was not considered ethical. A new sequence for skipping exon 53 and another for exon 45 are entering clinical trials (Table 1).

Handling and Storage

In lyophilized form, Morpholinos have been stored for 15 years and found to have good activity when dissolved. Some sequences have maintained good activity over years in aqueous solution. However, some sequences lose biological activity over time while maintaining UV absorbance (265nm) in the solution, while the UV activity of other sequences has been found to decline over time.

Chilling Morpholino solutions and especially putting the oligo solutions though repeated cycles of freezing and thawing, increases the chance that oligos will associate with the walls of the solution container. In this case the UV activity of the solution will decline over time. If the solution is removed from the container and the container is washed with a small quantity of 0.1N HCl, the missing UV activity can be found dissolved in the HCl solution.

If stored in aqueous solution at room temperature, some Morpholino sequences will maintain their biological activity over time while the activity of other sequences will decline. Solutions of soluble and problematic Morpholinos were assessed by size-exclusion HPLC and then stored for months. When the solutions were tested again on the size-exclusion HPLC system, the problematic sequences showed evidence of aggregation. After autoclaving the problematic sequence using the liquid cycle, the aggregates were mostly gone and the signal from the soluble sequence had grown. This shows that some oligos can complex in solution over time and that autoclaving can restore the single-stranded state of some problematic sequences (Hong Moulton, Pers. Comm.).

Recommended storage

To avoid the container adsorption linked to chilling or freezing Morpholinos, the solutions can be kept at room temperature. A solution concentration of 1mM will not exceed the solubility of most Morpholino solutions. If biological activity of the oligo solution decreases over time, autoclave the stock solution. Morpholino solutions in poorly-sealed containers can evaporate over time. Most Morpholinos that were in solution and then have dried out will not subsequently dissolve well (the exception is lyophilized oligos, which have tremendous surface area). To reduce evaporation, oligos can be kept in a humidor. A humidor is prepared using an open beaker of water in a sealed container; good options for the container are a bell jar or a sealed dessicator with the dessicant replaced with the beaker of water. Oligos should be placed into the humidor in sealed containers, but if a container is leaky the saturated water vapor in the humidor should prevent evaporation. Another option for long-term storage is lyophilization, after which the oligos should remain stable for many years dry at room temperature.

Sterilizing

Morpholino solutions can be sterilized by autoclaving or by

filtration. Morpholinos can associate with many filter media; polysulfone membrane filters have little affinity for Morpholinos and are available as single-use cartridges for use with syringes.

Photosensitivity (Photo-MO, fluorescent tags)

With exposure to light over time, Morpholinos with fluorescent tags can photobleach and photocleavable Morpholinos (Photo-Morpholinos) can degrade. While these oligos are typically delivered in amber vials, it is prudent to store them in the dark (wrapping the vial with aluminum foil is an easy solution).

Mutants and morphants (why don't all morpholinos copy mutants?)

The outcome of a knockdown using a Morpholino oligo may not be the same as is observed in a homozygous mutant organism [72]. Several reasons have been identified for the divergent outcomes. First, genetic compensation has been found for some mutations which obscure the lost function of the mutation through changes in the expression of other genes; this compensation was not observed for two knockdown techniques, Morpholinos and CRISPRi, which had outcomes similar to one another [73]. CRISPR knockdowns which were performed and assessed in the same individuals (F0 gene modification) were found to phenocopy Morpholino knockdowns [74]. Ascertaining that a particular mutation is a true genetic null can be difficult [75]. A high dose of a Morpholino can cause additional phenotypes to appear, likely due to interaction with off-target RNA driven by the high dose [76]. The different time scales of CRISPR gene modification (often assessed generations after the gene modification) and Morpholinos (generally assessed within days of the treatment) might lead to differences through slow genetic regulatory changes, such as epigenetic change, associated with a multi-generational time scale. Knockdowns and mutants sometimes present distinct outcomes, but this should not surprise us due to their different modes of interrupting expression (at RNA *vs*. DNA) and their different time scales (days versus generations).

Therapeutic potential

Morpholino have several clear advantages over other antisense types (including Locked Nucleic Acids (LNA), Peptide Nucleic Acids (PNA), phosphorothioate oligos and their derivatives and interfering RNAs such as siRNA, shRNA etc.). They are (1) specific, (2) water soluble, (3) non-toxic, (4) stable and (5) effective. Their full potential remains to be developed because there is no available system for systemic delivery that is both non-toxic and highly efficacious; however, for diseases such as Duchenne muscular dystrophy, which confers permeability to large molecules to muscle cells, Morpholinos are already proving effective in clinical trials. Because relatively few sequences have been tried in the clinic and access to trial information is limited, the statements in this section will be somewhat speculative, based mostly on experience with Morpholinos as research reagents. Here are the advantages of Morpholinos as therapeutic molecules as I see them.

First, Morpholinos do not use catalytic systems for their activity (RISC, RNase-H, etc.), improving their specificity relative to catalysis-dependent antisense; a translation-blocking Morpholino requires about 13 to 14 bases of complementarity to inhibit about half of the expression of a protein [2], while 8 bases is sufficient for a RNase-H competent oligo or an interfering RNA to alter protein expression, so a Morpholino will significantly interact with far fewer targets in the transcriptome. This specificity is crucial for development of safe therapeutics. Each new sequence must be carefully assessed for off-target RNA interactions to ensure a safe therapeutic but, while off-target interactions are nearly certain for RNase-H competent oligos and interfering RNAs, off-target interactions are far less common for Morpholinos. Other steric-blocking oligos with higher per-base affinity, such as LNAs or PNAs, have shorter active subsequences than Morpholinos and so have more interactions with unintended RNAs than do Morpholinos [2].

Second, most Morpholinos are water soluble. Most sequences are soluble at 1 mM over long periods (many months to years) at room temperature. The aqueous solubility of a Morpholino oligo varies with the sequence of the oligo. High G content can decrease the solubility of a Morpholino and as the G content of oligos rises over 40% G the oligos become difficult or impossible to dissolve. Solubility cannot yet reliably be predicted, though insoluble oligos with low G content are often found to have significant self-complementarity. While low solubility oligos are unusual, in a pharmaceutical development context solubility is a factor to watch and if an oligo does not dissolve or stay in solution well, an alternative sequence should be used. Autoclaving just before use can ensure sterility as well as disrupt oligo complexes, improving the single-stranded Morpholino activity in some solutions.

Third, the backbone chemistry of Morpholino oligos is very non-toxic, with mice dosed at 3g/Kg without clear toxicity [77]. Specific sequences can be toxic through base-pairing interactions, so each candidate therapeutic sequence should be screened carefully for cytotoxicity and organismal toxicity. As an additional precaution for first-in-man studies, keeping a complementary Morpholino available as an antidote would be a prudent strategy for rapidly removing oligo activity from the blood and, over time, for inactivating oligo in cells.

Fourth, the stability of Morpholinos will make the delivery of the molecules to healthcare providers simple and inexpensive. Lyophilized Morpholinos are stable long-term, while soluble sequences in buffers can be stored at room temperature. Because Morpholinos do not degrade in cells, repeated administration should lead to a slow increase in the oligo concentration resident in cells until, over time, it may be possible to decrease oligo doses for treatment of chronic conditions as the oligo already resident in cells provides some persistent activity.

Fifth, Morpholinos are effective. They can be targeted for many modes of action, including translation blocking, splice modification, miRNA inhibition, etc. If targeting to block translation, some sequences can make the band corresponding to the targeted RNA's protein product disappear from a Western blot. Many papers using Morpholinos to target splicing have reported complete conversion of an PCR product from the wild-spliced mass to the exon-skipped mass. Targeting miRNA, some sequences inhibit maturation effectively enough to cause disappearance of their targeted miRNA.

As specific, water soluble, non-toxic, stable and effective antisense, Morpholino oligos are the best available oligo choice for therapeutic development.

Acknowledgement

I thank Alexandra Vincent for her extensive help and Jeremy Bushman for his suggestions and edits.

References

1. Woolf TM, Jennings CG, Rebagliati M, Melton DA. The stability, toxicity and effectiveness of unmodified and phosphorothioate antisense oligodeoxynucleotides in Xenopus oocytes and embryos. Nucleic Acids Res. 1990; 18: 1763-1769.
2. Summerton J. Morpholino antisense oligomers: the case for an RNase H-independent structural type. Biochim Biophys Acta. 1999; 1489: 141-158.
3. Mende M, Christophorou NA, Streit A. Specific and effective gene knock-down in early chick embryos using morpholinos but not pRFPRNAi vectors. Mech Dev. 2008; 125: 947-962.
4. Summerton J. Morpholinos and PNAs Compared In: Peptide Nucleic Acids, Morpholinos and Related Antisense Biomolecules. Landes Biosciences. 2004.
5. Mendell JR, Goemans N, Lowes LP, Alfano LN. Longitudinal effect of eteplirsen versus historical control on ambulation in Duchenne muscular dystrophy. Ann Neurol. 2016; 79: 257-271.
6. Moulton JD, Yan YL. Using Morpholinos to control gene expression. Curr Protoc Mol Biol. 2008; 2: 26.
7. Hudziak RM, Barofsky E, Barofsky DF, Weller DL, Huang SB, Weller DD. Resistance of morpholino phosphorodiamidate oligomers to enzymatic degradation. Antisense Nucleic Acid Drug Dev. 1996; 6: 267-272.
8. Summerton J, Weller D. Morpholino antisense oligomers: design, preparation, and properties. Antisense Nucleic Acid Drug Dev. 1997; 7: 187-195.
9. Youngblood DS, Hatlevig SA, Hassinger JN, Iversen PL, Moulton HM. Stability of cell-penetrating peptide-morpholino oligomer conjugates in human serum and in cells. Bioconjug Chem. 2007; 18: 50-60.
10. Krieg AM, Stein CA. Phosphorothioate oligodeoxynucleotides: antisense or anti-protein? Antisense Res Dev. 1995; 5: 241.
11. Coffman JA, Dickey-Sims C, Haug JS, McCarthy JJ, Robertson AJ. Evaluation of developmental phenotypes produced by morpholino antisense targeting of a sea urchin Runx gene. BMC Biol. 2004; 2: 6.
12. Crosby JR, Zhao C, Zhang H, MacLeod AR, Guo S, Monia BP. Reversing Antisense Oligonucleotide Activity with a Sense Oligonucleotide Antidote: Proof of Concept Targeting Prothrombin. Nucleic Acid Ther. 2015; 25: 297-305.
13. Howard MT, Gesteland RF, Atkins JF. Efficient stimulation of site-specific ribosome frameshifting by antisense oligonucleotides. RNA. 2004; 10: 1653-1661.
14. Summerton J, Weller D. Antisense properties of morpholino oligomers. Nucleosides Nucleotides. 1997; 16: 889-898.
15. Aartsma-Rus A, Houlleberghs H, Van Deutekom JC, Van Ommen GJ, T Hoen PA. Exonic sequences provide better targets for antisense oligonucleotides than splice site sequences in the modulation of Duchenne muscular dystrophy splicing. Oligonucleotides. 2010; 20: 69-77.
16. Kloosterman WP, Lagendijk AK, Ketting RF, Moulton JD, Plasterk RH. Targeted inhibition of miRNA maturation with morpholinos reveals a role for miR-375 in pancreatic islet development. PLoS Biol. 2007; 5: e203.
17. Choi WY, Giraldez AJ, Schier AF. Target protectors reveal dampening and balancing of Nodal agonist and antagonist by miR-430. Science. 2007; 318: 271-274.
18. Yoon JH, Abdelmohsen K, Gorospe M. Posttranscriptional gene regulation by long noncoding RNA. J Mol Biol. 2013; 425: 3723-3730.
19. Kurian L, Aguirre A, Sancho-Martinez I, Benner C, Hishida T, Nguyen TB, et al. Identification of Novel Long Noncoding RNAs Underlying Vertebrate Cardiovascular Development. Circulation. 2015; 131: 1278-1290.
20. Lee N, Moss WN, Yario TA, Steitz JA. EBV noncoding RNA binds nascent RNA to drive host PAX5 to viral DNA. Cell. 2015; 160: 607-618.
21. Collart C, Christov CP, Smith JC, Krude T. The mid-blastula transition defines the onset of Y RNA-dependent DNA replication in Xenopus laevis. Mol Cell Biol. 2011; 31: 3857-3870.
22. Kowalski MP, Baylis HA, Krude T. Non-coding stem-bulge RNAs are required for cell proliferation and embryonic development in C. elegans. J Cell Sci. 2015; 128: 2118-2129.
23. Higa-Nakamine S, Suzuki T, Uechi T, Chakraborty A, Nakajima Y, Nakamura M, et al. Loss of ribosomal RNA modification causes developmental defects in zebrafish. Nucleic Acids Res. 2012; 40: 391-398.
24. Arthur PK, Claussen M, Koch S, Tarbashevich K, Jahn O, Pieler T. Participation of Xenopus Elr-type proteins in vegetal mRNA localization during oogenesis. J Biol Chem. 2009; 284: 19982-19992.
25. Rabinowitz JS, Lambert JD. Spiralian quartet developmental potential is regulated by specific localization elements that mediate asymmetric RNA segregation. Development. 2010; 137: 4039-4049.
26. Gilligan PC, Kumari P, Lim S, Cheong A, Chang A, Sampath K. Conservation defines functional motifs in the squint/nodal-related 1 RNA dorsal localization element. Nucleic Acids Res. 2011; 39: 3340-3349.
27. Garg A, Wesolowski D, Alonso D, Deitsch KW, Ben Mamoun C, Altman S. Targeting protein translation, RNA splicing, and degradation by morpholino-based conjugates in Plasmodium falciparum. Proc Natl Acad Sci U S A. 2015; 112: 11935-11940.
28. Wesolowski D, Alonso D, Altman S. Combined effect of a peptide-morpholino oligonucleotide conjugate and a cell-penetrating peptide as an antibiotic. Proc Natl Acad Sci USA. 2013; 110: 8686-8689.
29. Stein DA. Inhibition of RNA virus infections with peptide-conjugated morpholino oligomers. Curr Pharm Des. 2008; 14: 2619-2634.
30. Yen L, Svendsen J, Lee JS, Gray JT, Magnier M, Baba T, et al. Exogenous control of mammalian gene expression through modulation of RNA self-cleavage. Nature. 2004; 431: 471-476.
31. Marsollier AC, Ciszewski L, Mariot V, Popplewell L, Voit T, Dickson G, et al. Antisense targeting of 3'end elements involved in DUX4 mRNA processing is an efficient therapeutic strategy for Facioscapulohumeral Dystrophy: a new gene silencing approach. Hum Mol Genet. 2016.
32. Wheeler TM, Sobczak K, Lueck JD, Osborne RJ, Lin X, Dirksen RT, et al. Reversal of RNA dominance by displacement of protein sequestered on triplet repeat RNA. Science. 2009; 325: 336-339.
33. Lagendijk AK, Moulton JD, Bakkers J. Revealing details: whole mount microRNA in situ hybridization protocol for zebrafish embryos and adult tissues. Biol Open. 2012; 1: 566-569.
34. Chen J, Wu J, Hong Y. The morpholino molecular beacon for specific RNA visualization *in vivo*. Chem Commun (Camb). 2016; 52: 3191-3194.
35. Wages JM, Wages GM, Matthews P, Weller D, Summerton J. Affinity purification of RNA: sequence-specific capture by nonionic morpholino probes. Biotechniques. 1997; 23: 1116-1121.
36. Tercero N, Wang K, Gong P, Levicky R. Morpholino monolayers: preparation and label-free DNA analysis by surface hybridization. J Am Chem Soc. 2009; 131: 4953-4961.
37. Liu X, Wang Y, Hnatowich DJ. A nanoparticle for tumor targeted delivery of oligomers. Methods Mol Biol. 2011; 764: 91-105.
38. Chu TW, Zhang R, Yang J, Chao MP, Shami PJ, Kope A, et al. A Two-Step Pretargeted Nanotherapy for CD20 Crosslinking May Achieve Superior Anti-Lymphoma Efficacy to Rituximab. Theranostics. 2015; 5: 834-846.
39. Romaker D, Kumar V, Cerqueira DM, Cox RM, Wessely O. MicroRNAs are critical regulators of tuberous sclerosis complex and mTORC1 activity in the size control of the Xenopus kidney. Proc Natl Acad Sci U S A. 2014; 111: 6335-6340.
40. Bill BR, Petzold AM, Clark KJ, Schimmenti LA, Ekker SC. A primer for morpholino use in zebrafish. Zebrafish. 2009; 6: 69-77.

41. Robu ME, Larson JD, Nasevicius A, Beiraghi S, Brenner C, Farber SA, et al. p53 activation by knockdown technologies. PLoS Genet. 2007; 3: e78.

42. Partridge M, Vincent A, Matthews P, Puma J, Stein D, Summerton J. A simple method for delivering morpholino antisense oligos into the cytoplasm of cells. Antisense Nucleic Acid Drug Dev. 1996; 6: 169-175.

43. Kimmel CB, Law RD. Cell lineage of zebrafish blastomeres. I. Cleavage pattern and cytoplasmic bridges between cells. Dev Biol. 1985; 108: 78-85.

44. Kimmel CB, Law RD. Cell lineage of zebrafish blastomeres. II. Formation of the yolk syncytial layer. Dev Biol. 1985; 108: 86-93.

45. Kimmel CB, Law RD. Cell lineage of zebrafish blastomeres. III. Clonal analyses of the blastula and gastrula stages. Dev Biol. 1985; 108: 94-101.

46. Nutt SL, Bronchain OJ, Hartley KO, Amaya E. Comparison of morpholino based translational inhibition during the development of Xenopus laevis and Xenopus tropicalis. Genesis. 2001; 30: 110-113.

47. Okuda S, Tsutsui H, Shiina K, Sprunck S, Takeuchi H, Yui R, et al. Defensin-like polypeptide LUREs are pollen tube attractants secreted from synergid cells. Nature. 2009; 458: 357-361.

48. Norris A, Streit A. Morpholinos: studying gene function in the chick. Methods. 2014; 66: 454-465.

49. Hirose K, Shiomi T, Hozumi S, Kikuchi Y. Mechanistic target of rapamycin complex 1 signaling regulates cell proliferation, cell survival, and differentiation in regenerating zebrafish fins. BMC Dev Biol. 2014; 14: 42.

50. Bestman JE, Huang LC, Lee-Osbourne J, Cheung P, Cline HT. An *in vivo* screen to identify candidate neurogenic genes in the developing Xenopus visual system. Dev Biol. 2015; 408: 269-291.

51. Paredez AR, Nayeri A, Xu JW, Krtkova J, Cande WZ. Identification of obscure yet conserved actin-associated proteins in Giardia lamblia. Eukaryot Cell. 2014; 13: 776-784.

52. John JEP. Down regulation of gene expression in Mucor mucedo and Mucor circinelloides by transformation with antisense morpholino oligonucleotides. Dissertation. Friedrich-Schiller-Univeristat Jena. 2012.

53. Moulton HM, Hase MC, Smith KM, Iversen PL. HIV Tat peptide enhances cellular delivery of antisense morpholino oligomers. Antisense Nucleic Acid Drug Dev. 2003; 13: 31-43.

54. Abes R, Moulton HM, Clair P, Yang ST, Abes S, Melikov K, et al. Delivery of steric block morpholino oligomers by (R-X-R)4 peptides: structure-activity studies. Nucleic Acids Res. 2008; 36: 6343-6354.

55. Coenen-Stass AML, McClorey G, Manzano R, Betts CA, Blain A, Saleh AF, et al. Identification of novel, therapy-responsive protein biomarkers in a mouse model of Duchenne muscular dystrophy by aptamer-based serum proteomics. Sci Rep. 2015; 5: 17104.

56. Morcos PA, Li Y, Jiang S. Vivo-Morpholinos: a non-peptide transporter delivers Morpholinos into a wide array of mouse tissues. Biotechniques. 2008; 45: 613-614, 616, 618 passim.

57. Abes S, Moulton HM, Clair P, Prevot P, Youngblood DS, Wu RP, et al. Vectorization of morpholino oligomers by the (R-Ahx-R) (4) peptide allows efficient splicing correction in the absence of endosomolytic agents. J Control Release. 2006; 116: 304-313.

58. Oh-I S, Shimizu H, Sato T, Uehara Y, Okada S, Mori M. Molecular mechanisms associated with leptin resistance: n-3 polyunsaturated fatty acids induce alterations in the tight junction of the brain. Cell Metab. 2005; 1: 331-341.

59. Oh-I S, Shimizu H, Satoh T, Okada S, Adachi S, Inoue K, et al. Identification of nesfatin-1 as a satiety molecule in the hypothalamus. Nature. 2006; 443: 709-712.

60. Silvestre DC, Gil GA, Tomasini N, Bussolino DF, Caputto BL. Growth of peripheral and central nervous system tumors is supported by cytoplasmic c-Fos in humans and mice. PLoS One. 2010; 5: e9544.

61. McMillin M, Frampton G, Quinn M, Ashfaq S, De Los Santos M, Grant S, et al. Bile Acid Signaling Is Involved in the Neurological Decline in a Murine Model of Acute Liver Failure. Am J Pathol. 2016; 186: 312-323.

62. Koganti SR, Zhu Z, Subbotina E, GAO Z, Sierra A, Proenza M, et al. Disruption of KATP channel expression in skeletal muscle by targeted oligonucleotide delivery promotes activity-linked thermogenesis. Mol Ther. 2015; 23:707-716.

63. Gramlich M, Pane LS, Zhou Q, Chen Z, Murgia M, Schotterl S, et al. Antisense-mediated exon skipping: a therapeutic strategy for titin-based dilated cardiomyopathy. EMBO Mol Med. 2015; 7: 562-576.

64. Prasadan K, Tulachan S, Guo P, Shiota C, Shah S, Gittes G. Endocrine-committed progenitor cells retain their differentiation potential in the absence of neurogenin-3 expression. Biochem Biophys Res Commun. 2010; 396: 1036-1041.

65. Wu B, Lu P, Benrashid E, Malik S, Ashar J, Doran TJ, et al. Dose-dependent restoration of dystrophin expression in cardiac muscle of dystrophic mice by systemically delivered morpholino. Gene Ther. 2010; 17: 132-140.

66. Summerton JE. Endo-Porter: a novel reagent for safe, effective delivery of substances into cells. Ann N Y Acad Sci. 2005; 1058: 62-75.

67. Sung HJ, Ma W, Wang PY, Hynes J, O'Riordan TC, Combs CA, et al. Mitochondrial respiration protects against oxygen-associated DNA damage. Nat Commun. 2010; 1: 5.

68. Mutyam V, Puccetti MV, Frisbie J, Goldstein DL, Krane CM. Endo-Porter mediated delivery of Phosphorodiamidate Morpholino Oligos (PMOs) in erythrocyte suspension cultures from Cope's gray treefrog Hyla chrysoscelis. BioTechniques. 2011; 50: 329-332.

69. Wong TT, Zohar Y. Production of reproductively sterile fish by a non-transgenic gene silencing technology. Sci Rep. 2015; 5: 15822.

70. Shimada Y, Kuninaga S, Ariyoshi M, Zhang B, Shiina Y, Takahashi Y, et al. E2F8 promotes hepatic steatosis through FABP3 expression in diet-induced obesity in zebrafish. Nutr Metab (Lond). 2015; 12: 17.

71. Morcos PA. Achieving efficient delivery of morpholino oligos in cultured cells. Genesis. 2001; 30: 94-102.

72. Kok FO, Shin M, Ni C-W, Gupta A, Grosse AS, Van Impel A, et al. Reverse Genetic Screening Reveals Poor Correlation between Morpholino-Induced and Mutant Phenotypes in Zebrafish. Dev Cell. 2015; 32: 97-108.

73. Rossi A, Kontarakis Z, Gerri C, Nolte H, Holper S, Kruger M, et al. Genetic compensation induced by deleterious mutations but not gene knockdowns. Nature. 2015; 524: 230-233.

74. Bhattacharya D, Marfo CA, Li D, Lane M, Khokha MK. CRISPR/Cas9: An inexpensive, efficient loss of function tool to screen human disease genes in Xenopus. Dev Biol. 2015; 408: 196-204.

75. Blum M, De Robertis EM, Wallingford JB, Niehrs C. Morpholinos: Antisense and Sensibility. Dev Cell. 2015; 35: 145-149.

76. Morcos PA, Vincent AC, Moulton JD. Gene Editing Versus Morphants. Zebrafish. 2015; 12: 319.

77. Wu B, Lu P, Benrashid E, Malik S, Ashar J, Doran TJ, Lu QL. Dose-dependent restoration of dystrophin expression in cardiac muscle of dystrophic mice by systemically delivered morpholino. Gene Ther. 2010; 17: 132-140.

J Drug Discov Develop and Deliv - Volume 3 Issue 2 - 2016
ISSN : 2471-0288 | www.austinpublishinggroup.com
Moulton. © All rights reserved

Citation: Moulton JD. Guide for Morpholino Users: Toward Therapeutics. J Drug Discov Develop and Deliv. 2016; 3(2): 1023.