EXON SKIPPING OLIGOMER CONJUGATES FOR MUSCULAR DYSTROPHY

RELATED APPLICATIONS

This application claims priority to U.S. Provisional Application No. 62/779,028, filed December 13, 2018. The entire teachings of the above-referenced application are incorporated by reference in their entirety.

REFERENCE TO SEQUENCE LISTING SUBMITTED ELECTRONICALLY VIA EFS-WEB

The content of the electronically submitted sequence listing (Name: 8171_50_WO00_SL.txt; Size: 10,080 bytes; Date of Creation: November 6, 2019) filed concurrently herewith is herein incorporated by reference in its entirety.

FIELD OF THE DISCLOSURE

The present disclosure relates to novel antisense oligomers suitable for exon 50 skipping in the human dystrophin gene and pharmaceutical compositions thereof. The disclosure also provides methods for inducing exon 50 skipping using the novel antisense oligomers, methods for producing dystrophin in a subject having a mutation of the dystrophin gene that is amenable to exon 50 skipping, and methods for treating a subject having a mutation of the dystrophin gene that is amenable to exon 50 skipping.

BACKGROUND OF THE DISCLOSURE

Duchenne muscular dystrophy (DMD) is caused by a defect in the expression of the protein dystrophin. The gene encoding the protein contains 79 exons spread out over more than 2 million nucleotides of DNA. Any exonic mutation that changes the reading frame of the exon, or introduces a stop codon, or is characterized by removal of an entire out of frame exon or exons, or duplications of one or more exons, has the potential to disrupt production of functional dystrophin, resulting in DMD.

A less severe form of muscular dystrophy, Becker muscular dystrophy (BMD) has been found to arise where a mutation, typically a deletion of one or more exons, results in a correct reading frame along the entire dystrophin transcript, such that translation of mRNA into protein is not prematurely terminated. If the joining of the upstream and downstream exons in the processing of a mutated dystrophin pre-mRNA maintains the correct reading

frame of the gene, the result is an mRNA coding for a protein with a short internal deletion that retains some activity, resulting in a Becker phenotype.

There is a need for antisense oligomers that are suitable for exon 50 skipping and corresponding pharmaceutical compositions that are useful for therapeutic methods for producing dystrophin and treating DMD.

SUMMARY OF THE DISCLOSURE

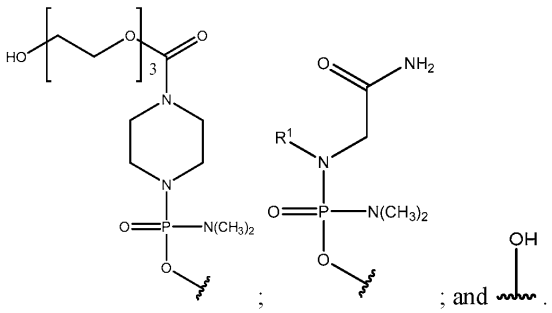
The antisense oligomers, or pharmaceutically acceptable salts thereof, are capable of binding a selected target to induce exon skipping in the human dystrophin gene, wherein the antisense oligomer comprises a sequence of bases that is complementary to an exon 50 target region of the dystrophin pre-mRNA designated as an annealing site, wherein the base sequence and annealing site are selected from:

Annealing Site	**Targeting Sequence [5' to 3']**	**SEQ ID NO:**
H50D(+04-18)	GGG ATC CAG TAT ACT TAC AGG C	SEQ ID NO: 1
H50D(+07-18)	GGG ATC CAG TAT ACT TAC AGG CTC C	SEQ ID NO: 2
H50D(+07-16)	GAT CCA GTA TAC TTA CAG GCT CC	SEQ ID NO: 3
H50D(+07-17)	GGA TCC AGT ATA CTT ACA GGC TCC	SEQ ID NO: 4
H50A(-19+07)	ACT TCC TCT TTA ACA GAA AAG CAT AC	SEQ ID NO: 5
H50D(+07-15)	ATC CAG TAT ACT TAC AGG CTC C	SEQ ID NO: 6
H50A(-02+23)	GAG CTC AGA TCT TCT AAC TTC CTC T	SEQ ID NO: 7
H50D(+06-18)	GGG ATC CAG TAT ACT TAC AGG CTC	SEQ ID NO: 8
H50D(+07-20)	ATG GGA TCC AGT ATA CTT ACA GGC TCC	SEQ ID NO: 9

wherein T is thymine or uracil. In one aspect, each T is thymine.

In some embodiments, each **Nu** from 1 to **n** and 5' to 3' corresponds to the nucleobases in one of the following: SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, or SEQ ID NO: 9. In some embodiments, each **Nu** from 1 to **n** and 5' to 3' corresponds to the nucleobases in SEQ ID NO: 3.

In one aspect, the antisense oligomer contains a **T** moiety attached to the 5' end of the antisense oligomer, wherein the **T** moiety is selected from:



; ; and .

In certain embodiments, the antisense oligomer is conjugated to one or more cell-penetrating peptides (referred to herein as "CPP"). In certain embodiments, one or more CPPs are attached to a terminus of the antisense oligomer. In certain embodiments, at least one CPP is attached to the 5' terminus of the antisense oligomer. In certain embodiments, at least one CPP is attached to the 3' terminus of the antisense oligomer. In certain embodiments, a first CPP is attached to the 5' terminus and a second CPP is attached to the 3' terminus of the antisense oligomer.

In some embodiments, the CPP is an arginine-rich peptide. The term "arginine-rich" refers to a CPP having at least 2, and preferably 2, 3, 4, 5, 6, 7, or 8 arginine residues, each optionally separated by one or more uncharged, hydrophobic residues, and optionally containing about 6-14 amino acid residues. As explained below, a CPP is preferably linked at its carboxy terminus to the 3' and/or 5' end of an antisense oligonucleotide through a linker, which may also be one or more amino acids, and is preferably also capped at its amino terminus by a substituent R^a with R^a selected from H, acyl, acetyl, benzoyl, or stearoyl. In some embodiments, R^a is acetyl.

As seen in the table below, non-limiting examples of CPP's for use herein include -$(RXR)_4$-R^a (SEQ ID NO: 15), -R-$(FFR)_3$-R^a (SEQ ID NO: 16), -B-X-$(RXR)_4$-R^a (SEQ ID NO: 17), -B-X-R-$(FFR)_3$-R^a (SEQ ID NO: 18), -GLY-R-$(FFR)_3$-R^a (SEQ ID NO: 19), -GLY-R_5-R^a (SEQ ID NO: 20), –R_5-R^a (SEQ ID NO: 21), -GLY-R_6-R^a (SEQ ID NO: 11) and –R_6-R^a (SEQ ID NO: 10), wherein R^a is selected from H, acyl, acetyl, benzoyl, and stearoyl, and wherein R is arginine, X is 6-aminohexanoic acid, B is β-alanine, F is phenylalanine and GLY (or G) is glycine. The CPP "R_5 (SEQ ID NO: 21)" is meant to indicate a peptide of five (5) arginine residues linked together via amide bonds (and not a single substituent *e.g.*, R^5 (SEQ ID NO: 21)). The CPP "R_6 (SEQ ID NO: 10)" is meant to

indicate a peptide of six (6) arginine residues linked together via amide bonds (and not a single substituent *e.g.*, R^6 (SEQ ID NO: 10)). In some embodiments, R^a is acetyl.

Exemplary CPPs are provided in **Table 1** (SEQ ID NOS: 10, 11, and 15-21).

Table 1: Exemplary Cell-Penetrating Peptides

Name	Sequence	SEQ ID NO:
R_6G	RRRRRRG	11
R_6	RRRRRR	10
$(RXR)_4$	RXRRXRRXRRXR	15
$(RFF)_3R$	RFFRFFRFFR	16
$(RXR)_4XB$	RXRRXRRXRRXRXB	17
$(RFF)_3RXB$	RFFRFFRFFRXB	18
$(RFF)_3RG$	RFFRFFRFFRG	19
R_5G	RRRRRG	20
R_5	RRRRR	21
R is arginine; X is 6-aminohexanoic acid; B is β-alanine; F is phenylalanine; G is glycine		

CPPs, their synthesis, and methods of conjugating to an oligomer are further described in U.S. Application Publication No. US 2012/0289457 and International Patent Application Publication Nos. WO 2004/097017, WO 2009/005793, and WO 2012/150960, the disclosures of which are incorporated herein by reference in their entirety.

In some embodiments, an antisense oligonucleotide comprises a substituent "Z," defined as the combination of a CPP and a linker. The linker bridges the CPP at its carboxy terminus to the 3'-end and/or the 5'-end of the oligonucleotide. In various embodiments, an antisense oligonucleotide may comprise only one CPP linked to the 3' end of the oligomer. In other embodiments, an antisense oligonucleotide may comprise only one CPP linked to the 5' end of the oligomer.

The linker within Z may comprise, for example, 1, 2, 3, 4, or 5 amino acids.

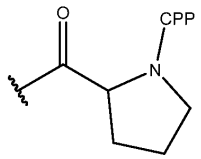
In particular embodiments, Z is selected from:

-C(O)$(CH_2)_5$NH-CPP;

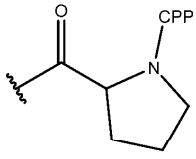
-C(O)$(CH_2)_2$NH-CPP;

-C(O)$(CH_2)_2$NHC(O)$(CH_2)_5$NH-CPP;

-C(O)CH_2NH-CPP; and the formula:

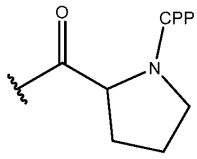


wherein the CPP is attached to the linker moiety by an amide bond at the CPP carboxy terminus.

In various embodiments, the CPP is an arginine-rich peptide as described herein and seen in Table 1. In various embodiments, the arginine-rich CPP is -R_5-R^a, (*i.e.*, five arginine residues; SEQ ID NO: 21), wherein R^a is selected from H, acyl, acetyl, benzoyl, and stearoyl. In certain embodiments, R^a is acetyl. In various embodiments, the CPP is SEQ ID NO: 21, and the linker is selected from the group consisting of -C(O)$(CH_2)_5$NH-, -C(O)$(CH_2)_2$NH-, -C(O)$(CH_2)_2$NHC(O)$(CH_2)_5$NH-, -C(O)CH_2NH-, and 

. In some embodiments, the linker comprises 1, 2, 3, 4, or 5 amino acids.

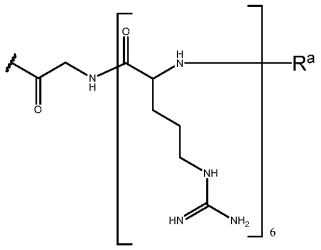
In some embodiments, the CPP is SEQ ID NO: 21 and the linker is Gly. In some embodiments, the CPP is SEQ ID NO: 20.

In certain embodiments, the arginine-rich CPP is -R_6-R^a, (*i.e.*, six arginine residues; SEQ ID NO: 10), wherein R^a is selected from H, acyl, acetyl, benzoyl, and stearoyl. In certain embodiments, R^a is acetyl. In various embodiments, the CPP is selected from SEQ ID NOS: 10, 15, or 16, and the linker is selected from the group consisting of -C(O)$(CH_2)_5$NH-, -C(O)$(CH_2)_2$NH-, -C(O)$(CH_2)_2$NHC(O)$(CH_2)_5$NH-, -C(O)CH_2NH-, and 

. In some embodiments, the linker comprises 1, 2, 3, 4, or 5 amino acids.

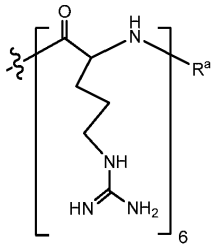
In some embodiments, the CPP is SEQ ID NO: 10 and the linker is Gly. In some embodiments, the CPP is SEQ ID NO: 11.

In certain embodiments, Z is -C(O)CH_2NH-R_6-R^a ("R_6" is disclosed as SEQ ID NO: 10) covalently bonded to an antisense oligomer of the disclosure at the 5' and/or 3' end of the oligomer, wherein R^a is H, acyl, acetyl, benzoyl, or stearoyl to cap the amino terminus of the R_6 (SEQ ID NO: 10). In certain embodiments, R^a is acetyl. In these non-limiting examples, the CPP is –R_6-R^a (SEQ ID NO: 10) and the linker is -C(O)CH_2NH-, (*i.e.* GLY). This particular example of Z = -C(O)CH_2NH-R_6-R^a ("R_6" is disclosed as SEQ ID NO: 10) is also exemplified by the following structure:



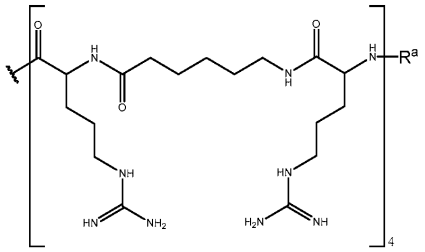
wherein R^a is selected from H, acyl, acetyl, benzoyl, and stearoyl. In some embodiments, R^a is acetyl.

In various embodiments, the CPP is -R_6-R^a (SEQ ID NO: 10), also exemplified as the following formula:

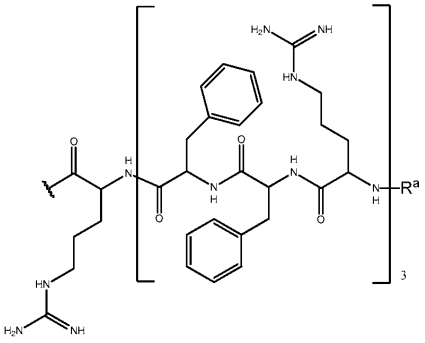


wherein R^a is selected from H, acyl, acetyl, benzoyl, and stearoyl. In certain embodiments, the CPP is SEQ ID NO: 11. In some embodiments, R^a is acetyl.

In some embodiments, the CPP is –(RXR)$_4$-R^a (SEQ ID NO: 15), also exemplified as the following formula:



In various embodiments, the CPP is –R-(FFR)$_3$-R^a (SEQ ID NO: 16), also exemplified as the following formula:



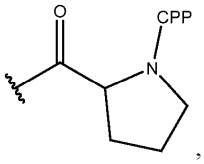
In various embodiments, Z is selected from:

-C(O)(CH$_2$)$_5$NH-CPP;

-C(O)(CH$_2$)$_2$NH-CPP;

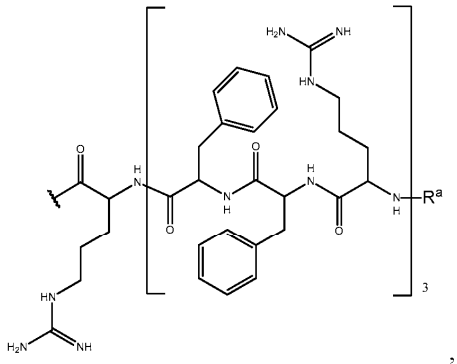
-C(O)(CH$_2$)$_2$NHC(O)(CH$_2$)$_5$NH-CPP;

-C(O)CH$_2$NH-CPP, and the formula:

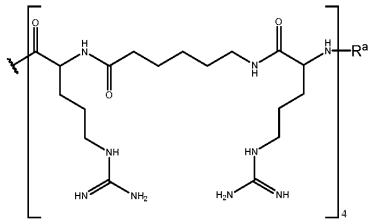


,

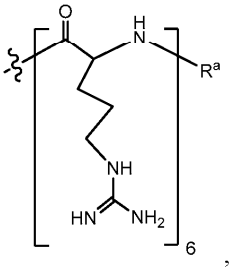
wherein the CPP is attached to the linker moiety by an amide bond at the CPP carboxy terminus, and wherein the CPP is selected from:



, (-R-(FFR)$_3$-R^a) (SEQ ID NO: 16),



, (-(RXR)$_4$-R^a) (SEQ ID NO: 15),



, and (-R$_6$-R^a) (SEQ ID NO: 10). In some embodiments, R^a is acetyl.

In some embodiments, each **Nu** from 1 to **n** and 5' to 3' corresponds to SEQ ID NO. 3.

In some aspects, the nucleobases of the modified antisense oligomer are linked to morpholino ring structures, wherein the morpholino ring structures are joined by phosphorous-containing intersubunit linkages joining a morpholino nitrogen of one ring structure to a 5' exocyclic carbon of an adjacent ring structure.

In some aspects, the nucleobases of the antisense oligomer are linked to a peptide nucleic acid (PNA), wherein the phosphate-sugar polynucleotide backbone is replaced by a flexible pseudo-peptide polymer to which the nucleobases are linked.

In some aspects, at least one of the nucleobases of the antisense oligomer is linked to a locked nucleic acid (LNA), wherein the locked nucleic acid structure is a nucleotide analog that is chemically modified where the ribose moiety has an extra bridge connecting the 2′ oxygen and the 4′ carbon.

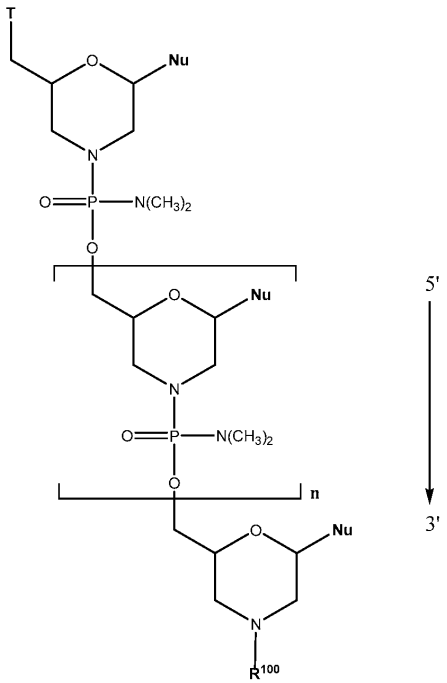
In some aspects, at least one of the nucleobases of the antisense oligomer is linked to a bridged nucleic acid (BNA), wherein the sugar conformation is restricted or locked by introduction of an additional bridged structure to the furanose skeleton. In some aspects, at least one of the nucleobases of the antisense oligomer is linked to a 2'-O,4'-C-ethylene-bridged nucleic acid (ENA).

In some aspects, the modified antisense oligomer may contain unlocked nucleic acid (UNA) subunits. UNAs and UNA oligomers are an analogue of RNA in which the C2′-C3' bond of the subunit has been cleaved.

In some aspects, the modified antisense oligomer contains one or more phosphorothioates (or S-oligos), in which one of the nonbridging oxygens is replaced by a sulfur. In some aspects the modified antisense oligomer contains one or more 2' O-Methyl, 2' O-MOE, MCE, and 2'-F in which the 2'-OH of the ribose is substituted with a methyl, methoxyethyl, 2-(N-methylcarbamoyl)ethyl, or fluoro group, respectively.

In some aspects, the modified antisense oligomer is a tricyclo-DNA (tc-DNA) which is a constrained DNA analog in which each nucleotide is modified by the introduction of a cyclopropane ring to restrict conformational flexibility of the backbone and to optimize the backbone geometry of the torsion angle γ.

In various aspects, the disclosure provides antisense oligomers according to Formula (I):

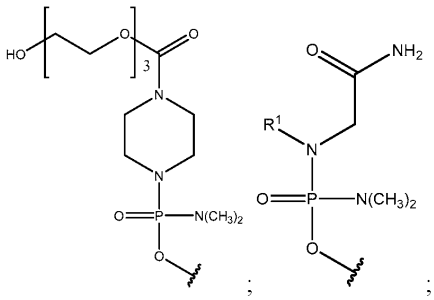


(I)

or a pharmaceutically acceptable salt thereof, wherein:

each **Nu** is a nucleobase, which taken together form a targeting sequence;

T is a moiety selected from:



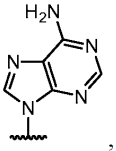
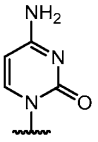
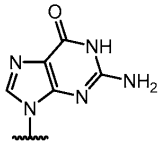
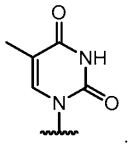
; ; and 

; and the distal –OH or –NH_2 of the T moiety is optionally linked to a cell-penetrating peptide.

$\mathbf{R^{100}}$ is hydrogen or a cell-penetrating peptide;

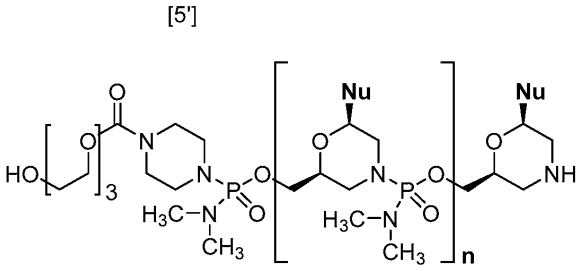
each **Nu** from 1 to **n** and 5' to 3' corresponds to the nucleobases in one of the following:

Annealing Site	Targeting Sequence [5' to 3']	SEQ ID NO:
H50D(+04-18)	GGG ATC CAG TAT ACT TAC AGG C	SEQ ID NO: 1
H50D(+07-18)	GGG ATC CAG TAT ACT TAC AGG CTC C	SEQ ID NO: 2
H50D(+07-16)	GAT CCA GTA TAC TTA CAG GCT CC	SEQ ID NO: 3
H50D(+07-17)	GGA TCC AGT ATA CTT ACA GGC TCC	SEQ ID NO: 4
H50A(-19+07)	ACT TCC TCT TTA ACA GAA AAG CAT AC	SEQ ID NO: 5
H50D(+07-15)	ATC CAG TAT ACT TAC AGG CTC C	SEQ ID NO: 6
H50A(-02+23)	GAG CTC AGA TCT TCT AAC TTC CTC T	SEQ ID NO: 7
H50D(+06-18)	GGG ATC CAG TAT ACT TAC AGG CTC	SEQ ID NO: 8
H50D(+07-20)	ATG GGA TCC AGT ATA CTT ACA GGC TCC	SEQ ID NO: 9

wherein A is , C is , G is , and T is .

In some embodiments, each **Nu** from 1 to **n** and 5' to 3' corresponds to the nucleobases in one of the following: SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, or SEQ ID NO: 9. In some embodiments, each **Nu** from 1 to **n** and 5' to 3' corresponds to SEQ ID NO. 3.

In another aspect, the disclosure provides antisense oligomers of Formula (II):

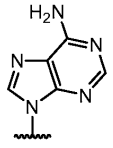
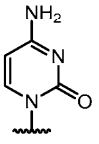
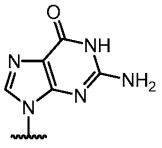
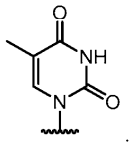


(II)

or a pharmaceutically acceptable salt thereof, where each **Nu** from 1 to **n** and 5' to 3' corresponds to the nucleobases in one of the following:

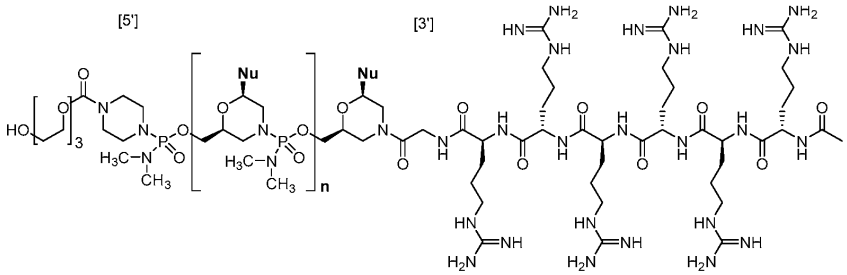
Annealing Site	Targeting Sequence [5' to 3']	SEQ ID NO:
H50D(+04-18)	GGG ATC CAG TAT ACT TAC AGG C	SEQ ID NO: 1
H50D(+07-18)	GGG ATC CAG TAT ACT TAC AGG CTC C	SEQ ID NO: 2
H50D(+07-16)	GAT CCA GTA TAC TTA CAG GCT CC	SEQ ID NO: 3
H50D(+07-17)	GGA TCC AGT ATA CTT ACA GGC TCC	SEQ ID NO: 4

H50A(-19+07)	ACT TCC TCT TTA ACA GAA AAG CAT AC	SEQ ID NO: 5
H50D(+07-15)	ATC CAG TAT ACT TAC AGG CTC C	SEQ ID NO: 6
H50A(-02+23)	GAG CTC AGA TCT TCT AAC TTC CTC T	SEQ ID NO: 7
H50D(+06-18)	GGG ATC CAG TAT ACT TAC AGG CTC	SEQ ID NO: 8
H50D(+07-20)	ATG GGA TCC AGT ATA CTT ACA GGC TCC	SEQ ID NO: 9

wherein A is , C is , G is , and T is . In some embodiments, the distal –OH of Formula (II) is linked to a cell-penetrating peptide.

In some embodiments, each **Nu** from 1 to **n** and 5' to 3' corresponds to the nucleobases in one of the following: SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, or SEQ ID NO: 9. In some embodiments, each **Nu** from 1 to **n** and 5' to 3' corresponds to SEQ ID NO. 3.

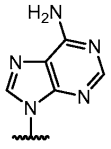
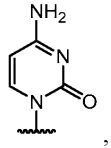
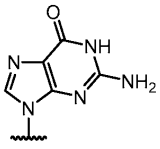
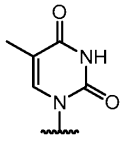
In another aspect, the disclosure provides antisense oligomers of Formula (III):



(III)

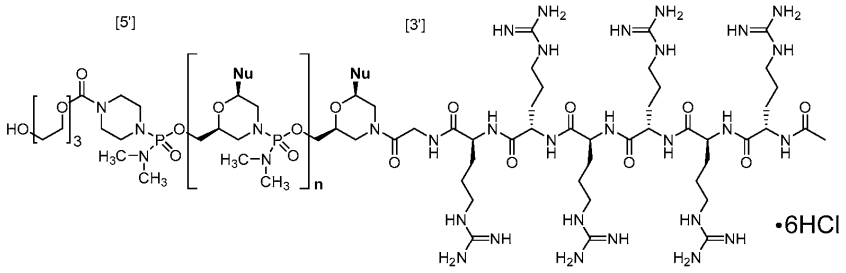
or a pharmaceutically acceptable salt thereof, where each **Nu** from 1 to **n** and 5' to 3' corresponds to the nucleobases in one of the following:

Annealing Site	Targeting Sequence [5' to 3']	SEQ ID NO:
H50D(+04-18)	GGG ATC CAG TAT ACT TAC AGG C	SEQ ID NO: 1
H50D(+07-18)	GGG ATC CAG TAT ACT TAC AGG CTC C	SEQ ID NO: 2
H50D(+07-16)	GAT CCA GTA TAC TTA CAG GCT CC	SEQ ID NO: 3
H50D(+07-17)	GGA TCC AGT ATA CTT ACA GGC TCC	SEQ ID NO: 4
H50A(-19+07)	ACT TCC TCT TTA ACA GAA AAG CAT AC	SEQ ID NO: 5
H50D(+07-15)	ATC CAG TAT ACT TAC AGG CTC C	SEQ ID NO: 6
H50A(-02+23)	GAG CTC AGA TCT TCT AAC TTC CTC T	SEQ ID NO: 7
H50D(+06-18)	GGG ATC CAG TAT ACT TAC AGG CTC	SEQ ID NO: 8
H50D(+07-20)	ATG GGA TCC AGT ATA CTT ACA GGC TCC	SEQ ID NO: 9

wherein A is , C is , G is , and T is . In some embodiments, the distal –OH of Formula (III) is linked to a cell-penetrating peptide.

In some embodiments, each **Nu** from 1 to **n** and 5' to 3' corresponds to SEQ ID NO. 3.

In another aspect, the disclosure provides antisense oligomers of Formula (IV):

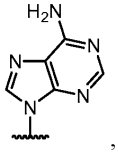
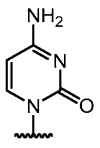
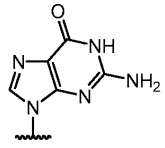
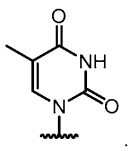


(IV)

where each **Nu** from 1 to **n** and 5' to 3' corresponds to the nucleobases in one of the following:

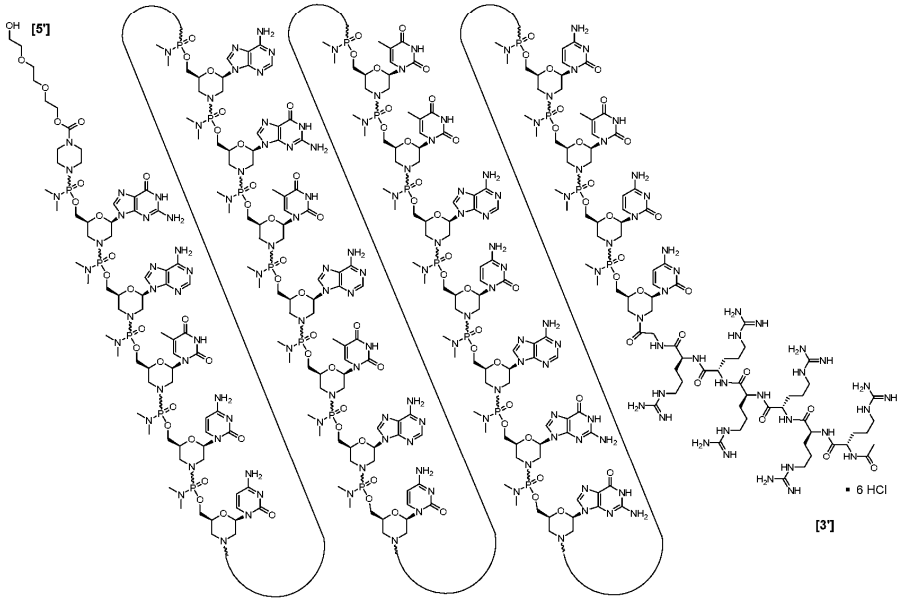
Annealing Site	Targeting Sequence [5' to 3']	SEQ ID NO:
H50D(+04-18)	GGG ATC CAG TAT ACT TAC AGG C	SEQ ID NO: 1

H50D(+07-18)	GGG ATC CAG TAT ACT TAC AGG CTC C	SEQ ID NO: 2
H50D(+07-16)	GAT CCA GTA TAC TTA CAG GCT CC	SEQ ID NO: 3
H50D(+07-17)	GGA TCC AGT ATA CTT ACA GGC TCC	SEQ ID NO: 4
H50A(-19+07)	ACT TCC TCT TTA ACA GAA AAG CAT AC	SEQ ID NO: 5
H50D(+07-15)	ATC CAG TAT ACT TAC AGG CTC C	SEQ ID NO: 6
H50A(-02+23)	GAG CTC AGA TCT TCT AAC TTC CTC T	SEQ ID NO: 7
H50D(+06-18)	GGG ATC CAG TAT ACT TAC AGG CTC	SEQ ID NO: 8
H50D(+07-20)	ATG GGA TCC AGT ATA CTT ACA GGC TCC	SEQ ID NO: 9

wherein A is , C is , G is , and T is .

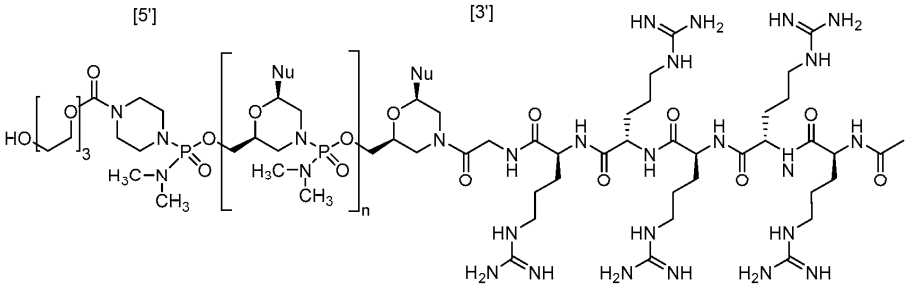
In some embodiments, each **Nu** from 1 to **n** and 5' to 3' corresponds to SEQ ID NO. 3.

In some embodiments for Formula (IV), the antisense oligomer is according to Formula (IVa)



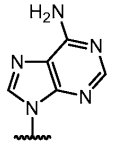
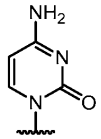
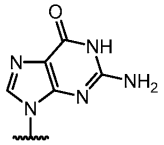
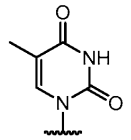
(IVa)

In another aspect, the disclosure provides antisense oligomers of Formula (V):



where each **Nu** from 1 to **n** and 5' to 3' corresponds to the nucleobases in one of the following:

Annealing Site	Targeting Sequence [5' to 3']	SEQ ID NO:
H50D(+04-18)	GGG ATC CAG TAT ACT TAC AGG C	SEQ ID NO: 1
H50D(+07-18)	GGG ATC CAG TAT ACT TAC AGG CTC C	SEQ ID NO: 2
H50D(+07-16)	GAT CCA GTA TAC TTA CAG GCT CC	SEQ ID NO: 3
H50D(+07-17)	GGA TCC AGT ATA CTT ACA GGC TCC	SEQ ID NO: 4
H50A(-19+07)	ACT TCC TCT TTA ACA GAA AAG CAT AC	SEQ ID NO: 5
H50D(+07-15)	ATC CAG TAT ACT TAC AGG CTC C	SEQ ID NO: 6
H50A(-02+23)	GAG CTC AGA TCT TCT AAC TTC CTC T	SEQ ID NO: 7
H50D(+06-18)	GGG ATC CAG TAT ACT TAC AGG CTC	SEQ ID NO: 8
H50D(+07-20)	ATG GGA TCC AGT ATA CTT ACA GGC TCC	SEQ ID NO: 9

wherein A is , C is , G is , and T is . In some embodiments, each **Nu** from 1 to **n** and 5' to 3' corresponds to SEQ ID NO. 3.

In another aspect, the disclosure provides a method for treating Duchenne muscular dystrophy (DMD) in a subject in need thereof wherein the subject has a mutation of the dystrophin gene that is amenable to exon 50 skipping, the method comprising administering to the subject an antisense oligomer of the disclosure. The disclosure also addresses the use of antisense oligomers of the disclosure for the manufacture of a medicament for treatment of Duchenne muscular dystrophy (DMD) in a subject in need thereof wherein the subject has a mutation of the dystrophin gene that is amenable to exon 50 skipping.

In another aspect, the disclosure provides a method of restoring an mRNA reading frame to induce dystrophin production in a subject having a mutation of the dystrophin gene that is amenable to exon 50 skipping, the method comprising administering to the subject an antisense oligomer of the disclosure. In another aspect, the disclosure provides a method of excluding exon 50 from dystrophin pre-mRNA during mRNA processing in a subject having a mutation of the dystrophin gene that is amenable to exon 50 skipping, the method comprising administering to the subject an antisense oligomer of the disclosure. In another aspect, the disclosure provides a method of binding exon 50, intron 49, and/or intron 50 of dystrophin pre-mRNA in a subject having a mutation of the dystrophin gene that is amenable to exon 50 skipping, the method comprising administering to the subject an antisense oligomer of the disclosure.

In another aspect, the disclosure provides an antisense oligomer of the disclosure herein for use in therapy. In certain embodiments, the disclosure provides an antisense oligomer of the disclosure for use in the treatment of Duchenne muscular dystrophy. In certain embodiments, the disclosure provides an antisense oligomer of the disclosure for use in the manufacture of a medicament for use in therapy. In certain embodiments, the disclosure provides an antisense oligomer of the disclosure for use in the manufacture of a medicament for the treatment of Duchenne muscular dystrophy.

In another aspect, the disclosure also provides kits for treating Duchenne muscular dystrophy (DMD) in a subject in need thereof wherein the subject has a mutation of the dystrophin gene that is amenable to exon 50 skipping, which kits comprise at least an antisense oligomer of the present disclosure, packaged in a suitable container and instructions for its use.

DETAILED DESCRIPTION OF THE DISCLOSURE

Embodiments of the present disclosure relate generally to improved antisense oligomers, and methods of use thereof, which are specifically designed to induce exon skipping in the human dystrophin gene. Dystrophin plays a vital role in muscle function, and various muscle-related diseases are characterized by mutated forms of this gene. Hence, in certain embodiments, the improved antisense oligomers described herein induce exon skipping in mutated forms of the human dystrophin gene, such as the mutated dystrophin

genes found in Duchenne muscular dystrophy (DMD) and Becker muscular dystrophy (BMD).

Due to aberrant mRNA splicing events caused by mutations, these mutated human dystrophin genes either express defective dystrophin protein or express no measurable dystrophin at all, a condition that leads to various forms of muscular dystrophy. To remedy this condition, the antisense oligomers of the present disclosure hybridize to selected regions of a pre-processed mRNA of a mutated human dystrophin gene, induce exon skipping and differential splicing in that otherwise aberrantly spliced dystrophin mRNA, and thereby allow muscle cells to produce an mRNA transcript that encodes a functional dystrophin protein. In certain embodiments, the resulting dystrophin protein is not necessarily the "wild-type" form of dystrophin, but is rather a truncated, yet functional, form of dystrophin.

By increasing the levels of functional dystrophin protein in muscle cells, these and related embodiments are useful in the prophylaxis and treatment of muscular dystrophy, especially those forms of muscular dystrophy, such as DMD and BMD, that are characterized by the expression of defective dystrophin proteins due to aberrant mRNA splicing. The specific antisense oligomers described herein further provide improved dystrophin-exon-specific targeting over other oligomers, and thereby offer significant and practical advantages over alternate methods of treating relevant forms of muscular dystrophy.

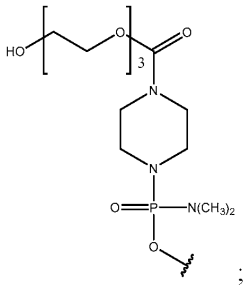
Thus, the disclosure relates to antisense oligomers, or pharmaceutically acceptable salts thereof, capable of binding a selected target to induce exon skipping in the human dystrophin gene, wherein the antisense oligomers comprise a sequence of bases that is complementary to an exon 50 target region of the dystrophin pre-mRNA designated as an annealing site, wherein the base sequence and annealing site are selected from:

Annealing Site	**Targeting Sequence [5' to 3']**	**SEQ ID NO:**
H50D(+04-18)	GGG ATC CAG TAT ACT TAC AGG C	SEQ ID NO: 1
H50D(+07-18)	GGG ATC CAG TAT ACT TAC AGG CTC C	SEQ ID NO: 2
H50D(+07-16)	GAT CCA GTA TAC TTA CAG GCT CC	SEQ ID NO: 3
H50D(+07-17)	GGA TCC AGT ATA CTT ACA GGC TCC	SEQ ID NO: 4
H50A(-19+07)	ACT TCC TCT TTA ACA GAA AAG CAT AC	SEQ ID NO: 5
H50D(+07-15)	ATC CAG TAT ACT TAC AGG CTC C	SEQ ID NO: 6
H50A(-02+23)	GAG CTC AGA TCT TCT AAC TTC CTC T	SEQ ID NO: 7
H50D(+06-18)	GGG ATC CAG TAT ACT TAC AGG CTC	SEQ ID NO: 8
H50D(+07-20)	ATG GGA TCC AGT ATA CTT ACA GGC TCC	SEQ ID NO: 9

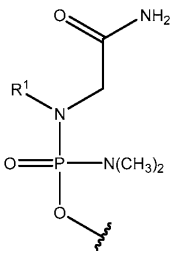
wherein T is thymine or uracil.

In some embodiments, each **Nu** from 1 to **n** and 5' to 3' corresponds to the nucleobases in one of the following: SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, or SEQ ID NO: 9. In some embodiments, each **Nu** from 1 to **n** and 5' to 3' corresponds to the nucleobases in SEQ ID NO: 3.

In one aspect, the antisense oligomer contains a **T** moiety attached to the 5' end of the antisense oligomer, wherein the **T** moiety is selected from:



;



;



and .

In certain embodiments, the antisense oligomer is conjugated to one or more cell-penetrating peptides (referred to herein as "CPP"). In certain embodiments, one or more CPPs are attached to a terminus of the antisense oligomer. In certain embodiments, at least one CPP is attached to the 5' terminus of the antisense oligomer. In certain embodiments, at least one CPP is attached to the 3' terminus of the antisense oligomer. In certain embodiments, a first CPP is attached to the 5' terminus and a second CPP is attached to the 3' terminus of the antisense oligomer.

In some embodiments, the CPP is an arginine-rich peptide. The term "arginine-rich" refers to a CPP having at least 2, and preferably 2, 3, 4, 5, 6, 7, or 8 arginine residues, each optionally separated by one or more uncharged, hydrophobic residues, and optionally containing about 6-14 amino acid residues. As explained below, a CPP is preferably linked at its carboxy terminus to the 3' and/or 5' end of an antisense oligonucleotide through a linker, which may also be one or more amino acids, and is preferably also capped at its amino terminus by a substituent R^a with R^a selected from H, acyl, acetyl, benzoyl, or stearoyl. In some embodiments, R^a is acetyl.

As seen in the table below, non-limiting examples of CPP's for use herein include –$(RXR)_4$-R^a (SEQ ID NO: 15), R-$(FFR)_3$-R^a (SEQ ID NO: 16), -B-X-$(RXR)_4$-R^a (SEQ ID NO: 17), -B-X-R-$(FFR)_3$-R^a (SEQ ID NO: 18), -GLY-R-$(FFR)_3$-R^a (SEQ ID NO: 19), -GLY-R_5-R^a (SEQ ID NO: 20), –R_5-R^a (SEQ ID NO: 21), -GLY-R_6-R^a (SEQ ID NO: 11) and –R_6-R^a (SEQ ID NO: 10), wherein R^a is selected from H, acyl, benzoyl, and stearoyl, and wherein R is arginine, X is 6-aminohexanoic acid, B is β-alanine, F is phenylalanine and GLY (or G) is glycine. The CPP "R_5 (SEQ ID NO: 21)" is meant to indicate a peptide of five (5) arginine residues linked together via amide bonds (and not a single substituent *e.g.*, R^5 (SEQ ID NO: 21)). The CPP "R_6 (SEQ ID NO: 10)" is meant to indicate a peptide of six (6) arginine residues linked together via amide bonds (and not a single substituent *e.g.* R^6 (SEQ ID NO: 10)). In some embodiments, R^a is acetyl.

Exemplary CPPs are provided in **Table 1** (SEQ ID NOS: 10, 11, and 15-21).

Table 1: Exemplary Cell-Penetrating Peptides

Name	Sequence	SEQ ID NO:
R_6G	RRRRRRG	11
R_6	RRRRRR	10
$(RXR)_4$	RXRRXRRXRRXR	15
$(RFF)_3$R	RFFRFFRFFR	16
$(RXR)_4$XB	RXRRXRRXRRXRXB	17
$(RFF)_3$RXB	RFFRFFRFFRXB	18
$(RFF)_3$RG	RFFRFFRFFRG	19
R_5G	RRRRRG	20
R_5	RRRRR	21
R is arginine; X is 6-aminohexanoic acid; B is β-alanine; F is phenylalanine; G is glycine		

CPPs, their synthesis, and methods of conjugating to an oligomer are further described in U.S. Application Publication No. US 2012/0289457 and International Patent Application Publication Nos. WO 2004/097017, WO 2009/005793, and WO 2012/150960, the disclosures of which are incorporated herein by reference in their entirety.

In some embodiments, an antisense oligonucleotide comprises a substituent "Z," defined as the combination of a CPP and a linker. The linker bridges the CPP at its carboxy terminus to the 3'-end and/or the 5'-end of the oligonucleotide. In various embodiments, an

antisense oligonucleotide may comprise only one CPP linked to the 3' end of the oligomer. In other embodiments, an antisense oligonucleotide may comprise only one CPP linked to the 5' end of the oligomer.

The linker within Z may comprise, for example, 1, 2, 3, 4, or 5 amino acids.

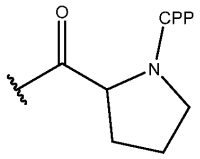
In particular embodiments, Z is selected from:

-C(O)$(CH_2)_5$NH-CPP;

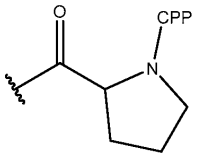
-C(O)$(CH_2)_2$NH-CPP;

-C(O)$(CH_2)_2$NHC(O)$(CH_2)_5$NH-CPP;

-C(O)CH_2NH-CPP, and the formula:



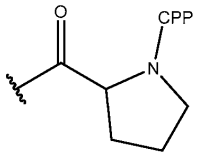
wherein the CPP is attached to the linker moiety by an amide bond at the CPP carboxy terminus.

In various embodiments, the CPP is an arginine-rich peptide as described herein and seen in Table 1. In certain embodiments, the arginine-rich CPP is -R_5-R^a, (*i.e.*, five arginine residues; SEQ ID NO: 21), wherein R^a is selected from H, acyl, acetyl, benzoyl, and stearoyl. In certain embodiments, R^a is acetyl. In various embodiments, the CPP is selected from SEQ ID NOS: 15, 16, or 21, and the linker is selected from the group consisting of -C(O)$(CH_2)_5$NH-, -C(O)$(CH_2)_2$NH-, -C(O)$(CH_2)_2$NHC(O)$(CH_2)_5$NH-, -C(O)CH_2NH-, and 

. In some embodiments, the linker comprises 1, 2, 3, 4, or 5 amino acids.

In some embodiments, the CPP is SEQ ID NO: 21 and the linker is Gly. In some embodiments, the CPP is SEQ ID NO: 20.

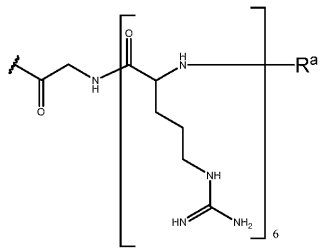
In certain embodiments, the arginine-rich CPP is -R_6-R^a, (*i.e.*, six arginine residues; SEQ ID NO: 10), wherein R^a is selected from H, acyl, acetyl, benzoyl, and stearoyl. In

certain embodiments, R^a is acetyl. In various embodiments, the CPP is selected from SEQ ID NOS: 10, 15, or 16, and the linker is selected from the group consisting of -C(O)$(CH_2)_5$NH-, -C(O)$(CH_2)_2$NH-, -C(O)$(CH_2)_2$NHC(O)$(CH_2)_5$NH-, -C(O)CH_2NH-, and 

. In some embodiments, the linker comprises 1, 2, 3, 4, or 5 amino acids.

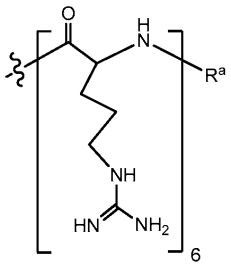
In some embodiments, the CPP is SEQ ID NO: 10 and the linker is Gly. In some embodiments, the CPP is SEQ ID NO: 11.

In certain embodiments, Z is -C(O)CH_2NH-R_6-R^a ("R_6" is disclosed as SEQ ID NO: 10) covalently bonded to an antisense oligomer of the disclosure at the 5' and/or 3' end of the oligomer, wherein R^a is H, acyl, acetyl, benzoyl, or stearoyl to cap the amino terminus of the R_6 (SEQ ID NO: 10). In certain embodiments, R^a is acetyl. In these non-limiting examples, the CPP is –R_6-R^a (SEQ ID NO: 10) and the linker is -C(O)CH_2NH-, (*i.e.* GLY). This particular example of Z = -C(O)CH_2NH-R_6-R^a ("R_6" is disclosed as SEQ ID NO: 10) is also exemplified by the following structure:



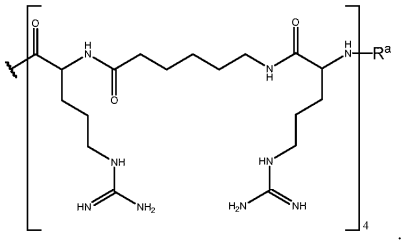
wherein R^a is selected from H, acyl, acetyl, benzoyl, and stearoyl.

In various embodiments, the CPP is -R_6-R^a (SEQ ID NO: 10), also exemplified as the following formula:

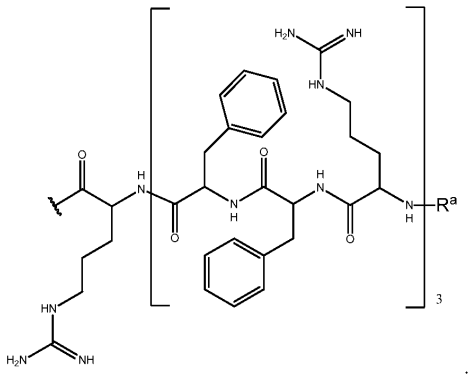


wherein R^a is selected from H, acyl, acetyl, benzoyl, and stearoyl. In certain embodiments, the CPP is SEQ ID NO: 11. In some embodiments, R^a is acetyl.

In some embodiments, the CPP is $–(RXR)_4-R^a$ (SEQ ID NO: 15), also exemplified as the following formula:



In various embodiments, the CPP is $–R-(FFR)_3-R^a$ (SEQ ID NO: 16), also exemplified as the following formula:



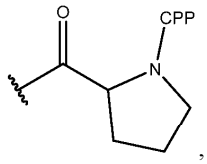
In various embodiments, Z is selected from:

$-C(O)(CH_2)_5NH$-CPP;

$-C(O)(CH_2)_2NH$-CPP;

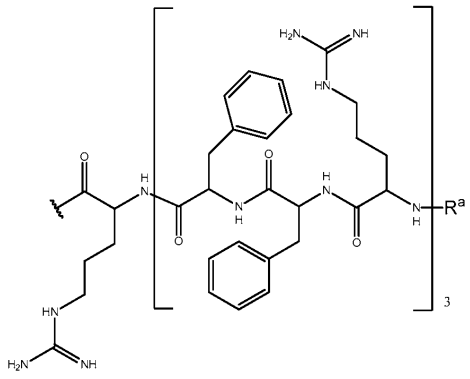
-C(O)$(CH_2)_2$NHC(O)$(CH_2)_5$NH-CPP;

-C(O)CH_2NH-CPP; and the formula:

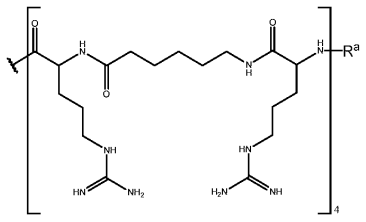


,

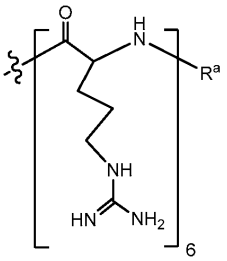
wherein the CPP is attached to the linker moiety by an amide bond at the CPP carboxy terminus, and wherein the CPP is selected from:



, (-R-(FFR)$_3$-R^a) (SEQ ID NO: 16),



, (-(RXR)$_4$-R^a) (SEQ ID NO: 15),



, or (-R_6-R^a) (SEQ ID NO: 10). In some embodiments, R^a is acetyl.

In some embodiments, each **Nu** from 1 to **n** and 5' to 3' corresponds SEQ ID NO. 3.

In some aspects, the nucleobases of the modified antisense oligomer are linked to morpholino ring structures, wherein the morpholino ring structures are joined by phosphorous-containing intersubunit linkages joining a morpholino nitrogen of one ring structure to a 5' exocyclic carbon of an adjacent ring structure. In such an aspect, T of each of SEQ ID NOS: 1-9 is preferably thymine.

In some aspects, the nucleobases of the antisense oligomer are linked to a peptide nucleic acid (PNA), wherein the phosphate-sugar polynucleotide backbone is replaced by a flexible pseudo-peptide polymer to which the nucleobases are linked.

In some aspects, the at least one of the nucleobases of the antisense oligomer is linked to locked nucleic acid (LNA), wherein the locked nucleic acid structures are nucleotide analogs that are chemically modified where the ribose moiety has an extra bridge connecting the 2′ oxygen and the 4′ carbon. In such an aspect, each nucleobase which is linked to an LNA in of each of SEQ ID NOS: 1-9 comprises a 5-methyl group.

In some aspects, at least one of the nucleobases of the antisense oligomer is linked to a bridged nucleic acid (BNA), wherein the sugar conformation is restricted or locked by introduction of an additional bridged structure to the furanose skeleton. In some aspects, at least one of the nucleobases of the antisense oligomer is linked to a 2'-O,4'-C-ethylene-bridged nucleic acid (ENA). In such aspects, each nucleobase which is linked to a BNA or ENA in of each of SEQ ID NOS: 1-9 comprises a 5-methyl group.

In some aspects, when the chemistry of the backbone allows, each thymine in SEQ ID NOS: 1-9 are uracil.

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by those of ordinary skill in the art to which the disclosure belongs. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present disclosure, preferred methods and materials are described. For the purposes of the present disclosure, the following terms are defined below.

I. Definitions

The term "alkyl," as used herein, unless otherwise specified, refers to a saturated straight or branched hydrocarbon. In certain embodiments, the alkyl group is a primary, secondary, or tertiary hydrocarbon. In certain embodiments, the alkyl group includes one to

ten carbon atoms, *i.e.*, C_1 to C_{10} alkyl. In certain embodiments, the alkyl group includes one to six carbon atoms, *i.e.*, C_1 to C_6 alkyl. The term includes both substituted and unsubstituted alkyl groups, including halogenated alkyl groups. In certain embodiments, the alkyl group is a fluorinated alkyl group. Non-limiting examples of moieties with which the alkyl group can be substituted are selected from the group consisting of halogen (fluoro, chloro, bromo, or iodo), hydroxyl, amino, alkylamino, arylamino, alkoxy, aryloxy, nitro, cyano, sulfonic acid, sulfate, phosphonic acid, phosphate, or phosphonate, either unprotected, or protected as necessary, as known to those skilled in the art, for example, as taught in Greene, *et al.*, Protective Groups in Organic Synthesis, John Wiley and Sons, Second Edition, 1991, hereby incorporated by reference. In certain embodiments, the alkyl group is selected from the group consisting of methyl, CF_3, CCl_3, $CFCl_2$, CF_2Cl, ethyl, CH_2CF_3, CF_2CF_3, propyl, isopropyl, butyl, isobutyl, sec-butyl, t-butyl, pentyl, isopentyl, neopentyl, hexyl, isohexyl, 3-methylpentyl, 2,2-dimethylbutyl, and 2,3-dimethylbutyl.

"Amenable to exon 50 skipping" as used herein with regard to a subject or patient is intended to include subjects and patients having one or more mutations in the dystrophin gene which, absent the skipping of exon 50 of the dystrophin pre-mRNA, causes the reading frame to be out-of-frame thereby disrupting translation of the pre-mRNA leading to an inability of the subject or patient to produce functional or semi-functional dystrophin. Determining whether a patient has a mutation in the dystrophin gene that is amenable to exon skipping is well within the purview of one of skill in the art (see, e.g., Aartsma-Rus et al. (2009) Hum Mutat. 30:293-299; Gurvich et al., Hum Mutat. 2009; 30(4) 633-640; and Fletcher et al. (2010) Molecular Therapy 18(6) 1218-1223.).

The term "oligomer" as used herein refers to a sequence of subunits connected by intersubunit linkages. In certain instances, the term "oligomer" is used in reference to an "antisense oligomer." For "antisense oligomers," each subunit consists of: (i) a ribose sugar or a derivative thereof; and (ii) a nucleobase bound thereto, such that the order of the base-pairing moieties forms a base sequence that is complementary to a target sequence in a nucleic acid (typically an RNA) by Watson-Crick base pairing, to form a nucleic acid:oligomer heteroduplex within the target sequence with the proviso that either the subunit, the intersubunit linkage, or both are not naturally occurring. In certain embodiments, the antisense oligomer is a phosphorodiamidate morpholino oligomer (PMO). In other embodiments, the antisense oligomer is a 2'-O-methyl phosphorothioate.

In other embodiments, the antisense oligomer of the disclosure is a peptide nucleic acid (PNA), a locked nucleic acid (LNA), or a bridged nucleic acid (BNA) such as 2'-O,4'-C-ethylene-bridged nucleic acid (ENA). Additional exemplary embodiments are described herein.

The terms "complementary" and "complementarity" refer to two or more oligomers (i.e., each comprising a nucleobase sequence) that are related with one another by Watson-Crick base-pairing rules. For example, the nucleobase sequence "T-G-A (5'→3')," is complementary to the nucleobase sequence "A-C-T (3'→ 5')." Complementarity may be "partial," in which less than all of the nucleobases of a given nucleobase sequence are matched to the other nucleobase sequence according to base pairing rules. For example, in some embodiments, complementarity between a given nucleobase sequence and the other nucleobase sequence may be about 70%, about 75%, about 80%, about 85%, about 90% or about 95%. Or, there may be "complete" or "perfect" (100%) complementarity between a given nucleobase sequence and the other nucleobase sequence to continue the example. The degree of complementarity between nucleobase sequences has significant effects on the efficiency and strength of hybridization between the sequences.

The terms “effective amount” and “therapeutically effective amount” are used interchangeably herein and refer to an amount of therapeutic compound, such as an antisense oligomer, administered to a mammalian subject, either as a single dose or as part of a series of doses, which is effective to produce a desired therapeutic effect. For an antisense oligomer, this effect is typically brought about by inhibiting translation or natural splice-processing of a selected target sequence, or producing a clinically meaningful amount of dystrophin (statistical significance).

In some embodiments, an effective amount is about 1 mg/kg to about 200 mg/kg of a composition including an antisense oligomer for a period of time to treat the subject. In some embodiments, an effective amount is about 1 mg/kg to about 200 mg/kg of a composition including an antisense oligomer to increase the number of dystrophin-positive fibers in a subject. In certain embodiments, an effective amount is from about 1 mg/kg to about 200 mg/kg of a composition including an antisense oligomer to stabilize, maintain, or improve walking distance, for example in a 6 Minute Walk Test (6MWT), in a patient, relative to a healthy peer.

"Enhance" or "enhancing," or "increase" or "increasing," or "stimulate" or

"stimulating" refers generally to the ability of one or more antisense oligomers or pharmaceutical compositions of any of the foregoing to produce or cause a greater physiological response (i.e., downstream effects) in a cell or a subject, as compared to the response caused by either no antisense oligomer or a control compound. A greater physiological response may include increased expression of a functional form of a dystrophin protein, or increased dystrophin-related biological activity in muscle tissue, among other responses apparent from the understanding in the art and the description herein.

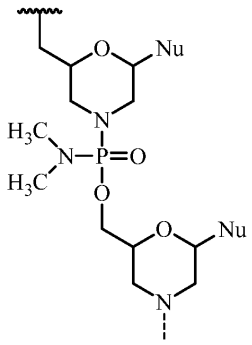
As used herein, the terms "function" and "functional" and the like refer to a biological, enzymatic, or therapeutic function.

A "functional" dystrophin protein refers generally to a dystrophin protein having sufficient biological activity to reduce the progressive degradation of muscle tissue that is otherwise characteristic of muscular dystrophy, typically as compared to the altered or "defective" form of dystrophin protein that is present in certain subjects with DMD or BMD. As one example, dystrophin-related activity in muscle cultures in vitro can be measured according to myotube size, myofibril organization (or disorganization), contractile activity, and spontaneous clustering of acetylcholine receptors (see, e.g., Brown et al., Journal of Cell Science. 112:209-216, 1999). Animal models are also valuable resources for studying the pathogenesis of disease, and provide a means to test dystrophin-related activity. Two of the most widely used animal models for DMD research are the mdx mouse and the golden retriever muscular dystrophy (GRMD) dog, both of which are dystrophin negative (see, e.g., Collins & Morgan, Int J Exp Pathol 84: 165-172, 2003). These and other animal models can be used to measure the functional activity of various dystrophin proteins. Included are truncated forms of dystrophin, such as those forms that are produced following the administration of certain of the exon-skipping antisense oligomers of the present disclosure.

The terms “mismatch” or “mismatches” refer to one or more nucleobases (whether contiguous or separate) in an oligomer nucleobase sequence that are not matched to a target pre-mRNA according to base pairing rules. While perfect complementarity is often desired, some embodiments can include one or more but preferably 6, 5, 4, 3, 2, or 1 mismatches with respect to the target pre-mRNA. Variations at any location within the oligomer are included. In certain embodiments, antisense oligomers of the disclosure include variations in nucleobase sequence near the termini, variations in the interior, and if present are typically within about 6, 5, 4, 3, 2, or 1 subunits of the 5' and/or 3' terminus. In certain embodiments,

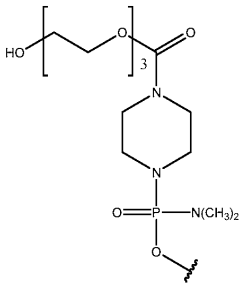
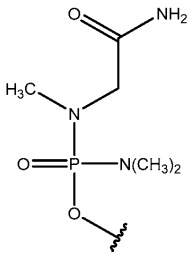
one, two, or three nucleobases can be removed and still provide on-target binding.

The terms "morpholino," "morpholino oligomer," and "PMO" refer to a phosphorodiamidate morpholino oligomer of the following general structure:



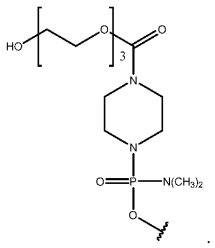
and as described in Figure 2 of Summerton, J., et al., *Antisense & Nucleic Acid Drug Development*, 7: 187-195 (1997). Morpholinos as described herein include all stereoisomers and tautomers of the foregoing general structure. The synthesis, structures, and binding characteristics of morpholino oligomers are detailed in U.S. Patent Nos.: 5,698,685; 5,217,866; 5,142,047; 5,034,506; 5,166,315; 5,521,063; 5,506,337; 8,076,476; and 8,299,206; all of which are incorporated herein by reference.

In certain embodiments, a morpholino is conjugated at the 5' or 3' end of the oligomer with a "tail" moiety to increase its stability and/or solubility. Exemplary tails include:

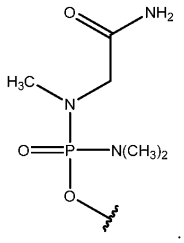

;

; and

. The distal –OH or –NH_2 of the T moiety is optionally linked to a cell-penetrating peptide.

Of the above exemplary tail moieties, "TEG" or "EG3" refers to the following tail moiety:

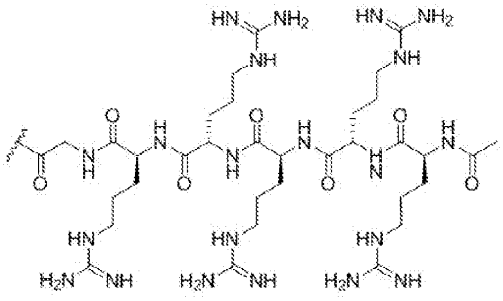


Of the above exemplary tail moieties, "GT" refers to the following tail moiety:

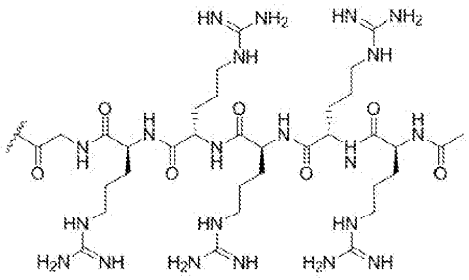


As used herein, the terms "-G-R_5 (SEQ ID NO: 20)" and "-G-R_5-Ac (SEQ ID NO: 20)" are used interchangeably and refer to a peptide moiety conjugated to an antisense oligomer of the disclosure. In various embodiments, "G" represents a glycine residue conjugated to "R_5 (SEQ ID NO: 21)" by an amide bond, and each "R" represents an arginine residue conjugated together by amide bonds such that "R_5 (SEQ ID NO: 21)" means five (5) arginine residues conjugated together by amide bonds. The arginine residues can have any stereo configuration, for example, the arginine residues can be L-arginine residues, D-arginine residues, or a mixture of D- and L-arginine residues. In certain embodiments, "-G-R_5 (SEQ ID NO: 20)" or "-G-R_5-Ac (SEQ ID NO: 20)" is linked to the distal –OH or NH_2 of the "tail" moiety. In certain embodiments, "-G-R_5 (SEQ ID NO: 20)" or "-G-R_5-Ac (SEQ ID NO: 20)" is conjugated to the morpholine ring nitrogen of the 3' most morpholino subunit of a PMO antisense oligomer of the disclosure. In some embodiments, "-G-R_5 (SEQ ID NO:

20)" or "-G-R_5-Ac (SEQ ID NO: 20)" is conjugated to the 3' end of an antisense oligomer of the disclosure and is of the following formula:



, or a pharmaceutically acceptable salt thereof, or

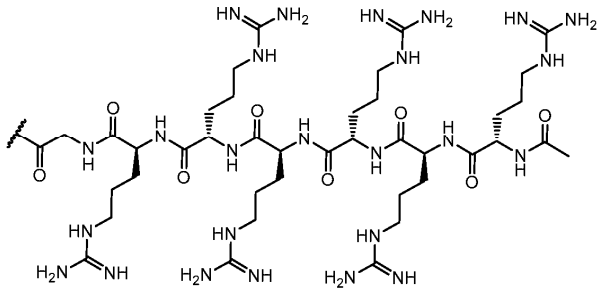


• 5HCl

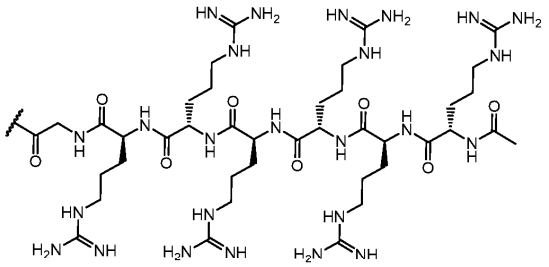
.

As used herein, the terms "-G-R_6 (SEQ ID NO: 11)" and "-G-R_6-Ac (SEQ ID NO: 11)" and "R_6G (SEQ ID NO: 11)" are used interchangeably and refer to a peptide moiety conjugated to an antisense oligomer of the disclosure. In various embodiments, "G" represents a glycine residue conjugated to "R_6 (SEQ ID NO: 10)" by an amide bond, and each "R" represents an arginine residue conjugated together by amide bonds such that "R_6 (SEQ ID NO: 10)" means six (6) arginine residues conjugated together by amide bonds. The arginine residues can have any stereo configuration, for example, the arginine residues can be L-arginine residues, D-arginine residues, or a mixture of D- and L-arginine residues. In certain embodiments, "-G-R_6 (SEQ ID NO: 11)" or "-G-R_6-Ac (SEQ ID NO: 11)" is linked to the distal –OH or $–NH_2$ of the "tail" moiety. In certain embodiments, "-G-R_6 (SEQ ID NO: 11)" or "-G-R_6-Ac (SEQ ID NO: 11)" is conjugated to the morpholine ring nitrogen of the 3' most morpholino subunit of a PMO antisense oligomer of the disclosure. In some embodiments,

"-G-R_6 (SEQ ID NO: 11)" or "-G-R_6-Ac (SEQ ID NO: 11)" is conjugated to the 3' end of an antisense oligomer of the disclosure and is of the following formula:



, or



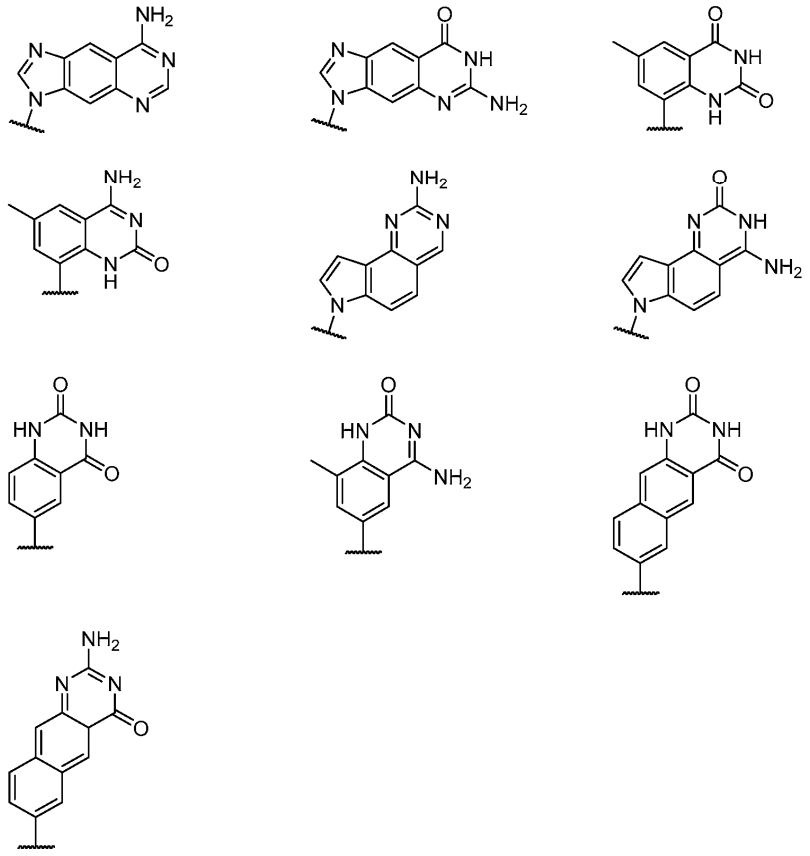
.6HCl

The terms "nucleobase" (Nu), "base pairing moiety" or "base" are used interchangeably to refer to a purine or pyrimidine base found in naturally occurring, or "native" DNA or RNA (e.g., uracil, thymine, adenine, cytosine, and guanine), as well as analogs of these naturally occurring purines and pyrimidines. These analogs may confer improved properties, such as binding affinity, to the oligomer. Exemplary analogs include hypoxanthine (the base component of inosine); 2,6-diaminopurine; 5-methyl cytosine; C5-propynyl-modified pyrimidines; 10-(9-(aminoethoxy)phenoxazinyl) (G-clamp) and the like.

Further examples of base pairing moieties include, but are not limited to, uracil, thymine, adenine, cytosine, guanine and hypoxanthine (inosine) having their respective amino groups protected by acyl protecting groups, 2-fluorouracil, 2-fluorocytosine, 5-bromouracil, 5-iodouracil, 2,6-diaminopurine, azacytosine, pyrimidine analogs such as pseudoisocytosine and pseudouracil and other modified nucleobases such as 8-substituted purines, xanthine, or hypoxanthine (the latter two being the natural degradation products). The modified nucleobases disclosed in: Chiu and Rana, RNA, 2003, 9, 1034-1048; Limbach *et al.* Nucleic Acids Research, 1994, 22, 2183-2196; and Revankar and Rao, Comprehensive

Natural Products Chemistry, vol. 7, 313; are also contemplated, the contents of which are incorporated herein by reference.

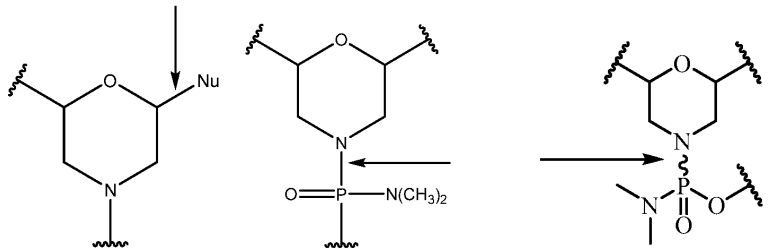
Further examples of base pairing moieties include, but are not limited to, expanded-size nucleobases in which one or more benzene rings has been added. Nucleic acid base replacements described in: the Glen Research catalog (www.glenresearch.com); Krueger AT *et al.*, Acc. Chem. Res., 2007, 40, 141-150; Kool, ET, Acc. Chem. Res., 2002, 35, 936-943; Benner S.A., *et al.*, Nat. Rev. Genet., 2005, 6, 553-543; Romesberg, F.E., *et al.*, Curr. Opin. Chem. Biol., 2003, 7, 723-733; and Hirao, I., Curr. Opin. Chem. Biol., 2006, 10, 622-627; the contents of which are incorporated herein by reference, are contemplated as useful in the antisense oligomers described herein. Examples of expanded-size nucleobases include those shown below, as well as tautomeric forms thereof.



The phrases "parenteral administration" and "administered parenterally" as used herein means modes of administration other than enteral and topical administration, usually by injection, and includes, without limitation, intravenous, intramuscular, intraarterial, intrathecal, intracapsular, intraorbital, intracardiac, intradermal, intraperitoneal, transtracheal, subcutaneous, subcuticular, intraarticular, subcapsular, subarachnoid, intraspinal and intrasternal injection and infusion.

As used herein, a set of brackets used within a structural formula indicate that the structural feature between the brackets is repeated. In some embodiments, the brackets used can be "[" and "]," and in certain embodiments, brackets used to indicate repeating structural features can be "(" and ")." In some embodiments, the number of repeat iterations of the structural feature between the brackets is the number indicated outside the brackets such as 2, 3, 4, 5, 6, 7, and so forth. In various embodiments, the number of repeat iterations of the structural feature between the brackets is indicated by a variable indicated outside the brackets such as "Z".

As used herein, a straight bond or a squiggly bond drawn to a chiral carbon or phosphorous atom within a structural formula indicates that the stereochemistry of the chiral carbon or phosphorous is undefined and is intended to include all forms of the chiral center and/or mixtures thereof. Examples of such illustrations are depicted below.



The phrase "pharmaceutically acceptable" means the substance or composition must be compatible, chemically and/or toxicologically, with the other ingredients comprising a formulation, and/or the subject being treated therewith.

The phrase "pharmaceutically-acceptable carrier" as used herein means a non-toxic, inert solid, semi-solid or liquid filler, diluent, encapsulating material, or formulation auxiliary of any type. Some examples of materials which can serve as pharmaceutically acceptable carriers are: sugars such as lactose, glucose, and sucrose; starches such as corn

starch and potato starch; cellulose and its derivatives such as sodium carboxymethyl cellulose, ethyl cellulose, and cellulose acetate; powdered tragacanth; malt; gelatin; talc; excipients such as cocoa butter and suppository waxes; oils such as peanut oil, cottonseed oil, safflower oil, sesame oil, olive oil, corn oil, and soybean oil; glycols such as propylene glycol; esters such as ethyl oleate and ethyl laurate; agar; buffering agents such as magnesium hydroxide and aluminum hydroxide; alginic acid; pyrogen-free water; isotonic saline; Ringer's solution; ethyl alcohol; phosphate buffer solutions; non-toxic compatible lubricants such as sodium lauryl sulfate and magnesium stearate; coloring agents; releasing agents; coating agents; sweetening agents; flavoring agents; perfuming agents; preservatives; and antioxidants; according to the judgment of the formulator.

The term "restoration" with respect to dystrophin synthesis or production refers generally to the production of a dystrophin protein including truncated forms of dystrophin in a patient with muscular dystrophy following treatment with an antisense oligomer described herein. The percent of dystrophin-positive fibers in a patient following treatment can be determined by a muscle biopsy using known techniques. For example, a muscle biopsy may be taken from a suitable muscle, such as the biceps brachii muscle in a patient.

Analysis of the percentage of positive dystrophin fibers may be performed pre-treatment and/or post-treatment or at time points throughout the course of treatment. In some embodiments, a post-treatment biopsy is taken from the contralateral muscle from the pre-treatment biopsy. Pre- and post-treatment dystrophin expression analysis may be performed using any suitable assay for dystrophin. In some embodiments, immunohistochemical detection is performed on tissue sections from the muscle biopsy using an antibody that is a marker for dystrophin, such as a monoclonal or a polyclonal antibody. For example, the MANDYS106 antibody can be used which is a highly sensitive marker for dystrophin. Any suitable secondary antibody may be used.

In some embodiments, the percent dystrophin-positive fibers are calculated by dividing the number of positive fibers by the total fibers counted. Normal muscle samples have 100% dystrophin-positive fibers. Therefore, the percent dystrophin-positive fibers can be expressed as a percentage of normal. To control for the presence of trace levels of dystrophin in the pretreatment muscle, as well as revertant fibers, a baseline can be set using sections of pre-treatment muscles from a patient when counting dystrophin-positive fibers in post-treatment muscles. This may be used as a threshold for counting dystrophin-positive

fibers in sections of post-treatment muscle in that patient. In other embodiments, antibody-stained tissue sections can also be used for dystrophin quantification using Bioquant image analysis software (Bioquant Image Analysis Corporation, Nashville, TN). The total dystrophin fluorescence signal intensity can be reported as a percentage of normal. In addition, Western blot analysis with monoclonal or polyclonal anti-dystrophin antibodies can be used to determine the percentage of dystrophin positive fibers. For example, the anti-dystrophin antibody NCL-Dys1 from Leica Biosystems may be used. The percentage of dystrophin-positive fibers can also be analyzed by determining the expression of the components of the sarcoglycan complex (β,γ) and/or neuronal NOS.

In some embodiments, treatment with an antisense oligomer of the disclosure slows or reduces the progressive respiratory muscle dysfunction and/or failure in patients with DMD that would be expected without treatment. In some embodiments, treatment with an antisense oligomer of the disclosure may reduce or eliminate the need for ventilation assistance that would be expected without treatment. In some embodiments, measurements of respiratory function for tracking the course of the disease, as well as the evaluation of potential therapeutic interventions include maximum inspiratory pressure (MIP), maximum expiratory pressure (MEP), and forced vital capacity (FVC). MIP and MEP measure the level of pressure a person can generate during inhalation and exhalation, respectively, and are sensitive measures of respiratory muscle strength. MIP is a measure of diaphragm muscle weakness.

In some embodiments, MEP may decline before changes in other pulmonary function tests, including MIP and FVC. In certain embodiments, MEP may be an early indicator of respiratory dysfunction. In certain embodiments, FVC may be used to measure the total volume of air expelled during forced exhalation after maximum inspiration. In patients with DMD, FVC increases concomitantly with physical growth until the early teens. However, as growth slows or is stunted by disease progression, and muscle weakness progresses, the vital capacity enters a descending phase and declines at an average rate of about 8 to 8.5 percent per year after 10 to 12 years of age. In certain embodiments, MIP percent predicted (MIP adjusted for weight), MEP percent predicted (MEP adjusted for age), and FVC percent predicted (FVC adjusted for age and height) are supportive analyses.

The terms "subject" and "patient" as used herein include any animal that exhibits a symptom, or is at risk for exhibiting a symptom, which can be treated with an antisense

oligomer of the disclosure, such as a subject (or patient) that has or is at risk for having DMD or BMD, or any of the symptoms associated with these conditions (e.g., muscle fiber loss). Suitable subjects (or patients) include laboratory animals (such as mouse, rat, rabbit, or guinea pig), farm animals, and domestic animals or pets (such as a cat or dog). Non-human primates and, preferably, human patients (or subjects), are included. Also included are methods of producing dystrophin in a subject (or patient) having a mutation of the dystrophin gene that is amenable to exon 50 skipping.

The phrases "systemic administration," "administered systemically," "peripheral administration" and "administered peripherally" as used herein mean the administration of a compound, drug or other material other than directly into the central nervous system, such that it enters the patient's system and, thus, is subject to metabolism and other like processes, for example, subcutaneous administration.

The phase “targeting sequence” or “base sequence” refers to a sequence of nucleobases of an oligomer that is complementary to a sequence of nucleotides in a target pre-mRNA. In some embodiments of the disclosure, the sequence of nucleotides in the target pre-mRNA is an exon 50, intron 49, and/or intron 50 annealing site in the dystrophin pre-mRNA designated as H50D(+04-18), H50D(+07-18), H50D(+07-16), H50D(+07-17), H50A(-19+07), H50D(+07-15), H50A(-02+23), H50D(+06-18), or H50D(+07-20). In one embodiment, the annealing site targeted by the antisense oligomer described herein is H50D(+07-16).

“Treatment” of a subject (e.g. a mammal, such as a human) or a cell is any type of intervention used in an attempt to alter the natural course of the subject or cell. Treatment includes, but is not limited to, administration of an oligomer or a pharmaceutical composition thereof, and may be performed either prophylactically or subsequent to the initiation of a pathologic event or contact with an etiologic agent. Treatment includes any desirable effect on the symptoms or pathology of a disease or condition associated with the dystrophin protein, as in certain forms of muscular dystrophy, and may include, for example, minimal changes or improvements in one or more measurable markers of the disease or condition being treated. Also included are "prophylactic" treatments, which can be directed to reducing the rate of progression of the disease or condition being treated, delaying the onset of that disease or condition, or reducing the severity of its onset. "Treatment" or "prophylaxis" does not necessarily indicate complete eradication, cure, or prevention of the

disease or condition, or associated symptoms thereof.

In some embodiments, treatment with an antisense oligomer of the disclosure increases novel dystrophin production, delays disease progression, slows or reduces the loss of ambulation, reduces muscle inflammation, reduces muscle damage, improves muscle function, reduces loss of pulmonary function, and/or enhances muscle regeneration that would be expected without treatment. In some embodiments, treatment maintains, delays, or slows disease progression. In some embodiments, treatment maintains ambulation or reduces the loss of ambulation. In some embodiments, treatment maintains pulmonary function or reduces loss of pulmonary function. In some embodiments, treatment maintains or increases a stable walking distance in a patient, as measured by, for example, the 6 Minute Walk Test (6MWT). In some embodiments, treatment maintains or reduces the time to walk/run 10 meters (i.e., the 10 meter walk/run test). In some embodiments, treatment maintains or reduces the time to stand from supine (*i.e.,* time to stand test). In some embodiments, treatment maintains or reduces the time to climb four standard stairs (*i.e.,* the four-stair climb test). In some embodiments, treatment maintains or reduces muscle inflammation in the patient, as measured by, for example, MRI (e.g., MRI of the leg muscles). In some embodiments, MRI measures T2 and/or fat fraction to identify muscle degeneration. MRI can identify changes in muscle structure and composition caused by inflammation, edema, muscle damage, and fat infiltration.

In some embodiments, treatment with an antisense oligomer of the disclosure increases novel dystrophin production and slows or reduces the loss of ambulation that would be expected without treatment. For example, treatment may stabilize, maintain, improve or increase walking ability (e.g., stabilization of ambulation) in the subject. In some embodiments, treatment maintains or increases a stable walking distance in a patient, as measured by, for example, the 6 Minute Walk Test (6MWT), described by McDonald, et al. (Muscle Nerve, 2010; 42:966-74, herein incorporated by reference). A change in the 6 Minute Walk Distance (6MWD) may be expressed as an absolute value, a percentage change or a change in the %-predicted value. The performance of a DMD patient in the 6MWT relative to the typical performance of a healthy peer can be determined by calculating a %-predicted value. For example, the %-predicted 6MWD may be calculated using the following equation for males: $196.72 + (39.81 \times \text{age}) - (1.36 \times \text{age}^2) + (132.28 \times \text{height in meters})$. For females, the %-predicted 6MWD may be calculated using the

following equation: 188.61 + (51.50 x age) – (1.86 x age^2) + (86.10 x height in meters) (Henricson et al. PLoS Curr., 2012, version 2, herein incorporated by reference).

Loss of muscle function in patients with DMD may occur against the background of normal childhood growth and development. Indeed, younger children with DMD may show an increase in distance walked during 6MWT over the course of about 1 year despite progressive muscular impairment. In some embodiments, the 6MWD from patients with DMD is compared to typically developing control subjects and to existing normative data from age and sex matched subjects. In some embodiments, normal growth and development can be accounted for using an age and height based equation fitted to normative data. Such an equation can be used to convert 6MWD to a percent-predicted (%-predicted) value in subjects with DMD. In certain embodiments, analysis of %-predicted 6MWD data represents a method to account for normal growth and development, and may show that gains in function at early ages (e.g., less than or equal to age 7) represent stable rather than improving abilities in patients with DMD (Henricson et al. PLoS Curr., 2012, version 2, herein incorporated by reference).

An antisense molecule nomenclature system was proposed and published to distinguish between the different antisense molecules (see Mann et al., (2002) J Gen Med 4, 644-654). This nomenclature became especially relevant when testing several slightly different antisense molecules, all directed at the same target region, as shown below:

H#A/D(x:y).

The first letter designates the species (e.g. H: human, M: murine, C: canine). "#" designates target dystrophin exon number. "A/D" indicates acceptor or donor splice site at the beginning and end of the exon, respectively. (x y) represents the annealing coordinates where "-" or "+" indicate intronic or exonic sequences respectively. For example, A(-6+18) would indicate the last 6 bases of the intron preceding the target exon and the first 18 bases of the target exon. The closest splice site would be the acceptor so these coordinates would be preceded with an "A". Describing annealing coordinates at the donor splice site could be D(+2-18) where the last 2 exonic bases and the first 18 intronic bases correspond to the annealing site of the antisense molecule. Entirely exonic annealing coordinates that would be represented by A(+65+85), that is the site between the 65th and 85th nucleotide from the start of that exon.

II. Antisense Oligomers

A. Antisense Oligomers Designed to Induce Exon 50 Skipping

In certain embodiments, antisense oligomers of the disclosure are complementary to an exon 50, intron 49, and/or intron 50 target region of the dystrophin gene and induce exon 50 skipping. In particular, the disclosure relates to antisense oligomers complementary to an exon 50, intron 49, and/or intron 50 target region of the dystrophin pre-mRNA designated as an annealing site. In some embodiments, the annealing site is selected from H50D(+04-18), H50D(+07-18), H50D(+07-16), H50D(+07-17), H50A(-19+07), H50D(+07-15), H50A(-02+23), H50D(+06-18), or H50D(+07-20). In some embodiments, the annealing site is H50D(+07-16).

Antisense oligomers of the disclosure target dystrophin pre-mRNA and induce skipping of exon 50, so it is excluded or skipped from the mature, spliced mRNA transcript. By skipping exon 50, the disrupted reading frame is restored to an in-frame mutation. While DMD is comprised of various genetic subtypes, antisense oligomers of the disclosure were specifically designed to skip exon 50 of dystrophin pre-mRNA. DMD mutations amenable to skipping exon 50 comprise a subgroup of DMD patients (4%).

The nucleobase sequence of an antisense oligomer that induces exon 50 skipping is designed to be complementary to a specific target sequence within exon 50, intron 49, and/or intron 50 of dystrophin pre-mRNA. In some embodiments, an antisense oligomer is a PMO wherein each morpholino ring of the PMO is linked to a nucleobase including, for example, nucleobases found in DNA (adenine, cytosine, guanine, and thymine).

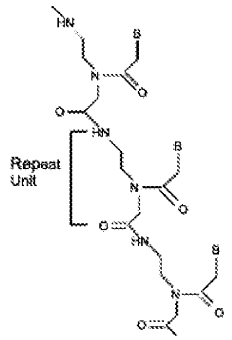
B. Oligomer Chemistry Features

The antisense oligomers of the disclosure can employ a variety of antisense oligomer chemistries. Examples of oligomer chemistries include, without limitation, morpholino oligomers, phosphorothioate modified oligomers, 2'-O-methyl modified oligomers, peptide nucleic acid (PNA), locked nucleic acid (LNA), phosphorothioate oligomers, 2'-O-MOE modified oligomers, 2'-fluoro-modified oligomer, 2'O,4'C-ethylene-bridged nucleic acids (ENAs), tricyclo-DNAs, tricyclo-DNA phosphorothioate subunits, 2'-O-[2-(N-methylcarbamoyl)ethyl] modified oligomers, including combinations of any of the foregoing. Phosphorothioate and 2'-O-Me-modified chemistries can be combined to generate a 2'-O-Me-phosphorothioate backbone. *See*, *e.g.*, PCT Publication Nos.

WO/2013/112053 and WO/2009/008725, which are hereby incorporated by reference in their entireties. As allowed by the chemistry utilized, each T in any of SEQ ID NOS: 1-9 can be uracil. As allowed by the chemistry utilized, relevant nucleobases in any of SEQ ID NOS: 1-9 can comprise a 5-methyl group. Exemplary embodiments of oligomer chemistries of the disclosure are further described below.

1. Peptide Nucleic Acids (PNAs)

Peptide nucleic acids (PNAs) are analogs of DNA in which the backbone is structurally homomorphous with a deoxyribose backbone, consisting of N-(2-aminoethyl) glycine units to which pyrimidine or purine bases are attached. PNAs containing natural pyrimidine and purine bases hybridize to complementary oligomers obeying Watson-Crick base-pairing rules, and mimic DNA in terms of base pair recognition . The backbone of PNAs is formed by peptide bonds rather than phosphodiester bonds, making them well-suited for antisense applications (see structure below). The backbone is uncharged, resulting in PNA/DNA or PNA/RNA duplexes that exhibit greater than normal thermal stability. PNAs are not recognized by nucleases or proteases. A non-limiting example of a PNA is depicted below.



PNA

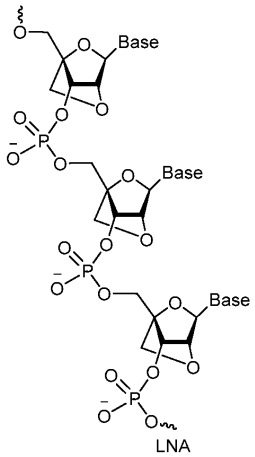
Despite a radical structural change to the natural structure, PNAs are capable of sequence-specific binding in a helix form to DNA or RNA. Characteristics of PNAs include a high binding affinity to complementary DNA or RNA, a destabilizing effect caused by single-base mismatch, resistance to nucleases and proteases, hybridization with DNA or RNA independent of salt concentration and triplex formation with homopurine DNA. PANAGENE™ has developed its proprietary Bts PNA monomers (Bts; benzothiazole-2-

sulfonyl group) and proprietary oligomerization process. The PNA oligomerization using Bts PNA monomers is composed of repetitive cycles of deprotection, coupling and capping. PNAs can be produced synthetically using any technique known in the art. *See*, *e.g.*, U.S. Pat. Nos.: 6,969,766; 7,211,668; 7,022,851; 7,125,994; 7,145,006; and 7,179,896. See also U.S. Pat. Nos.: 5,539,082; 5,714,331; and 5,719,262 for the preparation of PNAs. Further teaching of PNA compounds can be found in Nielsen *et al.*, *Science*, 254:1497-1500, 1991. Each of the foregoing is incorporated by reference in its entirety.

2. Locked Nucleic Acids (LNAs)

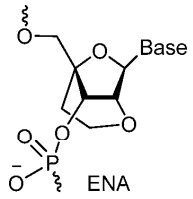
Antisense oligomers may also contain "locked nucleic acid" subunits (LNAs). "LNAs" are a member of a class of modifications called bridged nucleic acid (BNA). BNA is characterized by a covalent linkage that locks the conformation of the ribose ring in a C30-endo (northern) sugar pucker. For LNA, the bridge is composed of a methylene between the 2'-O and the 4'-C positions. LNA enhances backbone preorganization and base stacking to increase hybridization and thermal stability.

The structures of LNAs can be found, for example, in Wengel, *et al.*, *Chemical Communications* (1998) 455; Koshkin *et al.*, *Tetrahedron* (1998) 54:3607; Jesper Wengel, *Accounts of Chem. Research* (1999) 32:301; Obika, *et al.*, *Tetrahedron Letters* (1997) 38:8735; Obika, *et al.*, *Tetrahedron Letters* (1998) 39:5401; and Obika, *et al.*, *Bioorganic Medicinal Chemistry* (2008) 16:9230, which are hereby incorporated by reference in their entirety. A non-limiting example of an LNA is depicted below.



Antisense oligomers of the disclosure may incorporate one or more LNAs; in some cases, the antisense oligomers may be entirely composed of LNAs. Methods for the synthesis of individual LNA nucleoside subunits and their incorporation into oligomers are described, for example, in U.S. Pat.: Nos. 7,572,582; 7,569,575; 7,084,125; 7,060,809; 7,053,207; 7,034,133; 6,794,499; and 6,670,461; each of which is incorporated by reference in its entirety. Typical intersubunit linkers include phosphodiester and phosphorothioate moieties; alternatively, non-phosphorous containing linkers may be employed. Further embodiments include an LNA containing antisense oligomer where each LNA subunit is separated by a DNA subunit. Certain antisense oligomers are composed of alternating LNA and DNA subunits where the intersubunit linker is phosphorothioate.

2'O,4'C-ethylene-bridged nucleic acids (ENAs) are another member of the class of BNAs. A non-limiting example is depicted below.



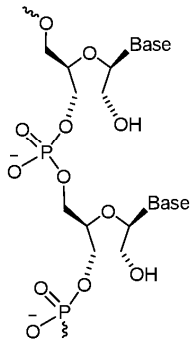
ENA

ENA oligomers and their preparation are described in Obika *et al.*, *Tetrahedron Lett* (1997) 38 (50): 8735, which is hereby incorporated by reference in its entirety. Antisense oligomers of the disclosure may incorporate one or more ENA subunits.

3. Unlocked nucleic acid (UNA)

Antisense oligomers may also contain unlocked nucleic acid (UNA) subunits. UNAs and UNA oligomers are an analogue of RNA in which the C2′-C3' bond of the subunit has been cleaved. Whereas LNA is conformationally restricted (relative to DNA and RNA),

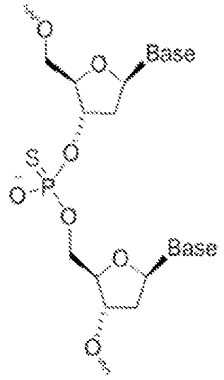
UNA is very flexible. UNAs are disclosed, for example, in WO 2016/070166. A non-limiting example of an UNA is depicted below.



Typical intersubunit linkers include phosphodiester and phosphorothioate moieties; alternatively, non-phosphorous containing linkers may be employed.

4. Phosphorothioates

"Phosphorothioates" (or S-oligos) are a variant of normal DNA in which one of the nonbridging oxygens is replaced by a sulfur. A non-limiting example of a phosphorothioate is depicted below.



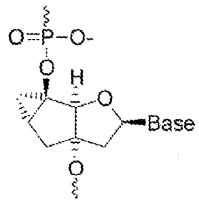
The sulfurization of the internucleotide bond reduces the action of endo-and exonucleases including 5' to 3' and 3' to 5' DNA POL 1 exonuclease, nucleases S1 and P1, RNases, serum nucleases and snake venom phosphodiesterase. Phosphorothioates are made by two principal routes: by the action of a solution of elemental sulfur in carbon disulfide on a hydrogen phosphonate, or by the method of sulfurizing phosphite triesters with either tetraethylthiuram disulfide (TETD) or 3H-1, 2-benzodithiol-3-one 1, 1-dioxide (BDTD)

(*see*, *e.g.*, Iyer *et al.*, *J. Org. Chem.* 55, 4693-4699, 1990, which is hereby incorporated by reference in its entirety). The latter methods avoid the problem of elemental sulfur's insolubility in most organic solvents and the toxicity of carbon disulfide. The TETD and BDTD methods also yield higher purity phosphorothioates.

5. Tricyclo-DNAs and Tricyclo-Phosphorothioate Subunits

Tricyclo-DNAs (tc-DNA) are a class of constrained DNA analogs in which each nucleotide is modified by the introduction of a cyclopropane ring to restrict conformational flexibility of the backbone and to optimize the backbone geometry of the torsion angle γ. Homobasic adenine- and thymine-containing tc-DNAs form extraordinarily stable A-T base pairs with complementary RNAs. Tricyclo-DNAs and their synthesis are described in International Patent Application Publication No. WO 2010/115993, which is hereby incorporated by reference in its entirety. Antisense oligomers of the disclosure may incorporate one or more tricycle-DNA subunits; in some cases, the antisense oligomers may be entirely composed of tricycle-DNA subunits.

Tricyclo-phosphorothioate subunits are tricyclo-DNA subunits with phosphorothioate intersubunit linkages. Tricyclo-phosphorothioate subunits and their synthesis are described in International Patent Application Publication No. WO 2013/053928, which is hereby incorporated by reference in its entirety. Antisense oligomers of the disclosure may incorporate one or more tricycle-DNA subunits; in some cases, the antisense oligomers may be entirely composed of tricycle-DNA subunits. A non-limiting example of a tricycle-DNA/tricycle- phosphorothioate subunit is depicted below.

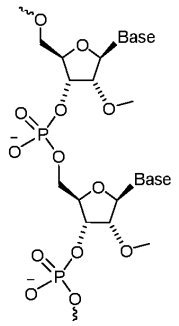


tricyclo-DNA

6. 2'-O-Methyl, 2'-O-MOE, and 2'-F Oligomers

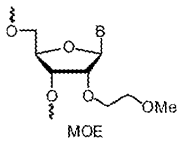
"2'-O-Me oligomer" molecules carry a methyl group at the 2'-OH residue of the ribose molecule. 2'-O-Me-RNAs show the same (or similar) behavior as DNA, but are protected against nuclease degradation. 2'-O-Me-RNAs can also be combined with

phosphorothioate oligomers (PTOs) for further stabilization. 2'O-Me oligomers (phosphodiester or phosphorothioate) can be synthesized according to routine techniques in the art (*see*, *e.g.*, Yoo *et al.*, *Nucleic Acids Res.* 32:2008-16, 2004, which is hereby incorporated by reference in its entirety). A non-limiting example of a 2'-O-Me oligomer is depicted below.



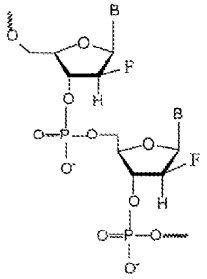
2'-O-Me

2'-O-Methoxyethyl Oligomers (2'-O-MOE) carry a methoxyethyl group at the 2'-OH residue of the ribose molecule and are discussed in Martin *et al.*, *Helv. Chim. Acta,* 78, 486-504, 1995, which is hereby incorporated by reference in its entirety. A non-limiting example of a 2'-O-MOE subunit is depicted below.



MOE

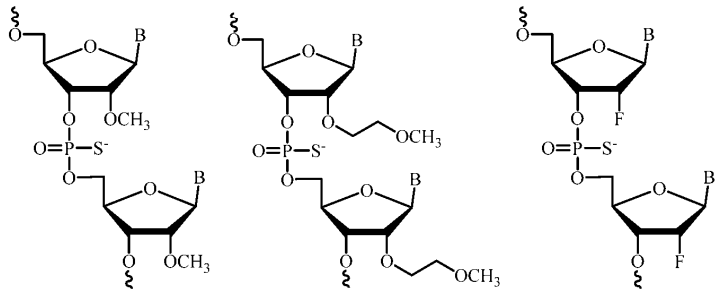
2'-Fluoro (2'-F) oligomers have a fluoro radical in at the 2' position in place of the 2'-OH. A non-limiting example of a 2'-F oligomer is depicted below.



2'-F

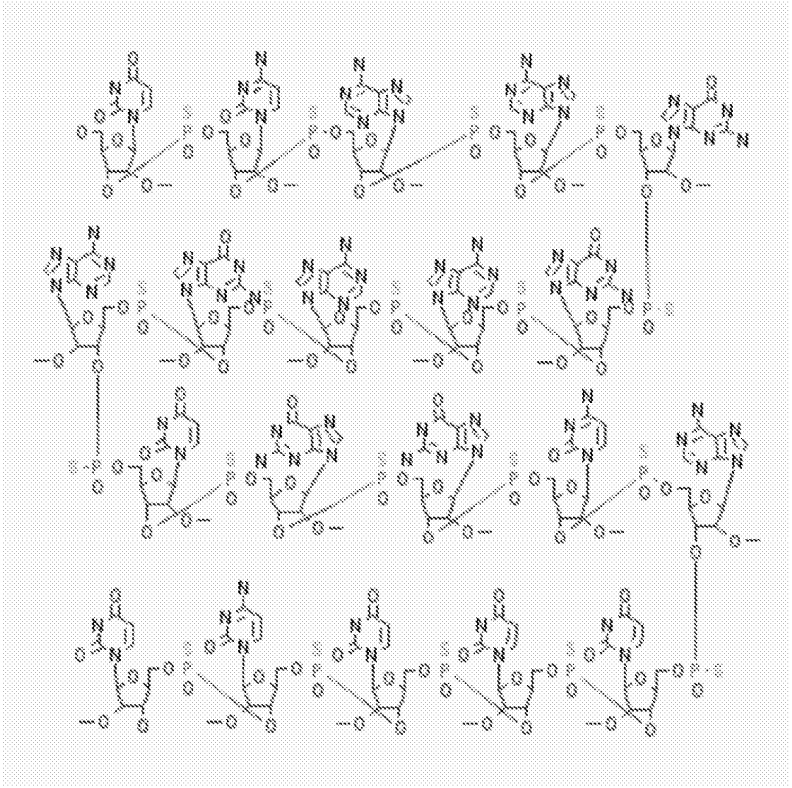
2'-fluoro oligomers are further described in WO 2004/043977, which is hereby incorporated by reference in its entirety.

2'-O-Methyl, 2'-O-MOE, and 2'-F oligomers may also comprise one or more phosphorothioate (PS) linkages as depicted below.

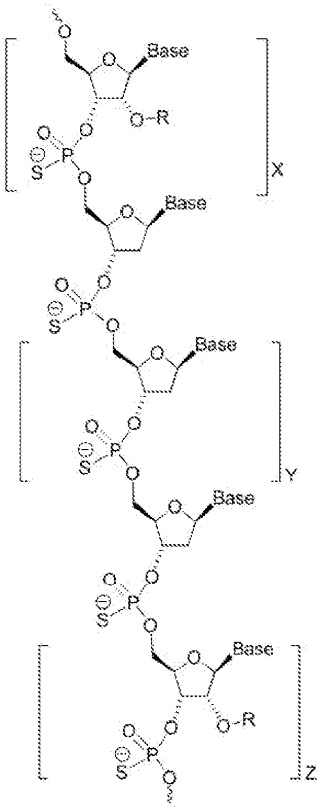


2'-O-Methyl PS 2'-O-MOE PS 2'-F PS

Additionally, 2'-O-Methyl, 2'-O-MOE, and 2'-F oligomers may comprise PS intersubunit linkages throughout the oligomer, for example, as in the 2'-O-methyl PS oligomer drisapersen depicted below.



Alternatively, 2'-O-Methyl, 2'-O-MOE, and/or 2'-F oligomers may comprise PS linkages at the ends of the oligomer, as depicted below.



where:

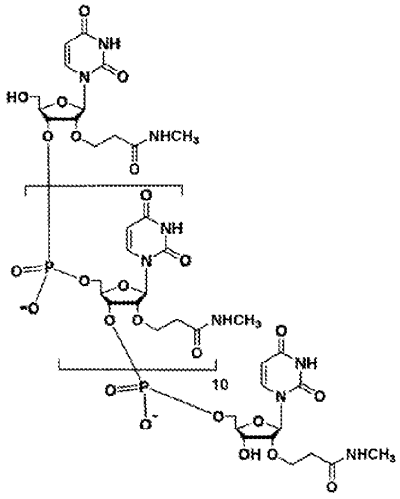
R is $CH_2CH_2OCH_3$ (methoxyethyl or MOE); and

X, Y, and Z denote the number of nucleotides contained within each of the designated 5'-wing, central gap, and 3'-wing regions, respectively.

Antisense oligomers of the disclosure may incorporate one or more 2'-O-Methyl, 2'-O-MOE, and 2'-F subunits and may utilize any of the intersubunit linkages described here. In some instances, an antisense oligomer of the disclosure may be composed of entirely 2'-O-Methyl, 2'-O-MOE, or 2'-F subunits. One embodiment of an antisense oligomers of the disclosure is composed entirely of 2'-O-methyl subunits.

7. 2'-O-[2-(N-methylcarbamoyl)ethyl] Oligomers (MCEs)

MCEs are another example of 2'-O modified ribonucleosides useful in the antisense oligomers of the disclosure. Here, the 2'-OH is derivatized to a 2-(N-methylcarbamoyl)ethyl moiety to increase nuclease resistance. A non-limiting example of an MCE oligomer is depicted below.

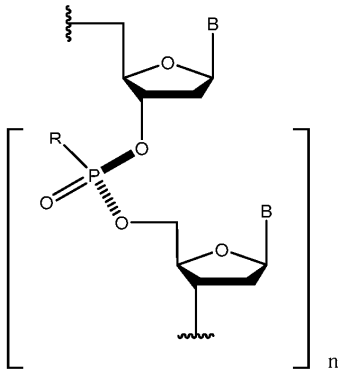


MCEs and their synthesis are described in Yamada *et al.*, *J. Org. Chem.* (2011) 76(9):3042-53, which is hereby incorporated by reference in its entirety. Antisense oligomers of the disclosure may incorporate one or more MCE subunits.

8. Stereo Specific Oligomers

Stereo specific oligomers are those in which the stereo chemistry of each phosphorous-containing linkage is fixed by the method of synthesis such that a substantially

stereo-pure oligomer is produced. A non-limiting example of a stereo specific oligomer is depicted below.



In the above example, each phosphorous of the oligomer has the same stereo configuration. Additional examples include the oligomers described herein. For example, LNAs, ENAs, Tricyclo-DNAs, MCEs, 2'-O-Methyl, 2'-O-MOE, 2'-F, and morpholino-based oligomers can be prepared with stereo-specific phosphorous-containing internucleoside linkages such as, for example, phosphorothioate, phosphodiester, phosphoramidate, phosphorodiamidate, or other phosphorous-containing internucleoside linkages. Stereo specific oligomers, methods of preparation, chiral controlled synthesis, chiral design, and chiral auxiliaries for use in preparation of such oligomers are detailed, for example, in WO2017192664, WO2017192679, WO2017062862, WO2017015575, WO2017015555, WO2015107425, WO2015108048, WO2015108046, WO2015108047, WO2012039448, WO2010064146, WO2011034072, WO2014010250, WO2014012081, WO20130127858, and WO2011005761, each of which is hereby incorporated by reference in its entirety.

Stereo specific oligomers can have phosphorous-containing internucleoside linkages in an R_P or S_P configuration. Chiral phosphorous-containing linkages in which the stereo configuration of the linkages is controlled is referred to as "stereopure," while chiral phosphorous-containing linkages in which the stereo configuration of the linkages is uncontrolled is referred to as "stereorandom." In certain embodiments, the oligomers of the disclosure comprise a plurality of stereopure and stereorandom linkages, such that the resulting oligomer has stereopure subunits at pre-specified positions of the oligomer. An example of the location of the stereopure subunits is provided in international patent

application publication number WO 2017/062862 A2 in Figures 7A and 7B. In an embodiment, all the chiral phosphorous-containing linkages in an oligomer are stereorandom. In an embodiment, all the chiral phosphorous-containing linkages in an oligomer are stereopure.

In an embodiment of an oligomer with n chiral phosphorous-containing linkages (where n is an integer of 1 or greater), all n of the chiral phosphorous-containing linkages in the oligomer are stereorandom. In an embodiment of an oligomer with n chiral phosphorous-containing linkages (where n is an integer of 1 or greater), all n of the chiral phosphorous-containing linkages in the oligomer are stereopure. In an embodiment of an oligomer with n chiral phosphorous-containing linkages (where n is an integer of 1 or greater), at least 10% (to the nearest integer) of the n phosphorous-containing linkages in the oligomer are stereopure. In an embodiment of an oligomer with n chiral phosphorous-containing linkages (where n is an integer of 1 or greater), at least 20% (to the nearest integer) of the n phosphorous-containing linkages in the oligomer are stereopure. In an embodiment of an oligomer with n chiral phosphorous-containing linkages (where n is an integer of 1 or greater), at least 30% (to the nearest integer) of the n phosphorous-containing linkages in the oligomer are stereopure. In an embodiment of an oligomer with n chiral phosphorous-containing linkages (where n is an integer of 1 or greater), at least 40% (to the nearest integer) of the n phosphorous-containing linkages in the oligomer are stereopure. In an embodiment of an oligomer with n chiral phosphorous-containing linkages (where n is an integer of 1 or greater), at least 50% (to the nearest integer) of the n phosphorous-containing linkages in the oligomer are stereopure. In an embodiment of an oligomer with n chiral phosphorous-containing linkages (where n is an integer of 1 or greater), at least 60% (to the nearest integer) of the n phosphorous-containing linkages in the oligomer are stereopure. In an embodiment of an oligomer with n chiral phosphorous-containing linkages (where n is an integer of 1 or greater), at least 70% (to the nearest integer) of the n phosphorous-containing linkages in the oligomer are stereopure. In an embodiment of an oligomer with n chiral phosphorous-containing linkages (where n is an integer of 1 or greater), at least 80% (to the nearest integer) of the n phosphorous-containing linkages in the oligomer are stereopure. In an embodiment of an oligomer with n chiral phosphorous-containing linkages (where n is an integer of 1 or greater), at least 90% (to the nearest integer) of the n phosphorous-containing linkages in the oligomer are stereopure.

In an embodiment of an oligomer with n chiral phosphorous-containing linkages (where n is an integer of 1 or greater), the oligomer contains at least 2 contiguous stereopure phosphorous-containing linkages of the same stereo orientation (*i.e.* either S_P or R_P). In an embodiment of an oligomer with n chiral phosphorous-containing linkages (where n is an integer of 1 or greater), the oligomer contains at least 3 contiguous stereopure phosphorous-containing linkages of the same stereo orientation (*i.e.* either S_P or R_P). In an embodiment of an oligomer with n chiral phosphorous-containing linkages (where n is an integer of 1 or greater), the oligomer contains at least 4 contiguous stereopure phosphorous-containing linkages of the same stereo orientation (*i.e.* either S_P or R_P). In an embodiment of an oligomer with n chiral phosphorous-containing linkages (where n is an integer of 1 or greater), the oligomer contains at least 5 contiguous stereopure phosphorous-containing linkages of the same stereo orientation (*i.e.* either S_P or R_P). In an embodiment of an oligomer with n chiral phosphorous-containing linkages (where n is an integer of 1 or greater), the oligomer contains at least 6 contiguous stereopure phosphorous-containing linkages of the same stereo orientation (*i.e.* either S_P or R_P). In an embodiment of an oligomer with n chiral phosphorous-containing linkages (where n is an integer of 1 or greater), the oligomer contains at least 7 contiguous stereopure phosphorous-containing linkages of the same stereo orientation (*i.e.* either S_P or R_P). In an embodiment of an oligomer with n chiral phosphorous-containing linkages (where n is an integer of 1 or greater), the oligomer contains at least 8 contiguous stereopure phosphorous-containing linkages of the same stereo orientation (*i.e.* either S_P or R_P). In an embodiment of an oligomer with n chiral phosphorous-containing linkages (where n is an integer of 1 or greater), the oligomer contains at least 9 contiguous stereopure phosphorous-containing linkages of the same stereo orientation (*i.e.* either S_P or R_P). In an embodiment of an oligomer with n chiral phosphorous-containing linkages (where n is an integer of 1 or greater), the oligomer contains at least 10 contiguous stereopure phosphorous-containing linkages of the same stereo orientation (*i.e.* either S_P or R_P). In an embodiment of an oligomer with n chiral phosphorous-containing linkages (where n is an integer of 1 or greater), the oligomer contains at least 11 contiguous stereopure phosphorous-containing linkages of the same stereo orientation (*i.e.* either S_P or R_P). In an embodiment of an oligomer with n chiral phosphorous-containing linkages (where n is an integer of 1 or greater), the oligomer contains at least 12 contiguous stereopure phosphorous-containing linkages of the same stereo orientation (*i.e.* either S_P or R_P). In an embodiment of an

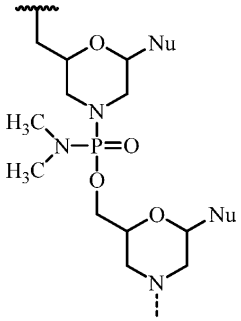
oligomer with n chiral phosphorous-containing linkages (where n is an integer of 1 or greater), the oligomer contains at least 13 contiguous stereopure phosphorous-containing linkages of the same stereo orientation (*i.e.* either S_P or R_P). In an embodiment of an oligomer with n chiral phosphorous-containing linkages (where n is an integer of 1 or greater), the oligomer contains at least 14 contiguous stereopure phosphorous-containing linkages of the same stereo orientation (*i.e.* either S_P or R_P). In an embodiment of an oligomer with n chiral phosphorous-containing linkages (where n is an integer of 1 or greater), the oligomer contains at least 15 contiguous stereopure phosphorous-containing linkages of the same stereo orientation (*i.e.* either S_P or R_P). In an embodiment of an oligomer with n chiral phosphorous-containing linkages (where n is an integer of 1 or greater), the oligomer contains at least 16 contiguous stereopure phosphorous-containing linkages of the same stereo orientation (*i.e.* either S_P or R_P). In an embodiment of an oligomer with n chiral phosphorous-containing linkages (where n is an integer of 1 or greater), the oligomer contains at least 17 contiguous stereopure phosphorous-containing linkages of the same stereo orientation (*i.e.* either S_P or R_P). In an embodiment of an oligomer with n chiral phosphorous-containing linkages (where n is an integer of 1 or greater), the oligomer contains at least 18 contiguous stereopure phosphorous-containing linkages of the same stereo orientation (*i.e.* either S_P or R_P). In an embodiment of an oligomer with n chiral phosphorous-containing linkages (where n is an integer of 1 or greater), the oligomer contains at least 19 contiguous stereopure phosphorous-containing linkages of the same stereo orientation (*i.e.* either S_P or R_P). In an embodiment of an oligomer with n chiral phosphorous-containing linkages (where n is an integer of 1 or greater), the oligomer contains at least 20 contiguous stereopure phosphorous-containing linkages of the same stereo orientation (*i.e.* either S_P or R_P).

In an embodiment of an oligomer with n chiral phosphorous-containing linkages (where n is an integer of 1 or greater), the oligomer contains at least 2 contiguous stereopure phosphorous-containing linkages of the same stereo orientation (*i.e.* either S_P or R_P) and at least 2 contiguous stereopure phosphorous-containing linkages of the other stereo orientation. For example, the oligomer can contain at least 2 contiguous stereopure phosphorous-containing linkages of the S_P orientation and at least 2 contiguous stereopure phosphorous-containing linkages of the R_P orientation.

In an embodiment of an oligomer with n chiral phosphorous-containing linkages (where n is an integer of 1 or greater), the oligomer contains at least 2 contiguous stereopure phosphorous-containing linkages of the same stereo orientation in an alternating pattern. For example, the oligomer can contain the following in order: 2 or more R_P, 2 or more S_P, and 2 or more R_P, etc.

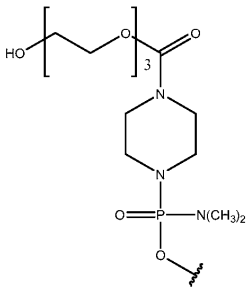
9. Morpholino Oligomers

Exemplary embodiments of the disclosure relate to phosphorodiamidate morpholino oligomers of the following general structure:

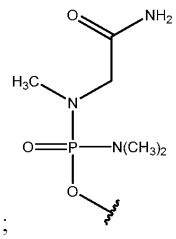


and as described in Figure 2 of Summerton, J., *et al.*, *Antisense & Nucleic Acid Drug Development*, 7: 187-195 (1997). Morpholinos as described herein are intended to cover all stereoisomers and tautomers of the foregoing general structure. The synthesis, structures, and binding characteristics of morpholino oligomers are detailed in U.S. Patent Nos.: 5,698,685; 5,217,866; 5,142,047; 5,034,506; 5,166,315; 5,521,063; 5,506,337; 8,076,476; and 8,299,206, all of which are incorporated herein by reference.

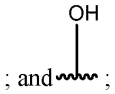
In certain embodiments, a morpholino is conjugated at the 5' or 3' end of the oligomer with a "tail" moiety to increase its stability and/or solubility. Exemplary tails include:



;



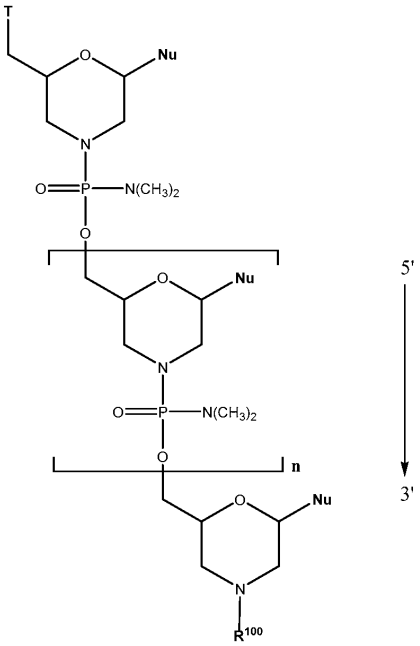
;



; and ;

and the distal –OH or –NH_2 of the "tail" moiety is optionally linked to a cell-penetrating peptide.

In various aspects, the disclosure provides antisense oligomers according to Formula (I):

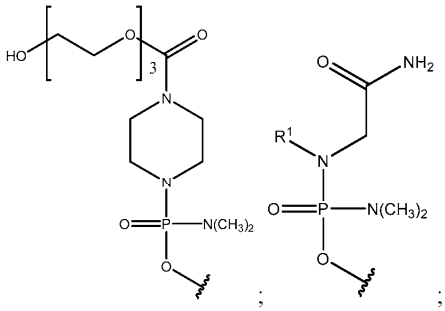


(I)

or a pharmaceutically acceptable salt thereof, wherein:

each **Nu** is a nucleobase which taken together form a targeting sequence;

T is a moiety selected from:

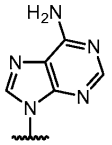
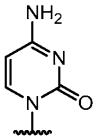
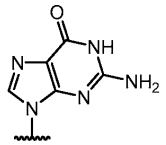
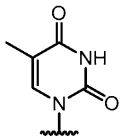


; ; and ; and the distal –OH or –NH_2 of the T moiety is optionally linked to a cell-penetrating peptide;

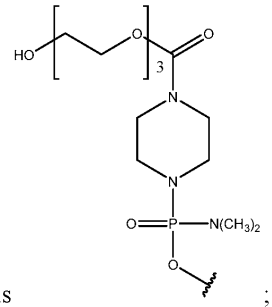
R^{100} is hydrogen or a cell-penetrating peptide;

each **Nu** from 1 to **n** and 5' to 3' corresponds to the nucleobases in one of the following:

Annealing Site	**Targeting Sequence [5' to 3']**	**SEQ ID NO:**
H50D(+04-18)	GGG ATC CAG TAT ACT TAC AGG C	SEQ ID NO: 1
H50D(+07-18)	GGG ATC CAG TAT ACT TAC AGG CTC C	SEQ ID NO: 2
H50D(+07-16)	GAT CCA GTA TAC TTA CAG GCT CC	SEQ ID NO: 3
H50D(+07-17)	GGA TCC AGT ATA CTT ACA GGC TCC	SEQ ID NO: 4
H50A(-19+07)	ACT TCC TCT TTA ACA GAA AAG CAT AC	SEQ ID NO: 5
H50D(+07-15)	ATC CAG TAT ACT TAC AGG CTC C	SEQ ID NO: 6
H50A(-02+23)	GAG CTC AGA TCT TCT AAC TTC CTC T	SEQ ID NO: 7
H50D(+06-18)	GGG ATC CAG TAT ACT TAC AGG CTC	SEQ ID NO: 8
H50D(+07-20)	ATG GGA TCC AGT ATA CTT ACA GGC TCC	SEQ ID NO: 9

wherein A is  , C is  , G is  , and T is  .

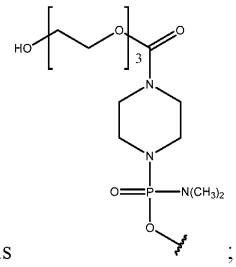
In some embodiments, each **Nu** from 1 to **n** and 5' to 3' corresponds to one of the following: SEQ ID NO. 1, SEQ ID NO. 3, SEQ ID NO. 4, SEQ ID NO. 5, SEQ ID NO. 6, SEQ ID NO. 7, SEQ ID NO. 8, or SEQ ID NO. 9. In some embodiments, each **Nu** from 1 to **n** and 5' to 3' corresponds SEQ ID NO. 3.



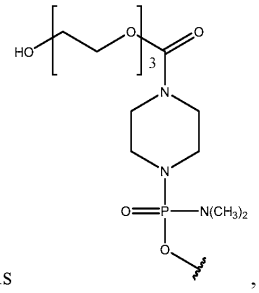
In various embodiments, T is ; and the distal –OH of the T moiety is optionally linked to a cell-penetrating peptide.

In various embodiments, R^{100} is hydrogen. In various other embodiments, R^{100} is a cell-penetrating peptide. In various embodiments, R^{100} is $–R^5$ (SEQ ID NO: 21). In various other embodiments, R^{100} is -G-R^5 (SEQ ID NO: 20). In various embodiments, R^{100} is -R^6 (SEQ ID NO: 10). In various other embodiments, R^{100} is -G-R^6 (SEQ ID NO: 11).

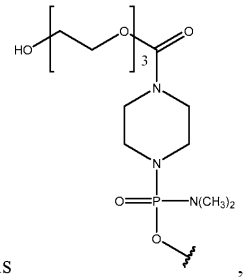
In some embodiments, an antisense oligomer of Formula (I) is in free base form. In some embodiments, an antisense oligomer of Formula (I) is a pharmaceutically acceptable salt thereof. In some embodiments, an antisense oligomer of Formula (I) is an HCl (hydrochloric acid) salt thereof. In certain embodiments, the HCl salt is a 1HCl, 2HCl, 3HCl, 4HCl, 5HCl, or 6HCl salt. In certain embodiments, the HCl salt is a 6HCl salt.



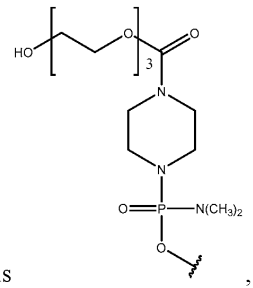
In various embodiments, T is ; and the distal –OH of the T moiety is optionally linked to a cell-penetrating peptide, and R^{100} is a cell-penetrating peptide.



In various embodiments, T is , and R^{100} is a cell-penetrating peptide.

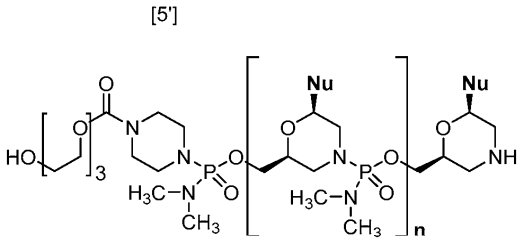


In various embodiments, T is , and R^{100} is -G-R^5 (SEQ ID NO: 20).



In various embodiments, T is , and R^{100} is -G-R^6 (SEQ ID NO: 11).

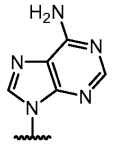
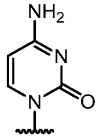
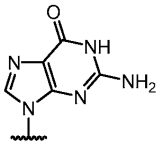
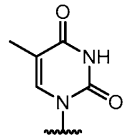
In some embodiments, an antisense oligomer of the disclosure is according to Formula (II):



(II)

or a pharmaceutically acceptable salt thereof, where each **Nu** from 1 to **n** and 5' to 3' corresponds to the nucleobases in one of the following:

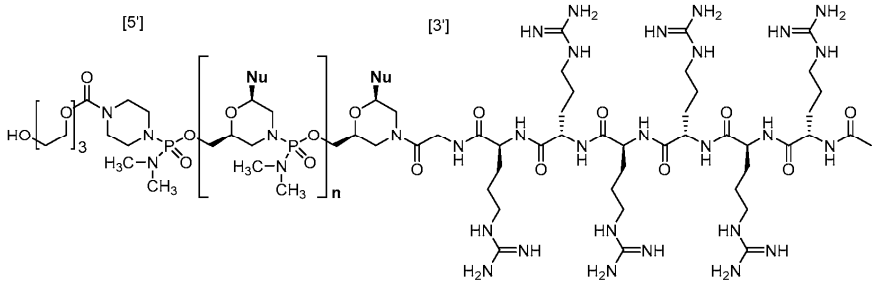
Annealing Site	**Targeting Sequence [5' to 3']**	**SEQ ID NO:**
H50D(+04-18)	GGG ATC CAG TAT ACT TAC AGG C	SEQ ID NO: 1
H50D(+07-18)	GGG ATC CAG TAT ACT TAC AGG CTC C	SEQ ID NO: 2
H50D(+07-16)	GAT CCA GTA TAC TTA CAG GCT CC	SEQ ID NO: 3
H50D(+07-17)	GGA TCC AGT ATA CTT ACA GGC TCC	SEQ ID NO: 4
H50A(-19+07)	ACT TCC TCT TTA ACA GAA AAG CAT AC	SEQ ID NO: 5
H50D(+07-15)	ATC CAG TAT ACT TAC AGG CTC C	SEQ ID NO: 6
H50A(-02+23)	GAG CTC AGA TCT TCT AAC TTC CTC T	SEQ ID NO: 7
H50D(+06-18)	GGG ATC CAG TAT ACT TAC AGG CTC	SEQ ID NO: 8
H50D(+07-20)	ATG GGA TCC AGT ATA CTT ACA GGC TCC	SEQ ID NO: 9

wherein A is , C is , G is , and T is . In some embodiments, the distal –OH of Formula (II) is linked to a cell-penetrating peptide.

In some embodiments, each **Nu** from 1 to **n** and 5' to 3' corresponds to one of the following: SEQ ID NO. 1, SEQ ID NO. 3, SEQ ID NO. 4, SEQ ID NO. 5, SEQ ID NO. 6, SEQ ID NO. 7, SEQ ID NO. 8, or SEQ ID NO. 9. In some embodiments, each **Nu** from 1 to **n** and 5' to 3' corresponds to SEQ ID NO. 3.

In some embodiments, an antisense oligomer of Formula (II) is in free base form. In some embodiments, an antisense oligomer of Formula (II) a pharmaceutically acceptable salt form thereof. In some embodiments, an antisense oligomer of Formula (II) is an HCl (hydrochloric acid) salt thereof. In certain embodiments, the HCl salt is a 1HCl, 2HCl, 3HCl, 4HCl, 5HCl, or 6HCl salt. In certain embodiments, the HCl salt is a 6HCl salt.

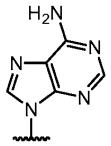
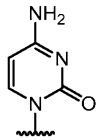
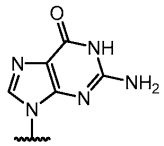
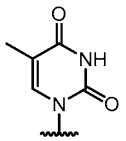
In some embodiments, an antisense oligomer of the disclosure is according to Formula (III):



(III)

or a pharmaceutically acceptable salt thereof, where each **Nu** from 1 to **n** and 5' to 3' corresponds to the nucleobases in one of the following:

Annealing Site	**Targeting Sequence [5' to 3']**	**SEQ ID NO:**
H50D(+04-18)	GGG ATC CAG TAT ACT TAC AGG C	SEQ ID NO: 1
H50D(+07-18)	GGG ATC CAG TAT ACT TAC AGG CTC C	SEQ ID NO: 2
H50D(+07-16)	GAT CCA GTA TAC TTA CAG GCT CC	SEQ ID NO: 3
H50D(+07-17)	GGA TCC AGT ATA CTT ACA GGC TCC	SEQ ID NO: 4
H50A(-19+07)	ACT TCC TCT TTA ACA GAA AAG CAT AC	SEQ ID NO: 5
H50D(+07-15)	ATC CAG TAT ACT TAC AGG CTC C	SEQ ID NO: 6
H50A(-02+23)	GAG CTC AGA TCT TCT AAC TTC CTC T	SEQ ID NO: 7
H50D(+06-18)	GGG ATC CAG TAT ACT TAC AGG CTC	SEQ ID NO: 8
H50D(+07-20)	ATG GGA TCC AGT ATA CTT ACA GGC TCC	SEQ ID NO: 9

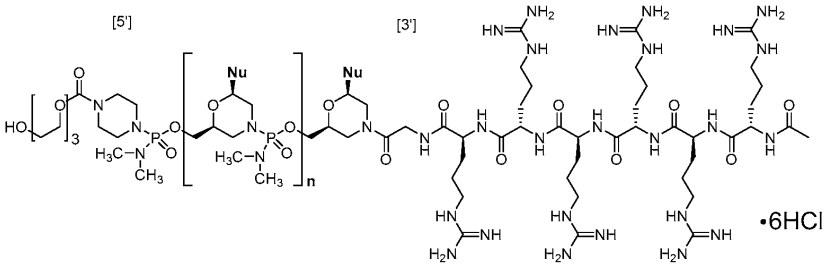
wherein A is , C is , G is , and T is . In some embodiments, the distal –OH of Formula (III) is linked to a cell-penetrating peptide.

In some embodiments, the distal –OH of Formula (III) is optionally linked to a cell-penetrating peptide.

In some embodiments, each **Nu** from 1 to **n** and 5' to 3' corresponds to one of the following: SEQ ID NO. 1, SEQ ID NO. 2, SEQ ID NO. 3, SEQ ID NO. 4, SEQ ID NO. 5, SEQ ID NO. 6, SEQ ID NO. 7, SEQ ID NO. 8, or SEQ ID NO. 9. In some embodiments, each **Nu** from 1 to **n** and 5' to 3' corresponds to SEQ ID NO. 3.

In some embodiments, an antisense oligomer of Formula (III) is in free base form. In some embodiments, an antisense oligomer of Formula (III) is a pharmaceutically acceptable salt thereof. In some embodiments, an antisense oligomer of Formula (III) is an HCl (hydrochloric acid) salt thereof. In certain embodiments, the HCl salt is a 6HCl salt.

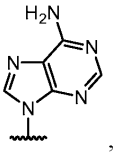
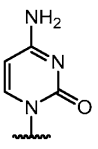
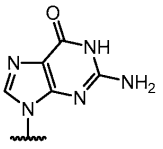
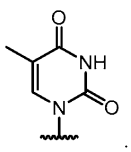
In some embodiments, an antisense oligomer of the disclosure is according to Formula (IV):



(IV)

where each **Nu** from 1 to **n** and 5' to 3' corresponds to the nucleobases in one of the following:

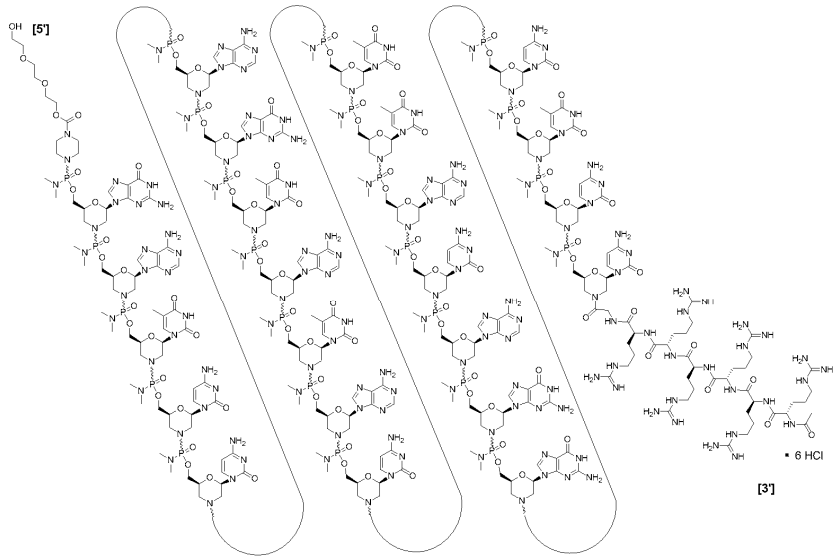
Annealing Site	**Targeting Sequence [5' to 3']**	**SEQ ID NO:**
H50D(+04-18)	GGG ATC CAG TAT ACT TAC AGG C	SEQ ID NO: 1
H50D(+07-18)	GGG ATC CAG TAT ACT TAC AGG CTC C	SEQ ID NO: 2
H50D(+07-16)	GAT CCA GTA TAC TTA CAG GCT CC	SEQ ID NO: 3
H50D(+07-17)	GGA TCC AGT ATA CTT ACA GGC TCC	SEQ ID NO: 4
H50A(-19+07)	ACT TCC TCT TTA ACA GAA AAG CAT AC	SEQ ID NO: 5
H50D(+07-15)	ATC CAG TAT ACT TAC AGG CTC C	SEQ ID NO: 6
H50A(-02+23)	GAG CTC AGA TCT TCT AAC TTC CTC T	SEQ ID NO: 7
H50D(+06-18)	GGG ATC CAG TAT ACT TAC AGG CTC	SEQ ID NO: 8
H50D(+07-20)	ATG GGA TCC AGT ATA CTT ACA GGC TCC	SEQ ID NO: 9

wherein A is , C is , G is , and T is .

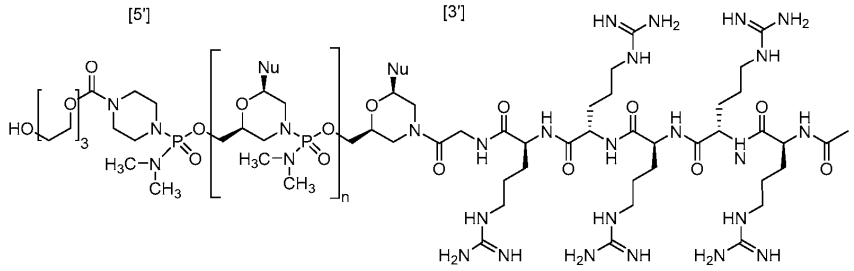
In some embodiments, the distal –OH of Formula (IV) is optionally linked to a cell-penetrating peptide.

In some embodiments, each **Nu** from 1 to **n** and 5' to 3' corresponds to one of the following: SEQ ID NO. 1, SEQ ID NO. 2, SEQ ID NO. 3, SEQ ID NO. 4, SEQ ID NO. 5, SEQ ID NO. 6, SEQ ID NO. 7, SEQ ID NO. 8, or SEQ ID NO. 9. In some embodiments, each **Nu** from 1 to **n** and 5' to 3' corresponds to SEQ ID NO. 3.

In some embodiments, including, for example, some embodiments of Formula (IV), the antisense oligomer is according to Formula (IVa):



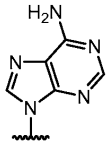
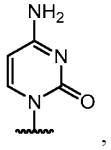
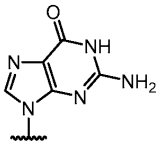
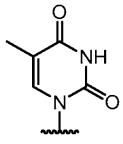
In some embodiments, an antisense oligomer of the disclosure is according to Formula (V):



(V)

or a pharmaceutically acceptable salt thereof, where each **Nu** from 1 to **n** and 5' to 3' corresponds to the nucleobases in one of the following:

Annealing Site	Targeting Sequence [5' to 3']	SEQ ID NO:
H50D(+04-18)	GGG ATC CAG TAT ACT TAC AGG C	SEQ ID NO: 1
H50D(+07-18)	GGG ATC CAG TAT ACT TAC AGG CTC C	SEQ ID NO: 2
H50D(+07-16)	GAT CCA GTA TAC TTA CAG GCT CC	SEQ ID NO: 3
H50D(+07-17)	GGA TCC AGT ATA CTT ACA GGC TCC	SEQ ID NO: 4
H50A(-19+07)	ACT TCC TCT TTA ACA GAA AAG CAT AC	SEQ ID NO: 5
H50D(+07-15)	ATC CAG TAT ACT TAC AGG CTC C	SEQ ID NO: 6
H50A(-02+23)	GAG CTC AGA TCT TCT AAC TTC CTC T	SEQ ID NO: 7
H50D(+06-18)	GGG ATC CAG TAT ACT TAC AGG CTC	SEQ ID NO: 8
H50D(+07-20)	ATG GGA TCC AGT ATA CTT ACA GGC TCC	SEQ ID NO: 9

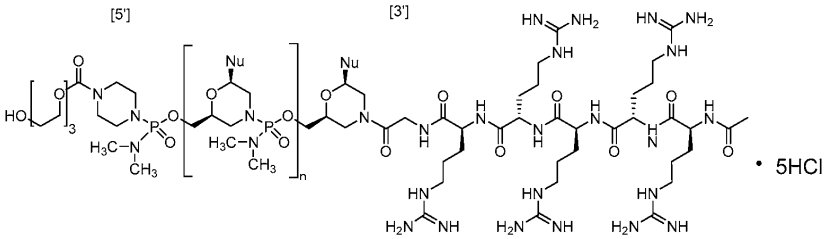
wherein A is , C is , G is , and T is . In some embodiments, the distal –OH of Formula (IV) is linked to a cell-penetrating peptide.

In some embodiments, the distal –OH of Formula (V) is optionally linked to a cell-penetrating peptide.

In some embodiments, each **Nu** from 1 to **n** and 5' to 3' corresponds to one of the following: SEQ ID NO. 1, SEQ ID NO. 2, SEQ ID NO. 3, SEQ ID NO. 4, SEQ ID NO. 5, SEQ ID NO. 6, SEQ ID NO. 7, SEQ ID NO. 8, or SEQ ID NO. 9. In some embodiments, each **Nu** from 1 to **n** and 5' to 3' corresponds to SEQ ID NO. 3.

In some embodiments, an antisense oligomer of Formula (V) is in free base form. In some embodiments, an antisense oligomer of Formula (V) is a pharmaceutically acceptable salt thereof. In some embodiments, an antisense oligomer of Formula (V) is an HCl (hydrochloric acid) salt thereof. In certain embodiments, the HCl salt is a 5HCl salt.

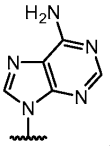
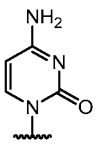
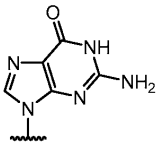
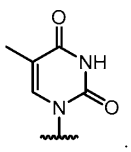
In some embodiments, an antisense oligomer of the disclosure is according to Formula (VI):



(VI)

where each **Nu** from 1 to **n** and 5' to 3' corresponds to the nucleobases in one of the following:

Annealing Site	Targeting Sequence [5' to 3']	SEQ ID NO:
H50D(+04-18)	GGG ATC CAG TAT ACT TAC AGG C	SEQ ID NO: 1
H50D(+07-18)	GGG ATC CAG TAT ACT TAC AGG CTC C	SEQ ID NO: 2
H50D(+07-16)	GAT CCA GTA TAC TTA CAG GCT CC	SEQ ID NO: 3
H50D(+07-17)	GGA TCC AGT ATA CTT ACA GGC TCC	SEQ ID NO: 4
H50A(-19+07)	ACT TCC TCT TTA ACA GAA AAG CAT AC	SEQ ID NO: 5
H50D(+07-15)	ATC CAG TAT ACT TAC AGG CTC C	SEQ ID NO: 6
H50A(-02+23)	GAG CTC AGA TCT TCT AAC TTC CTC T	SEQ ID NO: 7
H50D(+06-18)	GGG ATC CAG TAT ACT TAC AGG CTC	SEQ ID NO: 8
H50D(+07-20)	ATG GGA TCC AGT ATA CTT ACA GGC TCC	SEQ ID NO: 9

wherein A is , C is , G is , and T is .

In some embodiments, the distal –OH of Formula (VI) is optionally linked to a cell-penetrating peptide.

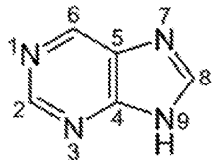
In some embodiments, each **Nu** from 1 to **n** and 5' to 3' corresponds to one of the following: SEQ ID NO. 1, SEQ ID NO. 2, SEQ ID NO. 3, SEQ ID NO. 4, SEQ ID NO. 5, SEQ ID NO. 6, SEQ ID NO. 7, SEQ ID NO. 8, or SEQ ID NO. 9. In some embodiments, each **Nu** from 1 to **n** and 5' to 3' corresponds to SEQ ID NO. 3.

10. Nucleobase Modifications and Substitutions

In certain embodiments, antisense oligomers of the disclosure are composed of RNA nucleobases and DNA nucleobases (often referred to in the art simply as "base"). RNA bases are commonly known as adenine (A), uracil (U), cytosine (C) and guanine (G). DNA bases are commonly known as adenine (A), thymine (T), cytosine (C) and guanine (G). In various embodiments, antisense oligomers of the disclosure are composed of cytosine (C), guanine (G), thymine (T), adenine (A), 5-methylcytosine (5mC), uracil (U), and hypoxanthine (I).

In certain embodiments, one or more RNA bases or DNA bases in an oligomer may be modified or substituted with a base other than a RNA base or DNA base. Oligomers containing a modified or substituted base include oligomers in which one or more purine or pyrimidine bases most commonly found in nucleic acids are replaced with less common or non-natural bases.

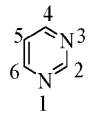
Purine bases comprise a pyrimidine ring fused to an imidazole ring, as described by the following general formula.



Purine

Adenine and guanine are the two purine nucleobases most commonly found in nucleic acids. Other naturally-occurring purines include, but not limited to, N^6-methyladenine, N^2-methylguanine, hypoxanthine, and 7-methylguanine.

Pyrimidine bases comprise a six-membered pyrimidine ring as described by the following general formula.



Pyrimidine Core

Cytosine, uracil, and thymine are the pyrimidine bases most commonly found in nucleic acids. Other naturally-occurring pyrimidines include, but not limited to, 5-methylcytosine,

5-hydroxymethylcytosine, pseudouracil, and 4-thiouracil. In one embodiment, the oligomers described herein contain thymine bases in place of uracil.

Other suitable bases include, but are not limited to: 2,6-diaminopurine, orotic acid, agmatidine, lysidine, 2-thiopyrimidines (*e.g.* 2-thiouracil, 2-thiothymine), G-clamp and its derivatives, 5-substituted pyrimidines (*e.g.* 5-halouracil, 5-propynyluracil, 5-propynylcytosine, 5-aminomethyluracil, 5-hydroxymethyluracil, 5-aminomethylcytosine, 5-hydroxymethylcytosine, Super T), 7-deazaguanine, 7-deazaadenine, 7-aza-2,6-diaminopurine, 8-aza-7-deazaguanine, 8-aza-7-deazaadenine, 8-aza-7-deaza-2,6-diaminopurine, Super G, Super A, and N4-ethylcytosine, or derivatives thereof; N^2-cyclopentylguanine (cPent-G), N^2-cyclopentyl-2-aminopurine (cPent-AP), and N^2-propyl-2-aminopurine (Pr-AP), pseudouracil, or derivatives thereof; and degenerate or universal bases, like 2,6-difluorotoluene or absent bases like abasic sites (*e.g.* 1-deoxyribose, 1,2-dideoxyribose, l-deoxy-2-O-methylribose; or pyrrolidine derivatives in which the ring oxygen has been replaced with nitrogen (azaribose)). Examples of derivatives of Super A, Super G, and Super T can be found in U.S. Patent 6,683,173 (Epoch Biosciences), which is incorporated here entirely by reference. cPent-G, cPent-AP, and Pr-AP were shown to reduce immunostimulatory effects when incorporated in siRNA (Peacock H. *et al. J. Am. Chem.* Soc. 2011, 133, 9200). Pseudouracil is a naturally occurring isomerized version of uracil, with a C-glycoside rather than the regular N-glycoside as in uridine. Pseudouridine-containing synthetic mRNA may have an improved safety profile compared to uridine-containing mPvNA (WO 2009127230, incorporated here in its entirety by reference).

Certain nucleobases are particularly useful for increasing the binding affinity of the antisense oligomers of the disclosure. These include 5-substituted pyrimidines, 6-azapyrimidines, and N-2, N-6, and O-6 substituted purines, including 2-aminopropyladenine, 5-propynyluracil, and 5-propynylcytosine. 5-methylcytosine substitutions have been shown to increase nucleic acid duplex stability by 0.6-1.2 °C and are presently preferred base substitutions, even more particularly when combined with 2'-O-methoxyethyl sugar modifications. Additional exemplary modified nucleobases include those wherein at least one hydrogen atom of the nucleobase is replaced with fluorine.

11. Pharmaceutically Acceptable Salts of Antisense oligomers

Certain embodiments of antisense oligomers described herein may contain a basic functional group, such as amino or alkylamino, and are, thus, capable of forming

pharmaceutically-acceptable salts with pharmaceutically-acceptable acids. The term "pharmaceutically-acceptable salts" in this respect, refers to the relatively non-toxic, inorganic and organic acid addition salts of antisense oligomers of the present disclosure. These salts can be prepared in situ in the administration vehicle or the dosage form manufacturing process, or by separately reacting a purified antisense oligomer of the disclosure in its free base form with a suitable organic or inorganic acid, and isolating the salt thus formed during subsequent purification. Representative salts include the hydrobromide, hydrochloride, sulfate, bisulfate, phosphate, nitrate, acetate, valerate, oleate, palmitate, stearate, laurate, benzoate, lactate, tosylate, citrate, maleate, fumarate, succinate, tartrate, naphthylate, mesylate, glucoheptonate, lactobionate, and laurylsulphonate salts and the like. (*See*, *e.g.*, Berge *et al.* (1977) "Pharmaceutical Salts", *J. Pharm. Sci.* 66:1-19).

The pharmaceutically acceptable salts of the subject antisense oligomers include the conventional nontoxic salts or quaternary ammonium salts of the antisense oligomers, *e.g.*, from non-toxic organic or inorganic acids. For example, such conventional nontoxic salts include those derived from inorganic acids such as hydrochloric, hydrobromic, sulfuric, sulfamic, phosphoric, nitric, and the like; and the salts prepared from organic acids such as acetic, propionic, succinic, glycolic, stearic, lactic, malic, tartaric, citric, ascorbic, palmitic, maleic, hydroxymaleic, phenylacetic, glutamic, benzoic, salicyclic, sulfanilic, 2-acetoxybenzoic, fumaric, toluenesulfonic, methanesulfonic, ethane disulfonic, oxalic, isothionic, and the like.

In certain embodiments, the antisense oligomers of the present disclosure may contain one or more acidic functional groups and, thus, are capable of forming pharmaceutically-acceptable salts with pharmaceutically-acceptable bases. The term "pharmaceutically-acceptable salts" in these instances refers to the relatively non-toxic, inorganic and organic base addition salts of antisense oligomers of the present disclosure. These salts can likewise be prepared in situ in the administration vehicle or the dosage form manufacturing process, or by separately reacting the purified antisense oligomer in its free acid form with a suitable base, such as the hydroxide, carbonate, or bicarbonate of a pharmaceutically-acceptable metal cation, with ammonia, or with a pharmaceutically-acceptable organic primary, secondary, or tertiary amine. Representative alkali or alkaline earth salts include the lithium, sodium, potassium, calcium, magnesium, and aluminum salts and the like. Representative organic amines useful for the formation of base addition salts

include ethylamine, diethylamine, ethylenediamine, ethanolamine, diethanolamine, piperazine and the like. (*See*, *e.g.*, Berge *et al.*, *supra*).

III. Formulations and Modes of Administration

In certain embodiments, the present disclosure provides formulations or pharmaceutical compositions suitable for the therapeutic delivery of antisense oligomers, as described herein. Hence, in certain embodiments, the present disclosure provides pharmaceutically acceptable compositions that comprise a therapeutically-effective amount of one or more of the antisense oligomers described herein, formulated together with one or more pharmaceutically acceptable carriers (additives) and/or diluents. While it is possible for an antisense oligomer of the present disclosure to be administered alone, it is preferable to administer the antisense oligomer as a pharmaceutical formulation (composition). In an embodiment, the antisense oligomer of the formulation is according to Formula (III) or a pharmaceutically acceptable salt thereof.

Methods for the delivery of nucleic acid molecules, which can be applicable to the antisense oligomers of the present disclosure, are described, for example, in: Akhtar *et al.*, 1992, *Trends Cell Bio.*, 2:139; Delivery Strategies for Antisense Oligonucleotide Therapeutics, ed. Akhtar, 1995, CRC Press; and Sullivan *et al.*, PCT WO 94/02595. These and other protocols can be utilized for the delivery of virtually any nucleic acid molecule, including the antisense oligomers of the present disclosure.

The pharmaceutical compositions of the present disclosure may be specially formulated for administration in solid or liquid form, including those adapted for the following: (1) oral administration, for example, drenches (aqueous or non-aqueous solutions or suspensions), tablets (targeted for buccal, sublingual, or systemic absorption), boluses, powders, granules, pastes for application to the tongue; (2) parenteral administration, for example, by subcutaneous, intramuscular, intravenous, or epidural injection as, for example, a sterile solution or suspension, or sustained-release formulation; (3) topical application, for example, as a cream, ointment, or a controlled-release patch or spray applied to the skin; (4) intravaginally or intrarectally, for example, as a pessary, cream, or foam; (5) sublingually; (6) ocularly; (7) transdermally; or (8) nasally.

Some examples of materials that can serve as pharmaceutically-acceptable carriers include, without limitation: (1) sugars, such as lactose, glucose and sucrose; (2) starches,

such as corn starch and potato starch; (3) cellulose, and its derivatives, such as sodium carboxymethyl cellulose, ethyl cellulose, and cellulose acetate; (4) powdered tragacanth; (5) malt; (6) gelatin; (7) talc; (8) excipients, such as cocoa butter and suppository waxes; (9) oils, such as peanut oil, cottonseed oil, safflower oil, sesame oil, olive oil, corn oil, and soybean oil; (10) glycols, such as propylene glycol; (11) polyols, such as glycerin, sorbitol, mannitol, and polyethylene glycol; (12) esters, such as ethyl oleate and ethyl laurate; (13) agar; (14) buffering agents, such as magnesium hydroxide and aluminum hydroxide; (15) alginic acid; (16) pyrogen-free water; (17) isotonic saline; (18) Ringer's solution; (19) ethyl alcohol; (20) pH buffered solutions; (21) polyesters, polycarbonates, and/or polyanhydrides; and (22) other non-toxic compatible substances employed in pharmaceutical formulations.

Additional non-limiting examples of agents suitable for formulation with the antisense oligomers of the instant disclosure include: PEG conjugated nucleic acids; phospholipid conjugated nucleic acids; nucleic acids containing lipophilic moieties; phosphorothioates; P-glycoprotein inhibitors (such as Pluronic P85) which can enhance entry of drugs into various tissues; biodegradable polymers, such as poly (D,L-lactide-coglycolide) microspheres for sustained release delivery after implantation (Emerich, D F *et al.*, 1999, Cell Transplant, 8, 47-58) Alkermes, Inc. Cambridge, Mass.; and loaded nanoparticles, such as those made of polybutylcyanoacrylate, which can deliver drugs across the blood brain barrier and can alter neuronal uptake mechanisms (Prog Neuropsychopharmacol Biol Psychiatry, 23, 941-949, 1999).

The disclosure also features the use of the composition comprising surface-modified liposomes containing poly(ethylene glycol) ("PEG") lipids (PEG-modified, branched and unbranched or combinations thereof, or long-circulating liposomes or stealth liposomes). Oligomer conjugates of the disclosure can also comprise covalently attached PEG molecules of various molecular weights. These formulations offer a method for increasing the accumulation of drugs in target tissues. This class of drug carriers resists opsonization and elimination by the mononuclear phagocytic system (MPS or RES), thereby enabling longer blood circulation times and enhanced tissue exposure for the encapsulated drug (Lasic *et al. Chem. Rev.* 1995, 95, 2601-2627; Ishiwata *et al.*, *Chem. Pharm. Bull.* 1995, 43, 1005-1011). Such liposomes have been shown to accumulate selectively in tumors, presumably by extravasation and capture in the neovascularized target tissues (Lasic *et al.*, *Science* 1995, 267, 1275-1276; Oku *et al.*, 1995, *Biochim. Biophys. Acta*, 1238, 86-90). The long-

circulating liposomes enhance the pharmacokinetics and pharmacodynamics of DNA and RNA, particularly compared to conventional cationic liposomes which are known to accumulate in tissues of the MPS (Liu *et al.*, *J. Biol. Chem.* 1995, 42, 24864-24870; Choi *et al.*, International PCT Publication No. WO 96/10391; Ansell *et al.*, International PCT Publication No. WO 96/10390; Holland *et al.*, International PCT Publication No. WO 96/10392). Long-circulating liposomes are also likely to protect drugs from nuclease degradation to a greater extent compared to cationic liposomes, based on their ability to avoid accumulation in metabolically aggressive MPS tissues such as the liver and spleen.

In a further embodiment, the present disclosure includes antisense oligomer pharmaceutical compositions prepared for delivery as described in U.S. Pat. Nos.: 6,692,911; 7,163,695; and 7,070,807. In this regard, in one embodiment, the present disclosure provides an antisense oligomer of the present disclosure in a composition comprising copolymers of lysine and histidine (HK) (as described in U.S. Pat. Nos.: 7,163,695; 7,070,807; and 6,692,911) either alone or in combination with PEG (*e.g.*, branched or unbranched PEG or a mixture of both), in combination with PEG and a targeting moiety, or any of the foregoing in combination with a crosslinking agent. In certain embodiments, the present disclosure provides antisense oligomers in pharmaceutical compositions comprising gluconic-acid-modified polyhistidine or gluconylated-polyhistidine/transferrin-polylysine. One skilled in the art will also recognize that amino acids with properties similar to His and Lys may be substituted within the composition.

Wetting agents, emulsifiers and lubricants (such as sodium lauryl sulfate and magnesium stearate), coloring agents, release agents, coating agents, sweetening agents, flavoring agents, perfuming agents, preservatives, and antioxidants can also be present in the compositions.

Examples of pharmaceutically-acceptable antioxidants include: (1) water soluble antioxidants, such as ascorbic acid, cysteine hydrochloride, sodium bisulfate, sodium metabisulfite, sodium sulfite and the like; (2) oil-soluble antioxidants, such as ascorbyl palmitate, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), lecithin, propyl gallate, alpha-tocopherol, and the like; and (3) metal chelating agents, such as citric acid, ethylenediamine tetraacetic acid (EDTA), sorbitol, tartaric acid, phosphoric acid, and the like.

Formulations of the present disclosure include those suitable for oral, nasal, topical (including buccal and sublingual), rectal, vaginal and/or parenteral administration. The formulations may conveniently be presented in unit dosage form and may be prepared by any methods well known in the art of pharmacy. The amount of active ingredient that can be combined with a carrier material to produce a single dosage form will vary depending upon the subject being treated and the particular mode of administration. The amount of active ingredient which can be combined with a carrier material to produce a single dosage form will generally be that amount of the active ingredient which produces a therapeutic effect. Generally this amount will range from about 0.1 percent to about ninety-nine percent of active ingredient, preferably from about 5 percent to about 70 percent, most preferably from about 10 percent to about 30 percent.

In certain embodiments, a formulation of the present disclosure comprises an excipient selected from cyclodextrins, celluloses, liposomes, micelle forming agents, *e.g.*, bile acids, and polymeric carriers, *e.g.*, polyesters and polyanhydrides; and an antisense oligomer of the present disclosure. In an embodiment, the antisense oligomer of the formulation is according to Formula (IV). In an embodiment, the antisense oligomer of the formulation is according to Formula (IVa). In certain embodiments, an aforementioned formulation renders orally bioavailable an antisense oligomer of the present disclosure.

Methods of preparing these formulations or pharmaceutical compositions include the step of bringing into association an antisense oligomer of the present disclosure with the carrier and, optionally, one or more accessory ingredients. In general, the formulations are prepared by uniformly and intimately bringing into association an antisense oligomer of the present disclosure with liquid carriers, or finely divided solid carriers, or both, and then, if necessary, shaping the product.

Formulations of the disclosure suitable for oral administration may be in the form of capsules, cachets, pills, tablets, lozenges (using a flavored basis, usually sucrose and acacia or tragacanth), powders, granules, or as a solution or a suspension in an aqueous or non-aqueous liquid, or as an oil-in-water or water-in-oil liquid emulsion, or as an elixir or syrup, or as pastilles (using an inert base, such as gelatin and glycerin, or sucrose and acacia) and/or as mouth washes and the like, each containing a predetermined amount of an antisense oligomer of the present disclosure as an active ingredient. An antisense oligomer of the present disclosure may also be administered as a bolus, electuary, or paste.

In solid dosage forms of the disclosure for oral administration (capsules, tablets, pills, dragees, powders, granules, trouches and the like), the active ingredient may be mixed with one or more pharmaceutically-acceptable carriers, such as sodium citrate or dicalcium phosphate, and/or any of the following: (1) fillers or extenders, such as starches, lactose, sucrose, glucose, mannitol, and/or silicic acid; (2) binders, such as, for example, carboxymethylcellulose, alginates, gelatin, polyvinyl pyrrolidone, sucrose and/or acacia; (3) humectants, such as glycerol; (4) disintegrating agents, such as agar-agar, calcium carbonate, potato or tapioca starch, alginic acid, certain silicates, and sodium carbonate; (5) solution retarding agents, such as paraffin; (6) absorption accelerators, such as quaternary ammonium compounds and surfactants, such as poloxamer and sodium lauryl sulfate; (7) wetting agents, such as, for example, cetyl alcohol, glycerol monostearate, and non-ionic surfactants; (8) absorbents, such as kaolin and bentonite clay; (9) lubricants, such as talc, calcium stearate, magnesium stearate, solid polyethylene glycols, sodium lauryl sulfate, zinc stearate, sodium stearate, stearic acid, and mixtures thereof; (10) coloring agents; and (11) controlled release agents such as crospovidone or ethyl cellulose. In the case of capsules, tablets and pills, the pharmaceutical compositions may also comprise buffering agents. Solid pharmaceutical compositions of a similar type may also be employed as fillers in soft and hard-shelled gelatin capsules using such excipients as lactose or milk sugars, as well as high molecular weight polyethylene glycols and the like.

A tablet may be made by compression or molding, optionally with one or more accessory ingredients. Compressed tablets may be prepared using binder (*e.g.*, gelatin or hydroxypropylmethyl cellulose), lubricant, inert diluent, preservative, disintegrant (for example, sodium starch glycolate or cross-linked sodium carboxymethyl cellulose), surface-active or dispersing agent. Molded tablets may be made by molding in a suitable machine a mixture of the powdered compound moistened with an inert liquid diluent.

The tablets, and other solid dosage forms of the pharmaceutical compositions of the present disclosure, such as dragees, capsules, pills and granules, may optionally be scored or prepared with coatings and shells, such as enteric coatings and other coatings well known in the pharmaceutical-formulating art. They may also be formulated so as to provide slow or controlled release of the active ingredient therein using, for example, hydroxypropylmethyl cellulose in varying proportions to provide the desired release profile, other polymer matrices, liposomes and/or microspheres. They may be formulated for rapid

release, *e.g.*, freeze-dried. They may be sterilized by, for example, filtration through a bacteria-retaining filter, or by incorporating sterilizing agents in the form of sterile solid pharmaceutical compositions which can be dissolved in sterile water, or some other sterile injectable medium immediately before use. These pharmaceutical compositions may also optionally contain opacifying agents and may be of a composition that they release the active ingredient(s) only, or preferentially, in a certain portion of the gastrointestinal tract, optionally, in a delayed manner. Examples of embedding compositions which can be used include polymeric substances and waxes. The active ingredient can also be in micro-encapsulated form, if appropriate, with one or more of the above-described excipients.

Liquid dosage forms for oral administration of the antisense oligomers of the disclosure include pharmaceutically acceptable emulsions, microemulsions, solutions, suspensions, syrups and elixirs. In addition to the active ingredient, the liquid dosage forms may contain inert diluents commonly used in the art, such as, for example, water or other solvents, solubilizing agents and emulsifiers, such as ethyl alcohol, isopropyl alcohol, ethyl carbonate, ethyl acetate, benzyl alcohol, benzyl benzoate, propylene glycol, 1,3-butylene glycol, oils (in particular, cottonseed, groundnut, corn, germ, olive, castor and sesame oils), glycerol, tetrahydrofuryl alcohol, polyethylene glycols and fatty acid esters of sorbitan, and mixtures thereof.

Besides inert diluents, the oral pharmaceutical compositions can also include adjuvants such as wetting agents, emulsifying and suspending agents, sweetening, flavoring, coloring, perfuming and preservative agents.

Suspensions, in addition to the active compounds, may contain suspending agents as, for example, ethoxylated isostearyl alcohols, polyoxyethylene sorbitol and sorbitan esters, microcrystalline cellulose, aluminum metahydroxide, bentonite, agar-agar and tragacanth, and mixtures thereof.

Formulations for rectal or vaginal administration may be presented as a suppository, which may be prepared by mixing one or more compounds of the disclosure with one or more suitable nonirritating excipients or carriers comprising, for example, cocoa butter, polyethylene glycol, a suppository wax or a salicylate, and which is solid at room temperature, but liquid at body temperature and, therefore, will melt in the rectum or vaginal cavity and release the active compound.

Formulations or dosage forms for the topical or transdermal administration of an oligomer as provided herein include powders, sprays, ointments, pastes, creams, lotions, gels, solutions, patches and inhalants. The active oligomer conjugates may be mixed under sterile conditions with a pharmaceutically-acceptable carrier, and with any preservatives, buffers, or propellants which may be required. The ointments, pastes, creams and gels may contain, in addition to an active compound of this disclosure, excipients, such as animal and vegetable fats, oils, waxes, paraffins, starch, tragacanth, cellulose derivatives, polyethylene glycols, silicones, bentonites, silicic acid, talc and zinc oxide, or mixtures thereof.

Powders and sprays can contain, in addition to an antisense oligomer of the present disclosure, excipients such as lactose, talc, silicic acid, aluminum hydroxide, calcium silicates and polyamide powder, or mixtures of these substances. Sprays can additionally contain customary propellants, such as chlorofluorohydrocarbons and volatile unsubstituted hydrocarbons, such as butane and propane.

Transdermal patches have the added advantage of providing controlled delivery of an antisense oligomer of the present disclosure to the body. Such dosage forms can be made by dissolving or dispersing the oligomer in the proper medium. Absorption enhancers can also be used to increase the flux of the agent across the skin. The rate of such flux can be controlled by either providing a rate controlling membrane or dispersing the agent in a polymer matrix or gel, among other methods known in the art.

Pharmaceutical compositions suitable for parenteral administration may comprise one or more oligomer conjugates of the disclosure in combination with one or more pharmaceutically-acceptable sterile isotonic aqueous or nonaqueous solutions, dispersions, suspensions or emulsions, or sterile powders which may be reconstituted into sterile injectable solutions or dispersions just prior to use, which may contain sugars, alcohols, antioxidants, buffers, bacteriostats, solutes which render the formulation isotonic with the blood of the intended recipient or suspending or thickening agents. Examples of suitable aqueous and nonaqueous carriers which may be employed in the pharmaceutical compositions of the disclosure include water, ethanol, polyols (such as glycerol, propylene glycol, polyethylene glycol, and the like), and suitable mixtures thereof, vegetable oils, such as olive oil, and injectable organic esters, such as ethyl oleate. Proper fluidity can be maintained, for example, by the use of coating materials, such as lecithin, by the maintenance of the required particle size in the case of dispersions, and by the use of

surfactants. In an embodiment, the antisense oligomer of the pharmaceutical composition is according to Formula (IV). In an embodiment, the antisense oligomer of the pharmaceutical composition is according to Formula (IVa)

These pharmaceutical compositions may also contain adjuvants such as preservatives, wetting agents, emulsifying agents, and dispersing agents. Prevention of the action of microorganisms upon the subject oligomer conjugates may be ensured by the inclusion of various antibacterial and antifungal agents, for example, paraben, chlorobutanol, phenol sorbic acid, and the like. It may also be desirable to include isotonic agents, such as sugars, sodium chloride, and the like into the compositions. In addition, prolonged absorption of the injectable pharmaceutical form may be brought about by the inclusion of agents which delay absorption such as aluminum monostearate and gelatin.

In some cases, in order to prolong the effect of a drug, it is desirable to slow the absorption of the drug from subcutaneous or intramuscular injection. This may be accomplished by the use of a liquid suspension of crystalline or amorphous material having poor water solubility, among other methods known in the art. The rate of absorption of the drug then depends upon its rate of dissolution which, in turn, may depend upon crystal size and crystalline form. Alternatively, delayed absorption of a parenterally-administered drug form is accomplished by dissolving or suspending the drug in an oil vehicle.

Injectable depot forms may be made by forming microencapsule matrices of the subject oligomer conjugates in biodegradable polymers such as polylactide-polyglycolide. Depending on the ratio of oligomer to polymer, and the nature of the particular polymer employed, the rate of oligomer release can be controlled. Examples of other biodegradable polymers include poly(orthoesters) and poly(anhydrides). Depot injectable formulations may also prepared by entrapping the drug in liposomes or microemulsions that are compatible with body tissues.

When the antisense oligomers of the present disclosure are administered as pharmaceuticals, to humans and animals, they can be given per se or as a pharmaceutical composition containing, for example, 0.1 to 99% (more preferably, 10 to 30%) of the antisense oligomer in combination with a pharmaceutically acceptable carrier.

The formulations or preparations of the present disclosure may be given orally, parenterally, topically, or rectally. They are typically given in forms suitable for each

administration route. For example, they are administered in tablets or capsule form, by injection, inhalation, eye lotion, ointment, suppository, or infusion; topically by lotion or ointment; or rectally by suppositories.

Regardless of the route of administration selected, the antisense oligomers of the present disclosure, which may be used in a suitable hydrated form, and/or the pharmaceutical compositions of the present disclosure, may be formulated into pharmaceutically-acceptable dosage forms by conventional methods known to those of skill in the art. Actual dosage levels of the active ingredients in the pharmaceutical compositions of this disclosure may be varied so as to obtain an amount of the active ingredient which is effective to achieve the desired therapeutic response for a particular patient, composition, and mode of administration, without being unacceptably toxic to the patient.

The selected dosage level will depend upon a variety of factors including the activity of the particular antisense oligomer of the present disclosure employed, or the ester, salt or amide thereof, the route of administration, the time of administration, the rate of excretion or metabolism of the particular oligomer being employed, the rate and extent of absorption, the duration of the treatment, other drugs, compounds and/or materials used in combination with the particular oligomer employed, the age, sex, weight, condition, general health and prior medical history of the patient being treated, and like factors well known in the medical arts.

A physician or veterinarian having ordinary skill in the art can readily determine and prescribe the effective amount of the pharmaceutical composition required. For example, the physician or veterinarian could start doses of the antisense oligomers of the disclosure employed in the pharmaceutical composition at levels lower than that required in order to achieve the desired therapeutic effect and gradually increase the dosage until the desired effect is achieved. In general, a suitable daily dose of an antisense oligomer of the disclosure will be that amount of the antisense oligomer which is the lowest dose effective to produce a therapeutic effect. Such an effective dose will generally depend upon the factors described herein. Generally, oral, intravenous, intracerebroventricular and subcutaneous doses of the antisense oligomers of this disclosure for a patient, when used for the indicated effects, will range from about 0.0001 to about 100 mg per kilogram of body weight per day.

In some embodiments, the antisense oligomers of the present disclosure are administered in doses generally from about 1 to about 200 mg/kg. In some embodiments, doses for i.v. administration are from about 0.5 mg to about 200 mg/kg.

In some embodiments, the antisense oligomer of Formula (I) is administered in doses generally from about 1 to about 200 mg/kg. In some embodiments, doses of the antisense oligomer of Formula (I) for i.v. administration are from about 0.5 mg to about 200 mg/kg. In some embodiments, the antisense oligomer of Formula (II) is administered in doses generally from about 1 to about 200 mg/kg. In some embodiments, doses of the antisense oligomer of Formula (II) for i.v. administration are from about 0.5 mg to about 200 mg/kg. In some embodiments, the antisense oligomer of Formula (III) is administered in doses generally from about 1 to about 200 mg/kg. In some embodiments, doses of the antisense oligomer of Formula (III) for i.v. administration are from about 0.5 mg to about 200 mg/kg. In some embodiments, the antisense oligomer of Formula (IV) is administered in doses generally from about 1 to about 200 mg/kg. In some embodiments, doses of the antisense oligomer of Formula (IV) for i.v. administration are from about 0.5 mg to about 200 mg/kg. In some embodiments, the antisense oligomer of Formula (IVa) is administered in doses generally from about 1 to about 200 mg/kg. In some embodiments, doses of the antisense oligomer of Formula (IVa) for i.v. administration are from about 0.5 mg to about 200 mg/kg. In some embodiments, the antisense oligomer of Formula (V) is administered in doses generally from about 1 to about 200 mg/kg. In some embodiments, doses of the antisense oligomer of Formula (V) for i.v. administration are from about 0.5 mg to about 200 mg/kg. In some embodiments, the antisense oligomer of Formula (VI) is administered in doses generally from about 1 to about 200 mg/kg. In some embodiments, doses of the antisense oligomer of Formula (VI) for i.v. administration are from about 0.5 mg to about 200 mg/kg.

As would be understood in the art, weekly, biweekly, every third week, or monthly administrations may be in one or more administrations or sub-doses as discussed herein.

Nucleic acid molecules and antisense oligomers described herein can be administered to cells by a variety of methods known to those familiar to the art, including, but not restricted to, encapsulation in liposomes, by iontophoresis, or by incorporation into other vehicles, such as hydrogels, cyclodextrins, biodegradable nanocapsules, and bioadhesive microspheres, as described herein and known in the art. In certain embodiments, microemulsification technology may be utilized to improve bioavailability of

lipophilic (water insoluble) pharmaceutical agents. Examples include Trimetrine (Dordunoo, S. K., *et al.*, *Drug Development and Industrial Pharmacy*, 17(12), 1685-1713, 1991) and REV 5901 (Sheen, P. C., *et al.*, *J Pharm Sci* 80(7), 712-714, 1991). Among other benefits, microemulsification provides enhanced bioavailability by preferentially directing absorption to the lymphatic system instead of the circulatory system, which thereby bypasses the liver, and prevents destruction of the compounds in the hepatobiliary circulation.

In one aspect of disclosure, the formulations contain micelles formed from an oligomer as provided herein and at least one amphiphilic carrier, in which the micelles have an average diameter of less than about 100 nm. More preferred embodiments provide micelles having an average diameter less than about 50 nm, and even more preferred embodiments provide micelles having an average diameter less than about 30 nm, or even less than about 20 nm.

While all suitable amphiphilic carriers are contemplated, the presently preferred carriers are generally those that have Generally-Recognized-as-Safe (GRAS) status, and that can both solubilize an antisense oligomer of the present disclosure and microemulsify it at a later stage when the solution comes into a contact with a complex water phase (such as one found in human gastro-intestinal tract). Usually, amphiphilic ingredients that satisfy these requirements have HLB (hydrophilic to lipophilic balance) values of 2-20, and their structures contain straight chain aliphatic radicals in the range of C-6 to C-20. Examples are polyethylene-glycolized fatty glycerides and polyethylene glycols.

Examples of amphiphilic carriers include saturated and monounsaturated polyethyleneglycolyzed fatty acid glycerides, such as those obtained from fully or partially hydrogenated various vegetable oils. Such oils may advantageously consist of tri-, di-, and mono-fatty acid glycerides and di- and mono-poly(ethylene glycol) esters of the corresponding fatty acids, with a particularly preferred fatty acid composition including capric acid 4-10%, capric acid 3-9%, lauric acid 40-50%, myristic acid 14-24%, palmitic acid 4-14%, and stearic acid 5-15%. Another useful class of amphiphilic carriers includes partially esterified sorbitan and/or sorbitol, with saturated or mono-unsaturated fatty acids (SPAN-series) or corresponding ethoxylated analogs (TWEEN-series).

Commercially available amphiphilic carriers may be particularly useful, including Gelucire-series, Labrafil, Labrasol, or Lauroglycol (all manufactured and distributed by

Gattefosse Corporation, Saint Priest, France), PEG-mono-oleate, PEG-di-oleate, PEG-mono-laurate and di-laurate, Lecithin, Polysorbate 80, etc. (produced and distributed by a number of companies in USA and worldwide).

In certain embodiments, the delivery may occur by use of liposomes, nanocapsules, microparticles, microspheres, lipid particles, vesicles, and the like, for the introduction of the pharmaceutical compositions of the present disclosure into suitable host cells. In particular, the pharmaceutical compositions of the present disclosure may be formulated for delivery either encapsulated in a lipid particle, a liposome, a vesicle, a nanosphere, a nanoparticle or the like. The formulation and use of such delivery vehicles can be carried out using known and conventional techniques.

Hydrophilic polymers suitable for use in the present disclosure are those which are readily water-soluble, can be covalently attached to a vesicle-forming lipid, and which are tolerated *in vivo* without toxic effects (*i.e.*, are biocompatible). Suitable polymers include poly(ethylene glycol) (PEG), polylactic (also termed polylactide), polyglycolic acid (also termed polyglycolide), a polylactic-polyglycolic acid copolymer, and polyvinyl alcohol. In certain embodiments, polymers have a weight average molecular weight of from about 100 or 120 daltons up to about 5,000 or 10,000 daltons, or from about 300 daltons to about 5,000 daltons. In other embodiments, the polymer is poly(ethylene glycol) having a weight average molecular weight of from about 100 to about 5,000 daltons, or having a weight average molecular weight of from about 300 to about 5,000 daltons. In certain embodiments, the polymer is a poly(ethylene glycol) having a weight average molecular weight of about 750 daltons, for example PEG(750). Polymers may also be defined by the number of monomers therein; a preferred embodiment of the present disclosure utilizes polymers of at least about three monomers, such PEG polymers consisting of three monomers have a molecular weight of approximately 132 daltons.

Other hydrophilic polymers which may be suitable for use in the present disclosure include polyvinylpyrrolidone, polymethoxazoline, polyethyloxazoline, polyhydroxypropyl methacrylamide, polymethacrylamide, polydimethylacrylamide, and derivatized celluloses such as hydroxymethylcellulose or hydroxyethylcellulose.

In certain embodiments, a formulation of the present disclosure comprises a biocompatible polymer selected from the group consisting of polyamides, polycarbonates, polyalkylenes, polymers of acrylic and methacrylic esters, polyvinyl polymers,

polyglycolides, polysiloxanes, polyurethanes and co-polymers thereof, celluloses, polypropylene, polyethylenes, polystyrene, polymers of lactic acid and glycolic acid, polyanhydrides, poly(ortho)esters, poly(butic acid), poly(valeric acid), poly(lactide-co-caprolactone), polysaccharides, proteins, polyhyaluronic acids, polycyanoacrylates, and blends, mixtures, or copolymers thereof.

Cyclodextrins are cyclic oligosaccharides, consisting of 6, 7, or 8 glucose units, designated by the Greek letter α, β, or γ, respectively. The glucose units are linked by α-1,4-glucosidic bonds. As a consequence of the chair conformation of the sugar units, all secondary hydroxyl groups (at C-2, C-3) are located on one side of the ring, while all the primary hydroxyl groups at C-6 are situated on the other side. As a result, the external faces are hydrophilic, making the cyclodextrins water-soluble. In contrast, the cavities of the cyclodextrins are hydrophobic, since they are lined by the hydrogen of atoms C-3 and C-5, and by ether-like oxygens. These matrices allow complexation with a variety of relatively hydrophobic compounds, including, for instance, steroid compounds such as 17α-estradiol (*see*, *e.g.*, van Uden *et al. Plant Cell Tiss. Org. Cult.* 38:1-3-113 (1994)). The complexation takes place by Van der Waals interactions and by hydrogen bond formation. For a general review of the chemistry of cyclodextrins, *see*, Wenz, Agnew. *Chem. Int. Ed. Engl.*, 33:803-822 (1994).

The physico-chemical properties of the cyclodextrin derivatives depend strongly on the kind and the degree of substitution. For example, their solubility in water ranges from insoluble (*e.g.*, triacetyl-beta-cyclodextrin) to 147% soluble (w/v) (G-2-beta-cyclodextrin). In addition, they are soluble in many organic solvents. The properties of the cyclodextrins enable the control over solubility of various formulation components by increasing or decreasing their solubility.

Numerous cyclodextrins and methods for their preparation have been described. For example, Parmeter (I), *et al.* (U.S. Pat. No. 3,453,259) and Gramera, *et al.* (U.S. Pat. No. 3,459,731) described electroneutral cyclodextrins. Other derivatives include cyclodextrins with cationic properties [Parmeter (II), U.S. Pat. No. 3,453,257], insoluble crosslinked cyclodextrins (Solms, U.S. Pat. No. 3,420,788), and cyclodextrins with anionic properties [Parmeter (III), U.S. Pat. No. 3,426,011]. Among the cyclodextrin derivatives with anionic properties, carboxylic acids, phosphorous acids, phosphinous acids, phosphonic acids, phosphoric acids, thiophosphonic acids, thiosulphinic acids, and sulfonic acids have been

appended to the parent cyclodextrin [*see*, Parmeter (III), U.S. Pat. No. 3,453,257]. Furthermore, sulfoalkyl ether cyclodextrin derivatives have been described by Stella, *et al.* (U.S. Pat. No. 5,134,127).

Liposomes consist of at least one lipid bilayer membrane enclosing an aqueous internal compartment. Liposomes may be characterized by membrane type and by size. Small unilamellar vesicles (SUVs) have a single membrane and typically range between 0.02 and 0.05 μm in diameter; large unilamellar vesicles (LUVS) are typically larger than 0.05 μm. Oligolamellar large vesicles and multilamellar vesicles have multiple, usually concentric, membrane layers and are typically larger than 0.1 μm. Liposomes with several nonconcentric membranes, *i.e.*, several smaller vesicles contained within a larger vesicle, are termed multivesicular vesicles.

One aspect of the present disclosure relates to formulations comprising liposomes containing an antisense oligomer of the present disclosure, where the liposome membrane is formulated to provide a liposome with increased carrying capacity. Alternatively or in addition, the antisense oligomer of the present disclosure may be contained within, or adsorbed onto, the liposome bilayer of the liposome. An antisense oligomer of the present disclosure may be aggregated with a lipid surfactant and carried within the liposome's internal space; in these cases, the liposome membrane is formulated to resist the disruptive effects of the active agent-surfactant aggregate.

According to one embodiment of the present disclosure, the lipid bilayer of a liposome contains lipids derivatized with poly(ethylene glycol) (PEG), such that the PEG chains extend from the inner surface of the lipid bilayer into the interior space encapsulated by the liposome, and extend from the exterior of the lipid bilayer into the surrounding environment.

Active agents contained within liposomes of the present disclosure are in solubilized form. Aggregates of surfactant and active agent (such as emulsions or micelles containing the active agent of interest) may be entrapped within the interior space of liposomes according to the present disclosure. A surfactant acts to disperse and solubilize the active agent, and may be selected from any suitable aliphatic, cycloaliphatic or aromatic surfactant, including but not limited to biocompatible lysophosphatidylcholines (LPGs) of varying chain lengths (for example, from about C14 to about C20). Polymer-derivatized lipids such as PEG-lipids may also be utilized for micelle formation as they will act to inhibit

micelle/membrane fusion, and as the addition of a polymer to surfactant molecules decreases the CMC of the surfactant and aids in micelle formation. Preferred are surfactants with CMOs in the micromolar range; higher CMC surfactants may be utilized to prepare micelles entrapped within liposomes of the present disclosure.

Liposomes according to the present disclosure may be prepared by any of a variety of techniques that are known in the art. *See, e.g.*, U.S. Pat. No. 4,235,871; Published PCT application WO 96/14057; New RRC, Liposomes: A practical approach, IRL Press, Oxford (1990), pages 33-104; and Lasic DD, Liposomes from physics to applications, Elsevier Science Publishers BV, Amsterdam, 1993. For example, liposomes of the present disclosure may be prepared by diffusing a lipid derivatized with a hydrophilic polymer into preformed liposomes, such as by exposing preformed liposomes to micelles composed of lipid-grafted polymers, at lipid concentrations corresponding to the final mole percent of derivatized lipid which is desired in the liposome. Liposomes containing a hydrophilic polymer can also be formed by homogenization, lipid-field hydration, or extrusion techniques, as are known in the art.

In another exemplary formulation procedure, the active agent is first dispersed by sonication in a lysophosphatidylcholine or other low CMC surfactant (including polymer grafted lipids) that readily solubilizes hydrophobic molecules. The resulting micellar suspension of active agent is then used to rehydrate a dried lipid sample that contains a suitable mole percent of polymer-grafted lipid, or cholesterol. The lipid and active agent suspension is then formed into liposomes using extrusion techniques as are known in the art, and the resulting liposomes separated from the unencapsulated solution by standard column separation.

In one aspect of the present disclosure, the liposomes are prepared to have substantially homogeneous sizes in a selected size range. One effective sizing method involves extruding an aqueous suspension of the liposomes through a series of polycarbonate membranes having a selected uniform pore size; the pore size of the membrane will correspond roughly with the largest sizes of liposomes produced by extrusion through that membrane. *See e.g.*, U.S. Pat. No. 4,737,323 (Apr. 12, 1988). In certain embodiments, reagents such as DharmaFECT® and Lipofectamine® may be utilized to introduce polynucleotides or proteins into cells.

The release characteristics of a formulation of the present disclosure depend on the encapsulating material, the concentration of encapsulated drug, and the presence of release modifiers. For example, release can be manipulated to be pH dependent, for example, using a pH sensitive coating that releases only at a low pH, as in the stomach, or a higher pH, as in the intestine. An enteric coating can be used to prevent release from occurring until after passage through the stomach. Multiple coatings or mixtures of cyanamide encapsulated in different materials can be used to obtain an initial release in the stomach, followed by later release in the intestine. Release can also be manipulated by inclusion of salts or pore forming agents, which can increase water uptake or release of drug by diffusion from the capsule. Excipients which modify the solubility of the drug can also be used to control the release rate. Agents which enhance degradation of the matrix or release from the matrix can also be incorporated. They can be added to the drug, added as a separate phase (*i.e.*, as particulates), or can be co-dissolved in the polymer phase depending on the compound. In most cases the amount should be between 0.1 and 30 percent (w/w polymer). Types of degradation enhancers include inorganic salts such as ammonium sulfate and ammonium chloride, organic acids such as citric acid, benzoic acid, and ascorbic acid, inorganic bases such as sodium carbonate, potassium carbonate, calcium carbonate, zinc carbonate, and zinc hydroxide, and organic bases such as protamine sulfate, spermine, choline, ethanolamine, diethanolamine, and triethanolamine and surfactants such as Tween® and Pluronic®. Pore forming agents which add microstructure to the matrices (*i.e.*, water soluble compounds such as inorganic salts and sugars) are added as particulates. The range is typically between one and thirty percent (w/w polymer).

Uptake can also be manipulated by altering residence time of the particles in the gut. This can be achieved, for example, by coating the particle with, or selecting as the encapsulating material, a mucosal adhesive polymer. Examples include most polymers with free carboxyl groups, such as chitosan, celluloses, and especially polyacrylates (as used herein, polyacrylates refers to polymers including acrylate groups and modified acrylate groups such as cyanoacrylates and methacrylates).

An antisense oligomer may be formulated to be contained within, or, adapted to release by a surgical or medical device or implant. In certain aspects, an implant may be coated or otherwise treated with an antisense oligomer. For example, hydrogels, or other polymers, such as biocompatible and/or biodegradable polymers, may be used to coat an

implant with the pharmaceutical compositions of the present disclosure (*i.e.*, the composition may be adapted for use with a medical device by using a hydrogel or other polymer). Polymers and copolymers for coating medical devices with an agent are well-known in the art. Examples of implants include, but are not limited to, stents, drug-eluting stents, sutures, prosthesis, vascular catheters, dialysis catheters, vascular grafts, prosthetic heart valves, cardiac pacemakers, implantable cardioverter defibrillators, IV needles, devices for bone setting and formation, such as pins, screws, plates, and other devices, and artificial tissue matrices for wound healing.

In addition to the methods provided herein, the antisense oligomers for use according to the disclosure may be formulated for administration in any convenient way for use in human or veterinary medicine, by analogy with other pharmaceuticals. The antisense oligomers and their corresponding formulations may be administered alone or in combination with other therapeutic strategies in the treatment of muscular dystrophy, such as myoblast transplantation, stem cell therapies, administration of aminoglycoside antibiotics, proteasome inhibitors, and up-regulation therapies (*e.g.*, upregulation of utrophin, an autosomal paralogue of dystrophin).

In some embodiments, the additional therapeutic may be administered prior, concurrently, or subsequently to the administration of the antisense oligomer of the present disclosure. For example, the antisense oligomers may be administered in combination with a steroid and/or antibiotic. In certain embodiments, the antisense oligomers are administered to a patient that is on background steroid theory (*e.g.*, intermittent or chronic/continuous background steroid therapy). For example, in some embodiments the patient has been treated with a corticosteroid prior to administration of an antisense oligomer and continues to receive the steroid therapy. In some embodiments, the steroid is glucocorticoid or prednisone.

The routes of administration described are intended only as a guide since a skilled practitioner will be able to determine readily the optimum route of administration and any dosage for any particular animal and condition. Multiple approaches for introducing functional new genetic material into cells, both *in vitro* and *in vivo* have been attempted (Friedmann (1989) *Science*, 244:1275-1280). These approaches include integration of the gene to be expressed into modified retroviruses (Friedmann (1989) *supra*; Rosenberg (1991) *Cancer Research* 51(18), suppl.: 5074S-5079S); integration into non-retrovirus vectors

(*e.g.*, adeno-associated viral vectors) (Rosenfeld, *et al.* (1992) *Cell*, 68:143-155; Rosenfeld, *et al.* (1991) *Science*, 252:431-434); or delivery of a transgene linked to a heterologous promoter-enhancer element via liposomes (Friedmann (1989), *supra*; Brigham, *et al.* (1989) *Am. J. Med. Sci.*, 298:278-281; Nabel, *et al.* (1990) *Science*, 249:1285-1288; Hazinski, *et al.* (1991) *Am. J. Resp. Cell Molec. Biol.*, 4:206-209; and Wang and Huang (1987) *Proc. Natl. Acad. Sci. (USA)*, 84:7851-7855); coupled to ligand-specific, cation-based transport systems (Wu and Wu (1988) *J. Biol. Chem.*, 263:14621-14624) or the use of naked DNA, expression vectors (Nabel *et al.* (1990), *supra*; Wolff *et al.* (1990) *Science*, 247:1465-1468). Direct injection of transgenes into tissue produces only localized expression (Rosenfeld (1992) *supra*; Rosenfeld *et al.* (1991) *supra*; Brigham *et al.* (1989) *supra*; Nabel (1990) *supra*; and Hazinski *et al.* (1991) *supra*). The Brigham *et al.* group (*Am. J. Med. Sci.* (1989) 298:278-281 and *Clinical Research* (1991) 39 (abstract)) have reported *in vivo* transfection only of lungs of mice following either intravenous or intratracheal administration of a DNA liposome complex. An example of a review article of human gene therapy procedures is: Anderson, *Science* (1992) 256:808-813.

In a further embodiment, pharmaceutical compositions of the disclosure may additionally comprise a carbohydrate as provided in Han *et al.*, *Nat. Comms.* 7, 10981 (2016) the entirety of which is incorporated herein by reference. In some embodiments, pharmaceutical compositions of the disclosure may comprise 5% of a hexose carbohydrate. For example, pharmaceutical composition of the disclosure may comprise 5% glucose, 5% fructose, or 5% mannose. In certain embodiments, pharmaceutical compositions of the disclosure may comprise 2.5% glucose and 2.5% fructose. In some embodiments, pharmaceutical compositions of the disclosure may comprises a carbohydrate selected from: arabinose present in an amount of 5% by volume, glucose present in an amount of 5% by volume, sorbitol present in an amount of 5% by volume, galactose present in an amount of 5% by volume, fructose present in an amount of 5% by volume, xylitol present in an amount of 5% by volume, mannose present in an amount of 5% by volume, a combination of glucose and fructose each present in an amount of 2.5% by volume, and a combination of glucose present in an amount of 5.7% by volume, fructose present in an amount of 2.86% by volume, and xylitol present in an amount of 1.4% by volume.

IV. Methods of Use

Restoration of the Dystrophin Reading Frame using Exon Skipping

A potential therapeutic approach to the treatment of DMD caused by out-of-frame mutations in the dystrophin gene is suggested by the milder form of dystrophinopathy known as BMD, which is caused by in-frame mutations. The ability to convert an out-of-frame mutation to an in-frame mutation would hypothetically preserve the mRNA reading frame and produce an internally shortened yet functional dystrophin protein. Antisense oligomers of the disclosure were designed to accomplish this.

Hybridization of the antisense oligomer of Formula (I), Formula (II), Formula (III), Formula (IV), Formula (IVa), Formula (V), Formula (VI) with the targeted pre-mRNA sequence interferes with formation of the pre-mRNA splicing complex and deletes exon 50 from the mature mRNA. The structure and conformation of antisense oligomers of the disclosure allow for sequence-specific base pairing to the complementary sequence.

Normal dystrophin mRNA containing all 79 exons will produce normal dystrophin protein. The shape of each exon depicts how codons are split between exons; of note, one codon consists of three nucleotides. Rectangular shaped exons start and end with complete codons. Arrow shaped exons start with a complete codon but end with a split codon, containing only nucleotide #1 of the codon. Nucleotides #2 and #3 of this codon are contained in the subsequent exon which will start with a chevron shape.

Clinical outcomes for analyzing the effect of an antisense oligomer that is complementary to a target region of exon 50, intron 49, and/or intron 50 of the human dystrophin pre-mRNA and induces exon 50 skipping include percent dystrophin positive fibers (PDPF), six-minute walk test (6MWT), loss of ambulation (LOA), North Star Ambulatory Assessment (NSAA), pulmonary function tests (PFT), ability to rise (from a supine position) without external support, *de novo* dystrophin production, and other functional measures.

In some embodiments, the present disclosure provides methods for producing dystrophin in a subject having a mutation of the dystrophin gene that is amenable to exon 50 skipping, the method comprising administering to the subject an antisense oligomer, or pharmaceutically acceptable salt thereof, as described herein. In certain embodiments, the present disclosure provides methods for restoring an mRNA reading frame to induce

dystrophin protein production in a subject with Duchenne muscular dystrophy (DMD) who has a mutation of the dystrophin gene that is amenable to exon 50 skipping. Protein production can be measured by reverse-transcription polymerase chain reaction (RT-PCR), western blot analysis, or immunohistochemistry (IHC).

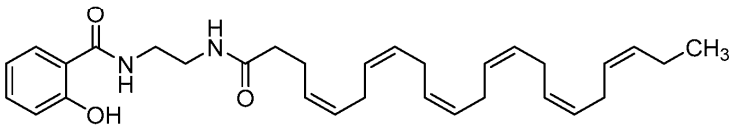
In some embodiments, the present disclosure provides methods for treating DMD in a subject in need thereof, wherein the subject has a mutation of the dystrophin gene that is amenable to exon 50 skipping, the method comprising administering to the subject an antisense oligomer, or pharmaceutically acceptable salt thereof, as described herein. In various embodiments, treatment of the subject is measured by delay of disease progression. In some embodiments, treatment of the subject is measured by maintenance of ambulation in the subject or reduction of loss of ambulation in the subject. In some embodiments, ambulation is measured using the 6 Minute Walk Test (6MWT). In certain embodiments, ambulation is measured using the North Start Ambulatory Assessment (NSAA).

In various embodiments, the present disclosure provides methods for maintaining pulmonary function or reducing loss of pulmonary function in a subject with DMD, wherein the subject has a mutation of the DMD gene that is amenable to exon 50 skipping, the method comprising administering to the subject an antisense oligomer, or pharmaceutically acceptable salt thereof, as described herein. In some embodiments, pulmonary function is measured as Maximum Expiratory Pressure (MEP). In certain embodiments, pulmonary function is measured as Maximum Inspiratory Pressure (MIP). In some embodiments, pulmonary function is measured as Forced Vital Capacity (FVC).

In a further embodiment, the pharmaceutical compositions of the disclosure may be co-administered with a carbohydrate in the methods of the disclosure, either in the same formulation or is a separate formulation, as provided in Han *et al.*, *Nat. Comms.* 7, 10981 (2016) the entirety of which is incorporated herein by reference. In some embodiments, pharmaceutical compositions of the disclosure may be co-administered with 5% of a hexose carbohydrate. For example, pharmaceutical compositions of the disclosure may be co-administered with 5% glucose, 5% fructose, or 5% mannose. In certain embodiments, pharmaceutical compositions of the disclosure may be co-administered with 2.5% glucose and 2.5% fructose. In some embodiments, pharmaceutical composition of the disclosure may be co-administered with a carbohydrate selected from: arabinose present in an amount of 5% by volume, glucose present in an amount of 5% by volume, sorbitol present in an

amount of 5% by volume, galactose present in an amount of 5% by volume, fructose present in an amount of 5% by volume, xylitol present in an amount of 5% by volume, mannose present in an amount of 5% by volume, a combination of glucose and fructose each present in an amount of 2.5% by volume, and a combination of glucose present in an amount of 5.7% by volume, fructose present in an amount of 2.86% by volume, and xylitol present in an amount of 1.4% by volume.

In various embodiments, an antisense oligomer of the disclosure is co-administered with a therapeutically effective amount of a non-steroidal anti-inflammatory compound. In some embodiments, the non-steroidal anti-inflammatory compound is an NF-kB inhibitor. For example, in some embodiments, the NF-kB inhibitor may be CAT-1004 or a pharmaceutically acceptable salt thereof. In various embodiments, the NF-kB inhibitor may be a conjugate of salicylate and DHA. In some embodiments, the NF-kB inhibitor is CAT-1041 or a pharmaceutically acceptable salt thereof. In certain embodiments, the NF-kB inhibitor is a conjugate of salicylate and EPA. In various embodiments, the NF-kB inhibitor is



, or a pharmaceutically acceptable salt thereof.

In some embodiments, non-steroidal anti-inflammatory compound is a TGF-b inhibitor. For example, in certain embodiments, the TGF-b inhibitor is HT-100.

In certain embodiments, there is described an antisense oligomer as described herein for use in therapy. In certain embodiments, there is described an antisense oligomer as described herein for use in the treatment of Duchenne muscular dystrophy. In certain embodiments, there is described an antisense oligomer as described herein for use in the manufacture of a medicament for use in therapy. In certain embodiments, there is described an antisense oligomer as described herein for use in the manufacture of a medicament for the treatment of Duchenne muscular dystrophy.

V. Kits

The disclosure also provides kits for treatment of a patient with a genetic disease which kit comprises at least an antisense molecule (*e.g.*, an antisense oligomer comprising

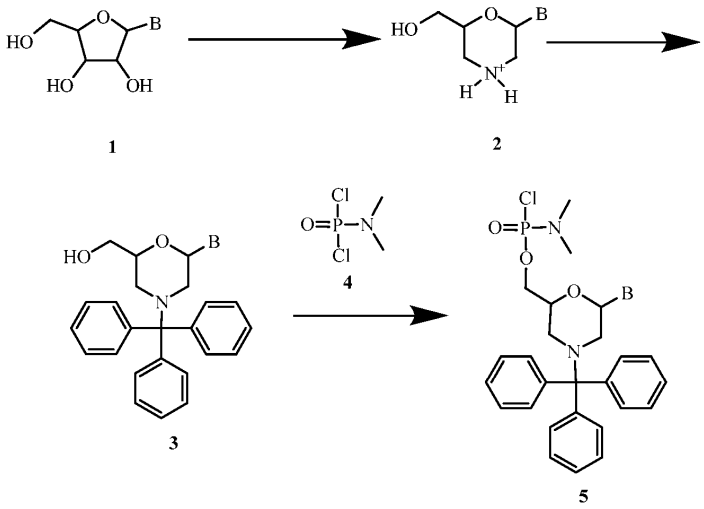
the base sequence set forth in any of SEQ ID NOS. 1-9), packaged in a suitable container, together with instructions for its use. The kits may also contain peripheral reagents such as buffers, stabilizers, etc. Those of ordinary skill in the field should appreciate that applications of the above method has wide application for identifying antisense molecules suitable for use in the treatment of many other diseases. In an embodiment, the kit comprises an antisense oligomer according to any of Formulas (I)-(VI).

Examples

Although the foregoing disclosure has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be readily apparent to one of ordinary skill in the art in light of the teachings of this disclosure that certain changes and modifications may be made thereto without departing from the spirit or scope of the appended claims. The following examples are provided by way of illustration only and not by way of limitation. Those of skill in the art will readily recognize a variety of noncritical parameters that could be changed or modified to yield essentially similar results.

Materials and Methods

Preparation of Morpholino Subunits



Scheme 1: General synthetic route to PMO Subunits

Referring to Scheme 1, wherein B represents a base pairing moiety, the morpholino subunits may be prepared from the corresponding ribonucleoside (**1**) as shown. The

morpholino subunit (**2**) may be optionally protected by reaction with a suitable protecting group precursor, for example trityl chloride. The 3' protecting group is generally removed during solid-state oligomer synthesis as described in more detail below. The base pairing moiety may be suitably protected for solid-phase oligomer synthesis. Suitable protecting groups include benzoyl for adenine and cytosine, phenylacetyl for guanine, and pivaloyloxymethyl for hypoxanthine (Inosine). The pivaloyloxymethyl group can be introduced onto the N1 position of the hypoxanthine heterocyclic base. Although an unprotected hypoxanthine subunit, may be employed, yields in activation reactions are far superior when the base is protected. Other suitable protecting groups include those disclosed in U.S. Patent No. 8,076,476, which is hereby incorporated by reference in its entirety.

Reaction of **3** with the activated phosphorous compound **4** results in morpholino subunits having the desired linkage moiety **5**.

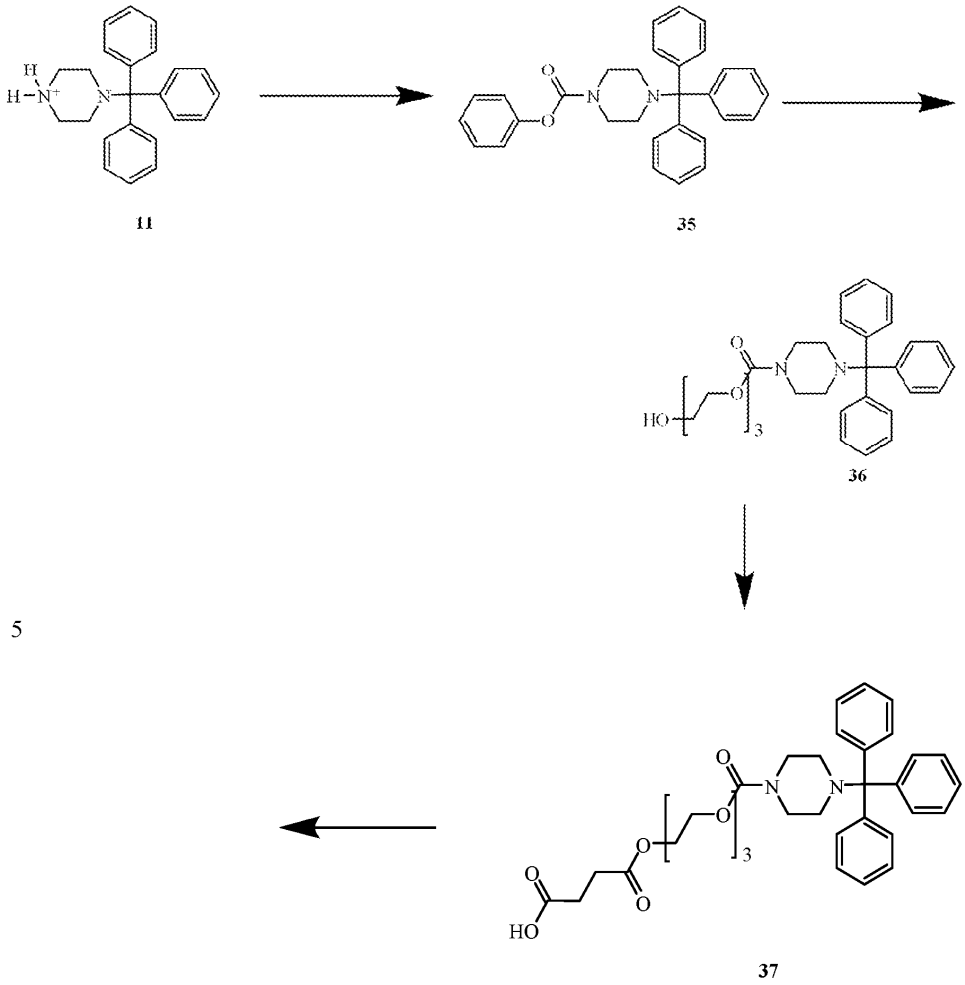
Compounds of structure **4** can be prepared using any number of methods known to those of skill in the art. Coupling with the morpholino moiety then proceeds as outlined above.

Compounds of structure **5** can be used in solid-phase oligomer synthesis for preparation of oligomers comprising the intersubunit linkages. Such methods are well known in the art. Briefly, a compound of structure **5** may be modified at the 5' end to contain a linker to a solid support. Once supported, the protecting group of **5** (*e.g.*, trityl) at 3'-end is removed and the free amine is reacted with an activated phosphorous moiety of a second compound of structure **5**. This sequence is repeated until the desired sequence oligo is obtained. The protecting group in the terminal 3' end may either be removed or left on if a 3' modification is desired. The oligo can be removed from the solid support using any number of methods, or example treatment with a base to cleave the linkage to the solid support.

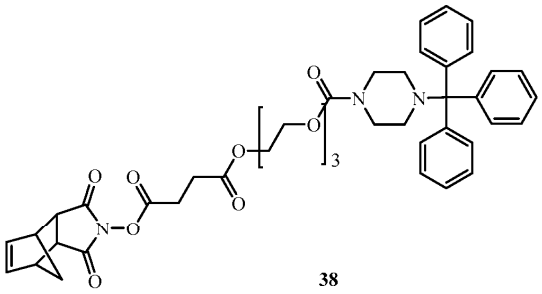
The preparation of morpholino oligomers in general and specific morpholino oligomers of the disclosure are described in more detail in the Examples.

Preparation of Morpholino Oligomers

The preparation of the compounds of the disclosure can be performed using the following protocol according to Scheme 2:



11 35 36 37



38

Scheme 2: Preparation of Activated Tail Acid

Preparation of trityl piperazine phenyl carbamate **35**: To a cooled suspension of compound **11** in dichloromethane (6 mL/g **11**) is added to a solution of potassium carbonate (3.2 eq) in water (4 mL/g potassium carbonate). To this two-phase mixture is slowly added a solution of phenyl chloroformate (1.03 eq) in dichloromethane (2 g/g phenyl chloroformate). The reaction mixture is warmed to 20 °C. Upon reaction completion (1-2 hr), the layers are separated. The organic layer is washed with water, and dried over anhydrous potassium carbonate. The product **35** is isolated by crystallization from acetonitrile.

Preparation of carbamate alcohol **36**: Sodium hydride (1.2 eq) is suspended in 1-methyl-2-pyrrolidinone (32 mL/g sodium hydride). Triethylene glycol (10.0 eq) and compound **35** (1.0 eq) can be added to this suspension. The resulting slurry is heated to 95 °C. Upon reaction completion (1-2 hr), the mixture is cooled to 20 °C. To this mixture is added 30% dichloromethane/methyl *tert*-butyl ether (v:v) and water. The product-containing organic layer is washed successively with aqueous NaOH, aqueous succinic acid, and saturated aqueous sodium chloride. The product 36 is isolated by crystallization from dichloromethane/methyl tert-butyl ether/heptane.

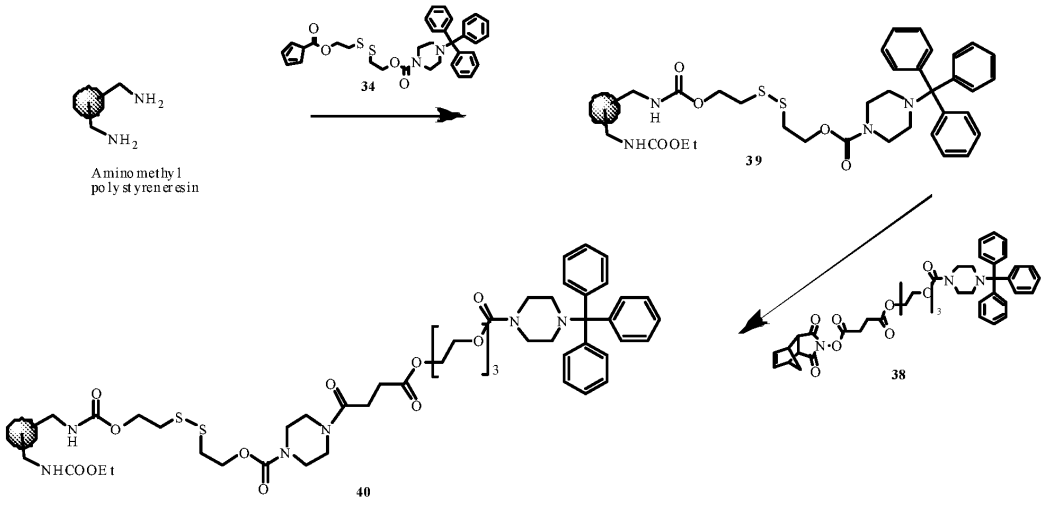
Preparation of Tail acid **37**: To a solution of compound **36** in tetrahydrofuran (7 mL/g **36**) succinic anhydride (2.0 eq) and DMAP (0.5 eq) are added. The mixture is heated to 50 °C. Upon reaction completion (5 hr), the mixture is cooled to 20 °C and adjusted to pH 8.5 with aqueous $NaHCO_3$. Methyl *tert*-butyl ether is added, and the product is extracted into the aqueous layer. Dichloromethane is added, and the aqueous layer mixture is adjusted to pH 3 with aqueous citric acid. The product-containing organic layer is washed with a

mixture of pH=3 citrate buffer and saturated aqueous sodium chloride. This dichloromethane solution of **37** is used without isolation in the preparation of compound **38**.

Preparation of **38**: N-hydroxy-5-norbornene-2,3-dicarboxylic acid imide (HONB) (1.02 eq), 4-dimethylaminopyridine (DMAP) (0.34 eq), and then 1-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (EDC) (1.1 eq) are added to the solution of compound **37**. The mixture is heated to 55 °C. Upon reaction completion (4-5 hr), the mixture is cooled to 20 °C and washed successively with 1:1 0.2 M citric acid/brine and brine. The dichloromethane solution undergoes solvent exchange to acetone and then to *N,N*-dimethylformamide, and the product is isolated by precipitation from acetone/ *N,N*-dimethylformamide into saturated aqueous sodium chloride. The crude product is reslurried several times in water to remove residual *N,N*-dimethylformamide and salts.

PMO Synthesis Method A: Use of Disulfide Anchor

Introduction of the activated "Tail" onto the anchor-loaded resin is performed in dimethyl imidazolidinone (DMI) by the procedure used for incorporation of the subunits during solid phase synthesis.



Scheme 3: Preparation of the Solid Support for Synthesis of Morpholino Oligomers

This procedure can be performed in a silanized, jacketed peptide vessel (ChemGlass, NJ, USA) with a coarse porosity (40-60 μm) glass frit, overhead stirrer, and 3-way Teflon stopcock to allow N_2 to bubble up through the frit or a vacuum extraction.

The resin treatment/wash steps in the following procedure consists of two basic operations: resin fluidization or stirrer bed reactor and solvent/solution extraction. For resin fluidization, the stopcock is positioned to allow N_2 flow up through the frit and the specified resin treatment/wash is added to the reactor and allowed to permeate and completely wet the resin. Mixing is then started and the resin slurry is mixed for the specified time. For solvent/solution extraction, mixing and N_2 flow are stopped and the vacuum pump is started and then the stopcock is positioned to allow evacuation of resin treatment/wash to waste. All resin treatment/wash volumes are 15 mL/g of resin unless noted otherwise.

1-methyl-2-pyrrolidinone (NMP; 20 mL/g resin) is added to aminomethylpolystyrene resin (100-200 mesh; ~1.0 mmol/g load based on nitrogen substitution; 75 g, 1 eq, Polymer Labs, UK, part #1464-X799) in a silanized, jacketed peptide vessel and the resin is allowed to swell with mixing for 1-2 hr. Following evacuation of the swell solvent, the resin is washed with dichloromethane (2 x 1-2 min), 5% diisopropylethylamine in 25% isopropanol/dichloromethane (2 x 3-4 min) and dichloromethane (2 x 1-2 min). After evacuation of the final wash, the resin is treated with a solution of disulfide anchor **34** in 1-methyl-2-pyrrolidinone (0.17 M; 15 mL/g resin, ~2.5 eq) and the resin/reagent mixture is heated at 45 °C for 60 hr. On reaction completion, heating is discontinued and the anchor solution is evacuated and the resin is washed with 1-methyl-2-pyrrolidinone (4 x 3-4 min) and dichloromethane (6 x 1-2 min). The resin is treated with a solution of 10% (v/v) diethyl dicarbonate (DEDC) in dichloromethane (16 mL/g; 2 x 5-6 min) and then washed with dichloromethane (6 x 1-2 min). The resin **39** is dried under a N_2 stream for 1-3 hr and then under vacuum to constant weight (± 2%).

Determination of the Loading of Aminomethylpolystyrene-disulfide resin: The loading of the resin (number of potentially available reactive sites) is determined by a spectrometric assay for the number of triphenylmethyl (trityl) groups per gram of resin.

A known weight of dried resin (25 ± 3 mg) is transferred to a silanized 25 mL volumetric flask and ~5 mL of 2% (v/v) trifluoroacetic acid in dichloromethane is added. The contents are mixed by gentle swirling and then allowed to stand for 30 min. The volume is brought up to 25 mL with additional 2% (v/v) trifluoroacetic acid in dichloromethane and the contents thoroughly mixed. Using a positive displacement pipette, an aliquot of the trityl-containing solution (500 µL) is transferred to a 10 mL volumetric flask and the volume brought up to 10 mL with methanesulfonic acid.

The trityl cation content in the final solution is measured by UV absorbance at 431.7 nm and the resin loading calculated in trityl groups per gram resin (μmol/g) using the appropriate volumes, dilutions, extinction coefficient (ε: 41 μmol-1cm-1), and resin weight. The assay is performed in triplicate and an average loading calculated.

The resin loading procedure in this example will provide resin with a loading of approximately 500 μmol/g. A loading of 300-400 in μmol/g is obtained if the disulfide anchor incorporation step is performed for 24 hr at room temperature.

Tail loading: Using the same setup and volumes as for the preparation of aminomethylpolystyrene-disulfide resin, the Tail can be introduced onto solid support. The anchor loaded resin is first deprotected under acidic conditions and the resulting material neutralized before coupling. For the coupling step, a solution of **38** (0.2 M) in DMI containing 4-ethylmorpholine (NEM, 0.4 M) is used instead of 1-methyl-2-pyrrolidinone for the disulfide anchor solution. After 2 hr at 45 °C, the resin **39** is washed twice with 5% diisopropylethylamine in 25% isopropanol/dichloromethane and once with DCM. A solution of benzoic anhydride (0.4 M) and NEM (0.4 M) is added to the resin. After 25 min, the reactor jacket is cooled to room temperature, and the resin is washed twice with 5% diisopropylethylamine in 25% isopropanol/dichloromethane and eight times with DCM. The resin **40** is filtered and dried under high vacuum. The loading for resin **40** is defined to be the loading of the original aminomethylpolystyrene-disulfide resin **39** used in the Tail loading.

Solid Phase Synthesis: Morpholino Oligomers are prepared on a custom-made BioAutomation 128AVB (Plano, TX) in 4 mL BioComma polypropylene reaction columns (Part # CT003-BC). An aluminum block with channels for water flow is placed around the columns as they sit on the synthesizer. The AVB128 alternatively adds reagent/wash solutions, hold for a specified time, and evacuate the columns using a vacuum.

For oligomers in the range up to about 25 subunits in length, aminomethylpolystyrene-disulfide resin with loading near 500 μmol/g of resin is preferred. For larger oligomers, aminomethylpolystyrene-disulfide resin with loading of 300-400 μmol/g of resin is preferred. If a molecule with 5'-Tail is desired, resin that has been loaded with Tail is chosen with the same loading guidelines.

The following reagent solutions are prepared:

Detritylation Solution: 1% 4 Cyanopyridine, and trifluoroacetic Acid (w/w) in 4:1 dichloromethane/triflouroethanol solution;

Neutralization Solution: 3% Diisopropylethylamine in 5:1 dichloromethane/isopropanol solution; and

Coupling Solution: 0.18 M (or 0.24 M for oligomers having grown longer than 20 subunits) activated Morpholino Subunit of the desired base and linkage type with 0.4 M N-ethylmorpholine, in 1,3-dimethylimidazolidinone (DMI) solution.

Dichloromethane (DCM) is used as a transitional wash separating the different reagent solution washes.

On the synthesizer, with the block set to 42 °C, 2 mL of 1-methyl-2-pyrrolidinone is added to each column containing 30 mg of aminomethylpolystyrene-disulfide resin (or Tail resin) is added and allowed to sit at room temperature for 30 min. After washing with 2 times 2 mL of dichloromethane, the following synthesis cycle can be employed:

Step	Volume	Delivery	Hold time
Detritylation	1.5 mL	Manifold	15 seconds
Detritylation	1.5 mL	Manifold	15 seconds
Detritylation	1.5 mL	Manifold	15 seconds
Detritylation	1.5 mL	Manifold	15 seconds
Detritylation	1.5 mL	Manifold	15 seconds
Detritylation	1.5 mL	Manifold	15 seconds
Detritylation	1.5 mL	Manifold	15 seconds
DCM	1.5 mL	Manifold	30 seconds
Neutralization	1.5 mL	Manifold	30 seconds
Neutralization	1.5 mL	Manifold	30 seconds
Neutralization	1.5 mL	Manifold	30 seconds
Neutralization	1.5 mL	Manifold	30 seconds
Neutralization	1.5 mL	Manifold	30 seconds
Neutralization	1.5 mL	Manifold	30 seconds
DCM	1.5 mL	Manifold	30 seconds
Coupling	350-500uL	Syringe	40 minutes
DCM	1.5 mL	Manifold	30 seconds
Neutralization	1.5 mL	Manifold	30 seconds
Neutralization	1.5 mL	Manifold	30 seconds
DCM	1.5 mL	Manifold	30 seconds
DCM	1.5 mL	Manifold	30 seconds
DCM	1.5 mL	Manifold	30 seconds

The sequences of the individual oligomers are programmed into the synthesizer so that each column receives the proper coupling solution (A,C,G,T, or I) in the proper sequence. When the oligomer in a column has completed incorporation of its final subunit, the column is removed from the block and a final cycle is performed manually using a coupling solution containing 4-methoxytriphenylmethyl chloride (0.32 M in DMI) and 0.89 M 4-ethylmorpholine.

Cleavage from the resin and removal of bases and backbone protecting groups: After methoxytritylation, the resin is washed 8 times with 2 mL 1-methyl-2-pyrrolidinone. One

mL of a cleavage solution consisting of 0.1 M 1,4-dithiothreitol (DTT) and 0.73 M triethylamine in 1-methyl-2-pyrrolidinone is added, the column is capped, and allowed to sit at room temperature for 30 min. After that time, the solution is drained into a 12 mL Wheaton vial. The greatly shrunken resin is washed twice with 300 µL of cleavage solution. To the solution is added 4.0 mL conc. aqueous ammonia (stored at -20 °C), the vial is capped tightly (with a Teflon lined screw cap), and the mixture is swirled to mix the solution. The vial is placed in a 45 °C oven for 16-24 hr to effect cleavage of base and backbone protecting groups.

Crude product purification: The vialed ammonolysis solution is removed from the oven and allowed to cool to room temperature. The solution is diluted with 20 mL of 0.28% aqueous ammonia and passed through a 2.5x10 cm column containing Macroprep HQ resin (BioRad). A salt gradient (A: 0.28% ammonia with B: 1 M sodium chloride in 0.28% ammonia; 0-100% B in 60 min) is used to elute the methoxytrityl protected oligomer. The combined fractions are pooled and further processed depending on the desired product.

Demethoxytritylation of Morpholino Oligomers: The pooled fractions from the Macroprep purification are treated with 1 M H_3PO_4 to lower the pH to 2.5. After initial mixing, the samples sit at room temperature for 4 min, at which time they are neutralized to pH 10-11 with 2.8% ammonia/water. The products are purified by solid phase extraction (SPE).

SPE column packing and conditioning: Amberchrome CG-300M (Dow Chemicals (Rohm and Haas) ; Midland, MI) (3 mL) is packed into 20 mL fritted columns (BioRad Econo-Pac Chromatography Columns (732-1011)) and the resin rinsed with 3 mL of the following: 0.28% NH_4OH / 80% acetonitrile; 0.5 M NaOH / 20% ethanol; water; 50 mM H_3PO_4 / 80% acetonitrile; water; 0.5 NaOH / 20% ethanol; water; 0.28% NH_4OH.

SPE purification: The solution from the demethoxytritylation is loaded onto the column and the resin is rinsed three times with 8 mL 0.28% aqueous ammonia. A Wheaton vial (12 mL) can be placed under the column and the product can be eluted by two washes with 2 mL of 45% acetonitrile in 0.28% aqueous ammonia.

Product isolation: The solutions are frozen in dry ice and the vials placed on a freeze dryer for at least two days to produce a fluffy white powder. The samples are then dissolved in water, filtered through a 0.22 micron filter (Pall Life Sciences, Acrodisc 25 mm syringe

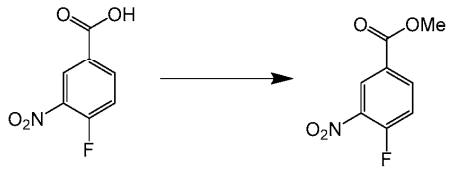
filter, with a 0.2 micron HT Tuffryn membrane) using a syringe and the Optical Density (OD) is measured on a UV spectrophotometer to determine the OD units of oligomer present, as well as dispense the sample for analysis. The solutions are then placed back in Wheaton vials for lyophilization.

Analysis of Morpholino Oligomers by MALDI: MALDI-TOF mass spectrometry can be used to determine the composition of fractions in purifications as well as provide evidence for identity (molecular weight) of the oligomers. Samples can be run following dilution with a solution of 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid), 3,4,5-trihydoxyacetophenone (THAP) or alpha-cyano-4-hydoxycinnamic acid (HCCA) as matrices.

PMO Synthesis Method B: Use of Nitrocarboxyphenylpropyl (NCP2) Anchor

NCP2 Anchor Synthesis:

1. Preparation of Methyl 4-fluoro-3-nitrobenzoate **(1)**

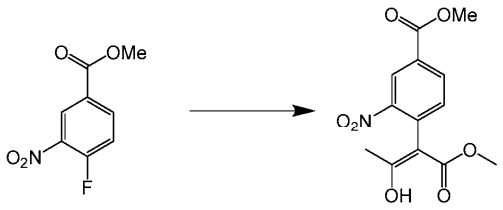


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12.7 kg of 4-fluoro-3-nitrobenzoic acid, 40 kg of methanol, and 2.82 kg of concentrated sulfuric acid can be added to a 100 L flask. The mixture is stirred at reflux (65 °C) for 36 hours. The reaction mixture is cooled to 0 °C. Crystals can form at about 38 °C. The mixture is held at 0° C for 4 hours and then filtered under nitrogen. The 100 L flask is washed and the filter cake is washed with 10 kg of methanol that had been cooled to 0 °C. The solid filter cake is dried on the funnel for 1 hour, transferred to trays, and dried in a vacuum oven at room temperature to a constant weight.

2. Preparation of 3-Nitro-4-(2-oxopropyl)benzoic Acid

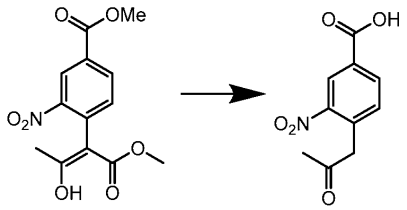
A. (Z)-Methyl 4-(3-hydroxy-1-methoxy-1-oxobut-2-en-2-yl)-3-nitrobenzoate (**2**)



1 **2**

3.98 kg of methyl 4-fluoro-3-nitrobenzoate (**1**) from the previous step, 9.8 kg DMF, and 2.81 kg methyl acetoacetate can be added to a 100 L flask. The mixture is stirred and cooled to 0 °C. 3.66 kg of DBU over about 4 hours is added while the temperature is maintained at or below 5 °C. The mixture is stirred an additional 1 hour. A solution of 8.15 kg of citric acid in 37.5 kg of purified water is added while the reaction temperature is maintained at or below 15 °C. After the addition, the reaction mixture is stirred an additional 30 minutes and then filtered under nitrogen. The wet filter cake is returned to the 100 L flask along with 14.8 kg of purified water. The slurry is stirred for 10 minutes and then filtered. The wet cake is again returned to the 100 L flask, slurried with 14.8 kg of purified water for 10 minutes, and filtered to crude (Z)-methyl 4-(3-hydroxy-1-methoxy-1-oxobut-2-en-2-yl)-3-nitrobenzoate.

B. 3-Nitro-4-(2-oxopropyl)benzoic Acid

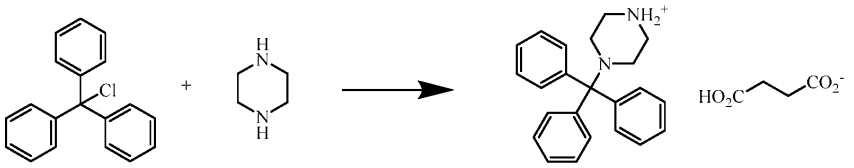


2 **3**

The crude (Z)-methyl 4-(3-hydroxy-1-methoxy-1-oxobut-2-en-2-yl)-3-nitrobenzoate can be charged to a 100 L reaction flask under nitrogen. 14.2 kg 1,4-dioxane is added and then stirred. A solution of 16.655 kg concentrated HCl and 13.33 kg purified water (6 M HCl) is added over 2 hours while the temperature of the reaction mixture is maintained below 15 °C. When the addition is complete, the reaction mixture is heated at reflux (80 °C)

for 24 hours, cooled to room temperature, and filtered under nitrogen. The solid filter cake is triturated with 14.8 kg of purified water, filtered, triturated again with 14.8 kg of purified water, and filtered. The solid is returned to the 100 L flask with 39.9 kg of DCM and refluxed with stirring for 1 hour. 1.5 kg of purified water is added to dissolve the remaining solids. The bottom organic layer is split to a pre-warmed 72 L flask, then returned to a clean dry 100 L flask. The solution is cooled to 0 °C, held for 1 hour, then filtered. The solid filter cake is washed twice each with a solution of 9.8 kg DCM and 5 kg heptane, then dried on the funnel. The solid is transferred to trays and dried to a constant weight of 1.855 kg 3-Nitro-4-(2-oxopropyl)benzoic Acid.

3. Preparation of N-Tritylpiperazine Succinate (NTP)



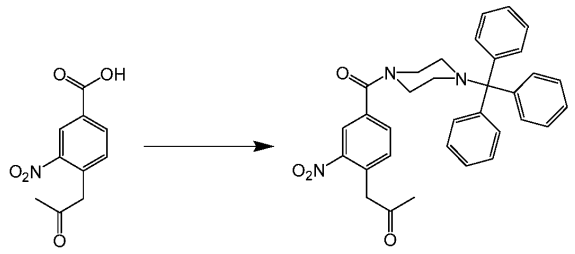
1.805 kg triphenylmethyl chloride and 8.3 kg of toluene (TPC solution) can be charged to a 72 L jacketed flask under nitrogen. The mixture is stirred until the solids dissolved. 5.61 kg piperazine, 19.9 kg toluene, and 3.72 kg methanol are added under nitrogen to a 100 L jacketed reaction flask. The mixture is stirred and cooled to 0 °C. TPC solution is slowly added over 4 hours in portions while the reaction temperature is maintained at or below 10 °C. The mixture is stirred for 1.5 hours at 10 °C, then allowed to warm to 14° C. 32.6 kg of purified water can be charged to the 72 L flask, then transferred to the 100 L flask while the internal batch temperature is maintained at 20 ± 5 °C. The layers are allowed to split and the bottom aqueous layer is separated and stored. The organic layer is extracted three times with 32 kg of purified water each, and the aqueous layers are separated and combined with the stored aqueous solution.

The remaining organic layer is cooled to 18 °C and a solution of 847 g of succinic acid in 10.87 kg of purified water is added slowly in portions to the organic layer. The mixture is stirred for 1.75 hours at 20 ± 5 °C. The mixture is filtered, and the solids are washed with 2 kg TBME and 2 kg of acetone and then dried on the funnel. The filter cake is triturated twice with 5.7 kg each of acetone and filtered and washed with 1 kg of acetone between

triturations. The solid is dried on the funnel, then transferred to trays, and dried in a vacuum oven at room temperature to a constant weight.

4. Preparation of (4-(2-Hydroxypropyl)-3-nitrophenyI)(4-tritylpiperazin-1-yl)methanone

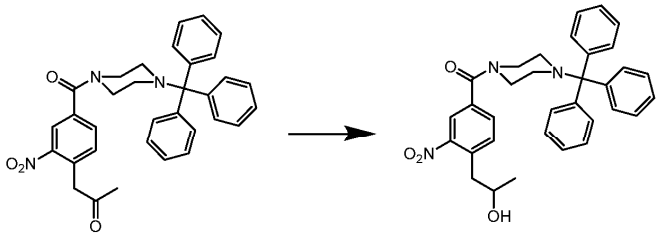
A. Preparation of 1-(2-Nitro-4(4-tritylpiperazine-1-carbonyl)phenyl)propan-2-one



3 4

2 kg of 3-Nitro-4-(2-oxopropyl)benzoic acid (**3**), 18.3 kg DCM, and 1.845 kg N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC.HCl) can be charged under nitrogen to a 100 L jacketed flask. The solution is stirred until a homogenous mixture forms. 3.048 kg of NTP is added over 30 minutes at room temperature and stirred for 8 hours. 5.44 kg of purified water is added to the reaction mixture and stirred for 30 minutes. The layers are allowed to separate and the bottom organic layer containing the product is drained and stored. The aqueous layer is extracted twice with 5.65 kg of DCM. The combined organic layers are washed with a solution of 1.08 kg sodium chloride in 4.08 kg purified water. The organic layer is dried over 1.068 kg of sodium sulfate and filtered. The sodium sulfate is washed with 1.3 kg of DCM. The combined organic layers are slurried with 252 g of silica gel and filtered through a filter funnel containing a bed of 252 g of silica gel. The silica gel bed is washed with 2 kg of DCM. The combined organic layers are evaporated on a rotary evaporator, and then 4.8 kg of THF is added to the residue and evaporated on the rotary evaporator until 2.5 volumes of the crude 1-(2-nitro-4(4-tritylpiperazine-1-carbonyl)phenyl)propan-2-one in THF is reached.

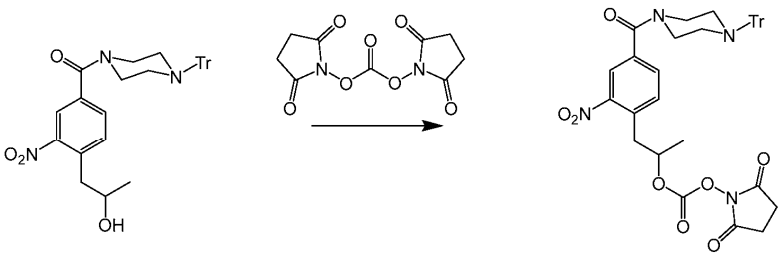
B. Preparation of (4-(2-Hydroxypropyl)-3-nitrophenyl)(4-tritylpiperazin-1-yl)methanone (**5**)



4 5

3600 g of **4** from the previous step and 9800 g THF can be charged under nitrogen into a 100 L jacketed flask. The stirred solution is cooled to ≤ 5 °C. The solution is diluted with 11525 g ethanol and 194 g of sodium borohydride is added over about 2 hours at ≤ 5 °C. The reaction mixture is stirred an additional 2 hours at ≤ 5 °C. The reaction is quenched with a solution of 1.1 kg ammonium chloride in 3 kg of water by slow addition to maintain the temperature at ≤ 10 °C. The reaction mixture is stirred an additional 30 minutes, filtered to remove inorganics, recharged to a 100 L jacketed flask, and extracted with 23 kg of DCM. The organic layer is separated and the aqueous layer is twice more extracted with 4.7 kg of DCM each. The combined organic layers are washed with a solution of 800 g of sodium chloride in 3 kg of water, then dried over 2.7 kg of sodium sulfate. The suspension is filtered and the filter cake is washed with 2 kg of DCM. The combined filtrates are concentrated to 2.0 volumes, diluted with 360 g of ethyl acetate, and evaporated. The crude product is loaded onto a silica gel column of 4 kg of silica packed with DCM under nitrogen and eluted with 2.3 kg ethyl acetate in 7.2 kg of DCM. The combined fractions are evaporated and the residue is taken up in 11.7 kg of toluene. The toluene solution is filtered and the filter cake is washed twice with 2 kg of toluene each. The filter cake is dried to a constant weight.

5. Preparation of 2,5-Dioxopyrrolidin-1-yl(1-(2-nitro-4-(4-triphenylmethylpiperazine-1 carbonyl)phenyl)propan-2-yl) carbonate (**NCP2 Anchor**)



5 **NCP2 Anchor**

4.3 kg of compound **5** (weight adjusted based on residual toluene by ^{1}H NMR; all reagents here after are scaled accordingly) and 12.7 kg pyridine can be charged to a 100 L jacketed flask under nitrogen. 3.160 kg of DSC (78.91 weight % by ^{1}H NMR) is added to this while the internal temperature is maintained at ≤ 35 °C. The reaction mixture is aged for about 22 hours at ambient room temperature and then filtered. The filter cake is washed with 200 g of pyridine. In two batches, each comprising ½ the filtrate volume, filtrate wash can be charged slowly to a 100 L jacketed flask containing a solution of 11 kg of citric acid and 50 kg of water, and stirred is for 30 minutes to allow for solid precipitation. The solid is collected with a filter funnel, washed twice with 4.3 kg of water per wash, and dried on the filter funnel under vacuum.

The combined solids can be charged to a 100 L jacketed flask and dissolved in 28 kg of DCM and washed with a solution of 900 g of potassium carbonate in 4.3 kg of water. After 1 hour, the layers are allowed to separate and the aqueous layer is removed. The organic layer is washed with 10 kg of water, separated, and dried over 3.5 kg of sodium sulfate. The DCM is filtered, evaporated, and dried under a vacuum to 6.16 kg of NCP2 Anchor.

NCP2 Anchor Loaded Resin Synthesis

About 52 L of NMP and 2300 g of aminomethyl polystyrene resin can be charged to a 75 L solid phase synthesis reactor with a Teflon stop cock. The resin is stirred in the NMP to swell for about 2 hours then drained. The resin is washed twice with 4 L DCM per wash, then twice with 39 L Neutralization Solution per wash, then twice with 39 L of DCM per wash. The NCP2 Anchor Solution is slowly added to the stirring resin solution, stirred

for 24 hours at room temperature, and drained. The resin is washed four times with 39 L of NMP per wash, and six times with 39 L of DCM per wash. The resin is treated and stirred with ½ the Diethyl Dicarbonate (DEDC) Capping Solution for 30 minutes, drained, and is treated and stirred with the 2nd half of the DEDC Capping Solution for 30 minutes and drained. The resin is washed six times with 39 L of DCM per wash and then dried in an oven to a constant weight of 3573.71 g of Anchor Loaded Resin.

Preparation of Morpholino Oligomer using NCP2 Anchor

50 L Solid-phase Synthesis of PMO Crude Drug Substance

1. Materials

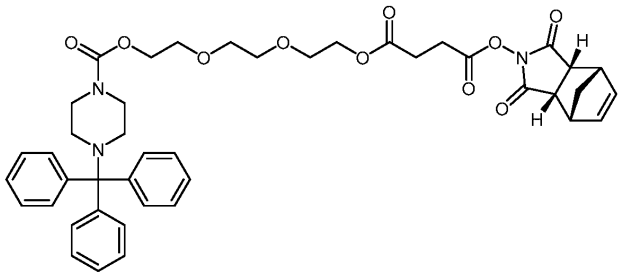
Table 2: Starting Materials

Material Name	Chemical Name	CAS Number	Chemical Formula	Molecular Weight
Activated A Subunit	Phosphoramidochloridic acid, *N*,*N*-dimethyl-,[6-[6-(benzoylamino)-9H-purin-9-yl]-4-(triphenylmethyl)-2-morpholinyl]methyl ester	1155373-30-0	$C_{38}H_{37}ClN_7O_4P$	722.2
Activated C Subunit	Phosphoramidochloridic acid, *N*,*N*-dimethyl-,[6-[4-(benzoylamino)-2-oxo-1(2H)-pyrimidinyl]-4-(triphenylmethyl)-2-morpholinyl]methyl ester	1155373-31-1	$C_{37}H_{37}ClN_5O_5P$	698.2
Activated DPG Subunit	Propanoic Acid, 2,2-dimethyl-,4-[[[9-[6-[[[chloro(dimethylamino)phosphinyl]oxy]methyl]-4-(triphenylmethyl)-2-morpholinyl]-2-[(2-phenylacetyl)amino]-9H-purin-6-yl]oxy]methyl]phenyl ester	1155309-89-9	$C_{51}H_{53}ClN_7O_7P$	942.2

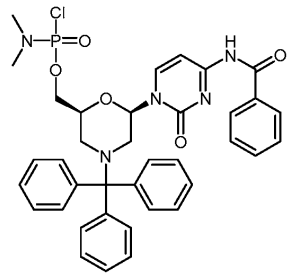
Activated T Subunit	Phosphoramidochloridic acid, *N*,*N*-dimethyl-,[6-(3,4-dihydro-5-methyl-2,4-dioxo-1(2H)-pyrimidinyl)]-4-(triphenylmethyl)-2-morpholinyl]methyl ester	1155373-34-4	$C_{31}H_{34}ClN_4O_5P$	609.1
Activated EG3 Tail	Butanedioic acid, 1-[3aR,4S,7R,7aS)-1,3,3a,4,7,7a-hexahydro-1,3-dioxo-4,7-methano-2H-isoindol-2-yl] 4-[2-[2-[2-[[[4-(triphenylmethyl)-1-piperazinyl]carbonyl]oxy]ethoxy]ethoxy]ethyl] ester	1380600-06-5	$C_{43}H_{47}N_3O_{10}$	765.9

Chemical Structures of Starting Materials:

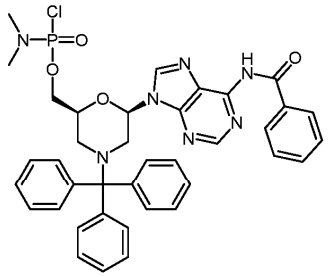
A. Activated EG3 Tail



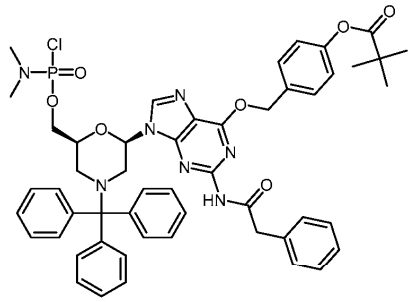
B. Activated C Subunit (For preparation, *see* U.S. Patent No. 8,067,571)



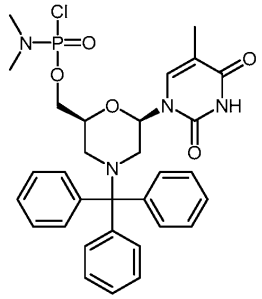
C. Activated A Subunit (For preparation, *see* U.S. Patent No. 8,067,571)



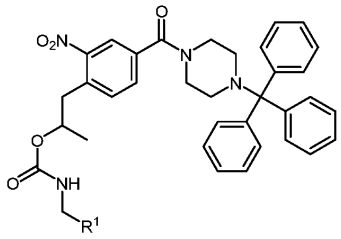
D. Activated DPG Subunit (For preparation, *see* WO 2009/064471)



E. Activated T Subunit (For preparation, *see* WO 2013/082551)



F. Anchor Loaded Resin



wherein R^1 is a support-medium.

Table 3: Description of Solutions for Solid Phase Oligomer Synthesis of PMO Crude Drug Substance

Solution Name	Solution Composition
NCP2 Anchor Solution	37.5 L NMP and 1292 g NCP2 Anchor
DEDC Capping Solution	4.16 L Diethyl Dicarbonate (DEDC), 3.64 L NEM, and 33.8 L DCM
CYTFA Solution	2.02 kg 4-cyanopyridine, 158 L DCM, 1.42 L TFA, 39 L TFE, and 2 L purified water
Neutralization Solution	35.3 L IPA, 7.5 L DIPEA, and 106.5 L DCM
Cleavage Solution	1,530.04 g DTT, 6.96 L NMP, and 2.98 L DBU

2. Synthesis of PMO Crude Drug Substance

A. Resin swelling

An aliquot of 750 g of Anchor Loaded Resin and 10.5 L of NMP can be charged to a 50 L silanized reactor and stirred for 3 hours. The NMP is drained and the Anchor Loaded Resin is washed twice with 5.5 L each of DCM and twice with 5.5 L each of 30% TFE/DCM.

B. Cycle 0: EG3 Tail Coupling

The Anchor Loaded Resin is washed three times with 5.5 L each of 30% TFE/DCM and drained, washed with 5.5 L of CYTFA solution for 15 minutes and drained, and again washed with 5.5 L of CYTFA Solution for 15 minutes without draining to which 122 mL of 1:1 NEM/DCM can be charged and the suspension stirred for 2 minutes and drained. The resin is washed twice with 5.5 L of Neutralization Solution for 5 minutes and drained, then twice with 5.5 L each of DCM and drained. A solution of 706.2 g of activated EG3 Tail and 234 mL of NEM in 3 L of DMI can be charged to the resin and stirred for 3 hours at RT and drained. The resin is washed twice with 5.5 L each of Neutralization Solution for 5 minutes per each wash, and then once with 5.5 L of DCM and drained. A solution of 374.8 g of benzoic anhydride and 195 mL NEM in 2680 mL NMP can be charged and stirred for 15

minutes and drained. The resin is stirred with 5.5 L of Neutralization Solution for 5 minutes, then washed once with 5.5 L of DCM and twice with 5.5 L each of 30% TFE/DCM. The resin is suspended in 5.5 L of 30% TFE/DCM and held for 14 hours.

C. Subunit Coupling Cycles 1-**n**

Table 4 - General Base Subunit Coupling

Cycle No.: Subunit (SU)	Pre-coupling Treatment				Coupling Cycle		Post-Coupling Treatment	
	1	2	3	4			1	2
	30% TFE/DCM Wash	CYTFA Solution[1]	Neutralization Solution	DCM Wash	Quantity SU (g) NEM (L) DMI (L)	RT Coupling Time (Hrs.)	DCM Wash	30% TFE/DCM Wash
1:C	5.5L	a) 5.5L b) 5.5L, 122mL	3x5.5L	5.5L	536.7g; 195 mL NEM; 3.2L DMI	5	5.5L	2x5.5L

[1] mL indicates the amount of 1:1 NEM/DCM

i. Pre-coupling treatments

Prior to each coupling cycle, the resin is: 1) washed with 30% TFE/DCM; 2) a) treated with CYTFA Solution for 15 minutes and drained, and b) treated with CYTFA solution for 15 minutes to which 1:1 NEM/DCM is added, stirred, and drained; 3) stirred three times with Neutralization Solution; and 4) washed twice with DCM.

ii. Post Coupling Treatments

After each subunit solution is drained, the resin is: 1) washed with DCM; and 2) washed two times with 30% TFE/DCM. If the resin is held for a time period prior to the next coupling cycle, the second TFE/DCM wash is not drained and the resin is retained in said TFE/DCM wash solution.

iii. Activated Subunit Coupling Cycles

Each coupling cycle is performed as generally described for the initial C (cytosine) monomer coupling in Table 2 for each base-containing subunit.

iv. Final IPA Washing

After the final coupling step is performed, the resin is washed 8 times with 19.5 L each of IPA, and dried under a vacuum at room temperature for about 63.5 hours to a dried weight of 5,579.8 g.

C. Cleavage

The above resin bound PMO Crude Drug Substance is divided into two lots, each lot is treated as follows. A 2,789.9 g lot of resin is: 1) stirred with 10 L of NMP for 2 hrs, then the NMP is drained; 2) washed three times with 10 L each of 30% TFE/DCM; 3) treated with 10 L CYTFA Solution for 15 minutes; and 4) treated with 10 L of CYTFA Solution for 15 minutes to which 130 mL 1:1 NEM/DCM is then added and stirred for 2 minutes and drained. The resin is treated three times with 10 L each of Neutralization Solution, washed six times with 10 L of DCM, and eight times with 10 L each of NMP. The resin is treated with a Cleaving Solution of 1530.4 g DTT and 2980 DBU in 6.96 L NMP for 2 hours to detach the PMO Crude Drug Substance from the resin. The Cleaving solution is drained and retained in a separate vessel. The reactor and resin are washed with 4.97 L of NMP which is combined with the Cleaving Solution.

D. Deprotection

The combined Cleaving Solution and NMP wash are transferred to a pressure vessel to which was added 39.8 L of NH_4OH ($NH_3 \cdot H_2O$) that is pre-chilled to a temperature of -10 °C to -25 °C in a freezer. The pressure vessel is sealed and heated to 45 °C for 16 hours then allowed to cool to 25 °C. This deprotection solution containing the PMO crude drug substance is diluted 3:1 with purified water and pH adjusted to 3.0 with 2 M phosphoric acid, then to pH 8.03 with NH_4OH.

E. Purification of PMO Crude Drug Substance

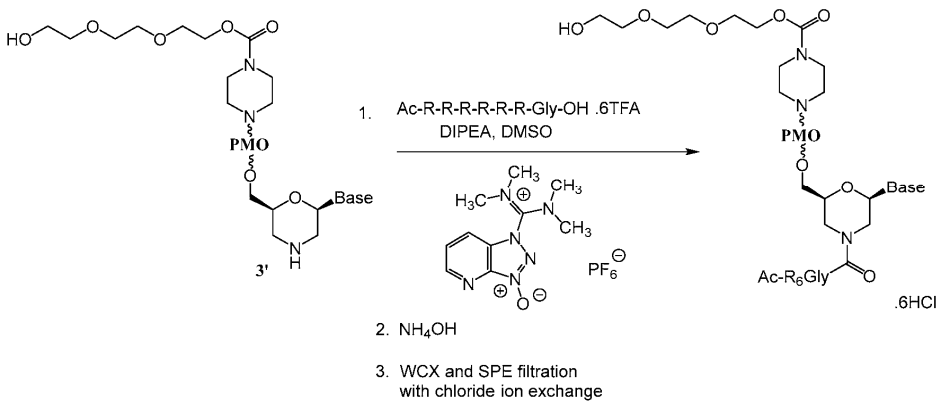
The deprotection solution from above part D, containing the PMO crude drug substance, is loaded onto a column of ToyoPearl Super-Q 650S anion exchange resin (Tosoh Bioscience) and eluted with a gradient of 0-35% B over 17 column volumes (Buffer A: 10 mM sodium hydroxide; Buffer B: 1 M sodium chloride in 10 mM sodium hydroxide) and fractions of acceptable purity (C18 and SCX HPLC) are pooled to a purified drug product solution.

The purified drug substance solution is desalted and lyophilized to purified PMO drug substance.

Table 5. Acronyms

Acronym	**Name**
CYTFA	4-Cyanopyridine Trifluoroacetic Acid
CPP	Cell Penetrating Peptide
DBU	1,8-Diazabicycloundec-7-ene
DCM	Dichloromethane
DEDC	Diethyl Dicarbonate
DIPEA	N,N-Diisopropylethylamine
DMI	1,3-Dimethyl-2-imidazolidinone
DMSO	Dimethyl Sulfoxide
DTT	DL-Dithiothreitol
HPLC	High performance Liquid Chromatography
IPA	Isopropyl alcohol
MW	Molecular weight
NEM	N-Ethylmorpholine
NMP	N-Methyl-2-pyrrolidone
SAX	Strong Anion Exchange
SCX	Strong Cation Exchange
SPE	Solid Phase Extraction
RT	Room temperature
TFA	2,2,2-Trifluoroacetic acid
TFE	2,2,2-Trifluoroethanol

CPP Conjugation ("R6Gly" is disclosed as SEQ ID NO: 11)



Analytical Procedures: Matrix-assisted LASER desorption ionization time-of-flight mass spectra (MALDI-TOF-MS) can be recorded on a Bruker AutoflexTM Speed, using a sinapinic acid (SA) matrix. SCX-HPLC can be performed on a Thermo Dionex UltiMate 3000 system equipped with a 3000 diode array detector and a ProPacTM SCX-20 column (250 x 4 mm) using a flow rate of 1.0 mL/min (pH = 2; 30 °C column temperature). The mobile phases can be **A** (25 % acetonitrile in water containing 24 mM H_3PO_4) and **B** (25 % acetonitrile in water containing 1 M KCl and 24 mM H_3PO_4). Gradient elution can be employed: 0 min, 35% **B**; 2 min, 35% **B**; 22 min, 80% **B**; 25 min, 80% **B**; 25.1 min, 35% **B**; 30 min, 35% **B**.

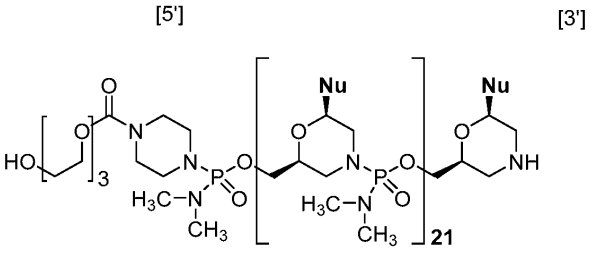
Ac-L-Arg-L-Arg-L-Arg-L-Arg-L-Arg-L-Arg-Gly-OH (SEQ ID NO: 11) hexatrifluoroacetate (614.7 mg, 0.354 mmol), and 1-[Bis(dimethylamino)methylene]-1*H*-1,2,3-triazolo[4,5-*b*]pyridinium 3-oxid hexafluorophosphate (HATU, 134.4 mg, 0.354 mmol) and dimethyl sulfoxide (DMSO, 20 mL) are added to a mixture of the PMO (freshly dried by lyophilization for two days). The mixture is stirred at room temperature for 3 minutes, then *N*,*N*-diisopropylethylamine (DIPEA, 68.5 mg, 0.530 mmol) is added. After 5 minutes, the cloudy mixture becomes a clear solution. The reaction can be monitored by SCX-HPLC. After 2 hours, 20 mL of 10% ammonium hydroxide solution (2.8% NH_3*H_2O) is added. The mixture is stirred at room temperature for an additional 2 hours. The reaction is terminated by the addition of 400 mL water. Trifluoroethanol (2.0 mL) is added to the solution.

The solution is divided into two portions and each portion can be purified by a WCX column (10 g resin per column). Each WCX column is first washed with 20 % acetonitrile in water (v/v) to remove the PMO starting material. The washings (225 mL for each column) can be stopped when MALDI-TOF mass spectrum analysis shows the absence of PMO signal. Each column is then washed with water (100 mL per column). The desired product can be eluted using 2.0 M guanidine HCl (140 mL for each column). The purified solutions are pooled together and then divided into two portions and each desalted by an SPE column (10 g resin for each column).

The SPE columns can be first washed with 1.0 M aqueous NaCl solution (100 mL for each column) to generate the hexahydrochloride salt form. Each SPE column is then washed with water (200 mL for each column). The final desalted product can be eluted using 50% acetonitrile in water (v/v, 150 mL for each column). The acetonitrile can be removed by evacuation at reduced pressure. The resulting aqueous solution can be lyophilized to obtain the desired product as a hexahydrochloride salt.

Example 1: PMOs

Using the PMO synthesis methods described above, PMO#1, PMO#2 and PMO#3 were synthesized as follows:



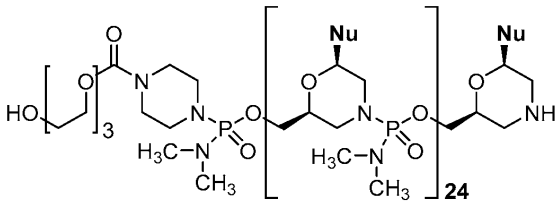
PMO#1

where each Nu from 1 to 22 and 5' to 3' is H50D(+04-18) (SEQ ID NO: 1):

Position No. 5' to 3'	Nu	Position No. 5' to 3'	Nu	Position No. 5' to 3'	Nu	Position No. 5' to 3'	Nu	Position No. 5' to 3'	Nu
1	G	6	C	11	A	16	T	21	G
2	G	7	C	12	T	17	A	22	C
3	G	8	A	13	A	18	C		

4	A	9	G	14	C	19	A		
5	T	10	T	15	T	20	G		

[5'] [3']

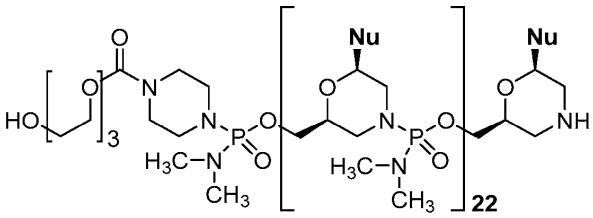


PMO#2

where each Nu from 1 to 25 and 5' to 3' is H50D(+07-18) (SEQ ID NO: 2):

Position No. 5' to 3'	Nu	Position No. 5' to 3'	Nu	Position No. 5' to 3'	Nu	Position No. 5' to 3'	Nu	Position No. 5' to 3'	Nu
1	G	6	C	11	A	16	T	21	G
2	G	7	C	12	T	17	A	22	C
3	G	8	A	13	A	18	C	23	T
4	A	9	G	14	C	19	A	24	C
5	T	10	T	15	T	20	G	25	C

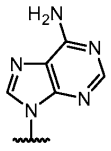
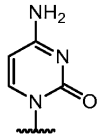
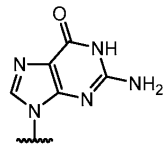
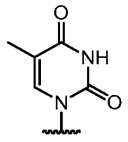
[5'] [3']



PMO#3

where each Nu from 1 to 23 and 5' to 3' is H50D(+07-16) (SEQ ID NO: 3):

Position No. 5' to 3'	Nu	Position No. 5' to 3'	Nu	Position No. 5' to 3'	Nu	Position No. 5' to 3'	Nu	Position No. 5' to 3'	Nu
1	G	6	A	11	A	16	C	21	T
2	A	7	G	12	C	17	A	22	C
3	T	8	T	13	T	18	G	23	C
4	C	9	A	14	T	19	G		
5	C	10	T	15	A	20	C		

wherein A is , C is , G is , and T is .

PMO#1 (SEQ ID NO: 1) yielded a product with solubility characteristic which were too limited to allow for drug product formulation while PMO#2 (SEQ ID NO: 2) yielded a product which could not be manufactured with sufficient yield and purity.

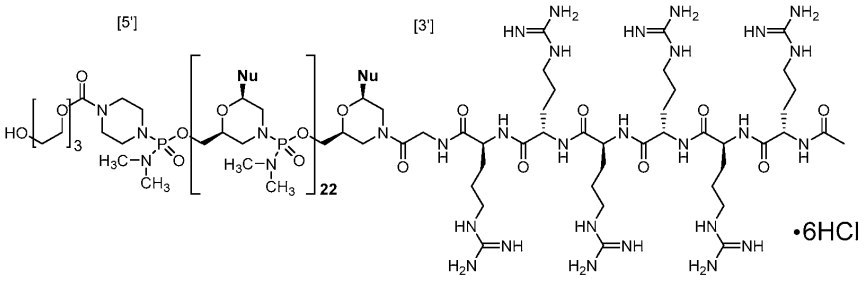
In contrast to the synthesis of PMO#1 and PMO#2, the synthesis of PMO#3 (which differs by only 2 bases from the 5' end PMO#2) provided no solubility or purification issues allowing for the subsequent synthesis of PPMO#3 from PMO#3 (Example 2 - below).

Targeting Sequence (5'-3')	SEQ ID NO:	PMO Manufacturing Issues:
GGGATCCAGTATACTTACAGGC	1	Solubility: Crude PMO precipitates out after storage displaying limited solubility and solution stability making subsequent PPMO synthesis unfeasible and to serve as a drug product candidate.
GGGATCCAGTATACTTACAGGCTCC	2	Purification: Crude PMO could not be purified to an appropriate purity level required for subsequent PPMO synthesis and to serve as a drug product candidate.
GATCCAGTATACTTACAGGCTCC	3	None (91% purity; dissolves in water)

A similar process was used to synthesize PMO#4, PMO#5, PMO#6, PMO#7, PMO#8, and PMO#9.

Example 2: PPMO#3

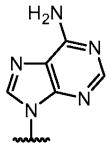
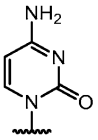
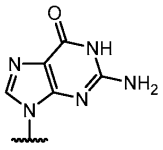
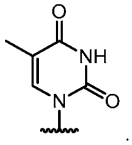
Using the protocol described above, PPMO#3 was synthesized from PMO#3 (SEQ ID NO: 3):



PPMO#3

where each Nu from 1 to 23 and 5' to 3' is SEQ ID NO: 3:

Position No. 5' to 3'	Nu	Position No. 5' to 3'	Nu	Position No. 5' to 3'	Nu	Position No. 5' to 3'	Nu	Position No. 5' to 3'	Nu
1	G	6	A	11	A	16	C	21	T
2	A	7	G	12	C	17	A	22	C
3	T	8	T	13	T	18	G	23	C
4	C	9	A	14	T	19	G		
5	C	10	T	15	A	20	C		

wherein A is , C is , G is , and T is .

Example 3: Exon 50 Skipping *in vitro*

Two compounds that target human dystrophin (*DMD*) exon 50 as described in the Table below, PMO#3 and PPMO#3 both of which contain the same sequence, were assessed for *DMD* exon 50 skipping in healthy human myotubes.

Sequences of PMO#3 and PPMO#3:

Name	Targeting Sequence (TS)	SEQ ID NO.	5'	3'
PMO#3	GATCCAGTATACTTACAGGCTCC	3	EG3	H
PPMO#3	GATCCAGTATACTTACAGGCTCC	3	EG3	-G-R_6 (SEQ ID NO: 11)

Specifically, healthy human myoblasts (passage 5-6, SKB-F-SL purchased from Zen-Bio, Inc.) were cultured to reach 80-90% confluency in SKM-M media prior to initiation of differentiation by incubating in low serum media (SKM-D, Zen-Bio, Inc.) Five-days after differentiation, mature myotubes were incubated with the above compounds at various concentrations (i.e., 40 μm, 20 μm, 10 μm, 5 μm, 2.5 μm, and 1.25 μm). After ninety-six hours of incubation, myotubes were washed with PBS and lysed by RLT buffer in the RNeasy Micro kit (Cat#74004, Qiagen) supplemented with 1% β-mercaptoethanol. Total RNA were isolated per manufacturer's recommendation, except that 20μL RNase-free water was used to elute RNA.

To determine *DMD* exon 50 skipping by both compounds, one-step end-point RT-PCR was performed. cDNA synthesis and PCR amplification was carried out by using 100ng of total RNA, gene-specific primers and SuperScript III One-step RT-PCR System with Platinum *Taq* DNA Polymerase (Cat#12574-026, Invitrogen). Gene-specific primers were designed to target at human *DMD* exons 49 and 52 (forward primer: CCA GCC ACT CAG CCA GTG AAG (SEQ ID NO: 12); reverse primer: CGA TCC GTA ATG ATT GTT CTA GCC(SEQ ID NO: 13)). cDNA synthesis and PCR amplification was performed by BioRad CFX96 real time thermocyclers using the program shown in Table 6. Expression of the skipped and non-skipped PCR products were assessed by loading 22μL PCR product onto the DNA Extended Range LabChip of a LabChip GX system prepared by the DNA 1K Reagent (Cat#760517 and CLS760673, Perkin Elmer) per manufacturer's instruction. Percentage of *DMD* exon 50 skipping is calculated as the percentage of the molarity (nmol/l) for exon 50 skipped band compared to the sum molarity for the skipped and the unskipped bands.

Two-tailed, unpaired Student's t-test (homoscedastic) was used to assess whether the means of the 2 groups are statistically different from each other at each dose. *P*-value < 0.05 is considered as statistically significant.

Table 6. Thermocycler program used to amplify *DMD* amplicons with or without exon 50 skipping.

Step	Temperature	Time
1. Reverse Transcription	55°C	30 min
2. Reverse transcriptase inactivation	94°C	2 min
3. Denature	94°C	45 sec
4. Anneal	59°C	45 sec
5. Extend	68°C	1 min
6. Repeat step 3-4	45 cycles	
7. Final extension	68°C	10 min
8. Storage	4°C	∞

The results, showing that PPMO#3 significantly increases *DMD* exon 50 skipping as compared to PMO#3, are presented in the table below (as the ratio of skipping of PPMO#3 to PMO#).

Table 7. Percentage of *DMD* exon 50 skipping by PMO#3 and PPMO#3 in human myotubes.

Compound/ Dose (μm)	Relative Exon Skipping					
	1.25	2.5	5	10	20	40
PMO#3	1	1	1	1	1	1
PPMO#3	2.6	3.2	3.0	2.4	2.1	1.9

The data in Table 7 above show that higher exon 50 skipping results in myotubes when the cells are treated with PPMO#3 as compared to PMO#3 at all concentrations. This improvement can be further demonstrated in an *in vivo* comparative test such as the non-human primate (NHP) study of Example 4 where NHPs are treated with PPMO#3 or PMO#3 and exon 50 skipping is measured in various relevant muscle tissues (see Example 4 for details).

Example 4: Exon 50 skipping in NHP

To further demonstrate the efficacy of exon skipping of PPMO antisense oligomers, non-human primates are utilized. Specifically, cynomolgus monkeys having intact muscle tissues are injected intravenously, with PPMO#3 (Example 2), PMO#3 (Example 1) or saline.

Animals are observed throughout the study, including clinical observations (e.g., evaluation of skin and fur, respiratory effects) and body weight measurements. Blood and urine samples are taken at least before testing begins, and 24 hours after the first dose and last dose (where applicable).

At each scheduled necropsy, or euthanized *in extremis*, sections of diaphragm, smooth muscle of the duodenum, esophagus, and aorta, quadriceps, deltoid, bicep, and heart are collected and snap frozen. Percent exon 50 skipping is determined using RT-PCR as described above.

Example 5: Exon 50 Skipping with PMOs In vitro

A series of PMOs (SEQ ID NOs 1-7; PMOs#1 – #7) were prepared and tested for exon 50 skipping efficiency. Briefly, human primary myoblasts were cultured using standard techniques. Lyophilized PMO were re-suspended in nuclease-free water; to verify molarity and PMO solutions were measured using a NanoDrop 2000 spectrophotometer (Thermo Scientific). A dose-range of PMOs were delivered to myoblasts cells (e.g., 0.625, 1.25, 2.5, 5, 10 and 20 μM) using nucleoporation according to the manufacturer's instructions and the P3 kit (Lonza) and allowed to incubate overnight in a 37°C, 5% CO2 incubator prior to RNA extraction. RNA was extracted from PMO-treated cells using the RNAspin 96 well RNA isolation kit from GE Healthcare and subjected to RT-PCR using standard techniques with primers that amplified human DMD exons 49-52. Skipping was measured using the Caliper LabChip bioanalyzer and the % exon skipping (i.e., band intensity of the exon-skipped product relative to the full length PCR product) was calculated by the equation: [exon 50 skipped product/(sum of exon 50 skipped and exon 50 unskipped products)*100] and EC50 values were calculated based on the percent skipping induced at each concentration. As shown in Table 8, PMO oligomers of the disclosure designed to target the splice acceptor or splice donor regions of exon 50 provided skipping of exon 50 with PMO#1, PMO#2 and PMO#3 providing the highest levels of exon 50 skipping activity (EC_{50} < 1.0 μM).

Table 8.

SEQ ID NO:	Compound	Activity (EC_{50} (1))
1	PMO#1 (+04-18)	****
2	PMO#2 (+07-18)	****
3	PMO#3 (+07-16)	****
4	PMO#4 (+07-17)	**
5	PMO#5 (-19+07)	**
6	PMO#6 (+07-15)	*
7	PMO#7 (-02+23)	*

(1) **** = EC_{50} < 1.0 μM; ** = EC_{50} 1.0 to 3.0 μM; * = EC_{50} 3.0 μM or greater.

It is understood that the foregoing detailed description and accompanying examples are merely illustrative and are not to be taken as limitations upon the scope of the invention, which is defined solely by the appended claims and their equivalents.

Various changes and modifications to the disclosed embodiments will be apparent to those skilled in the art. Such changes and modifications, including without limitation those relating to chemical structures, substituents, derivatives, intermediates, synthesis, compositions, formulations or methods of use of the invention, may be made without departing from the spirit or scope thereof.

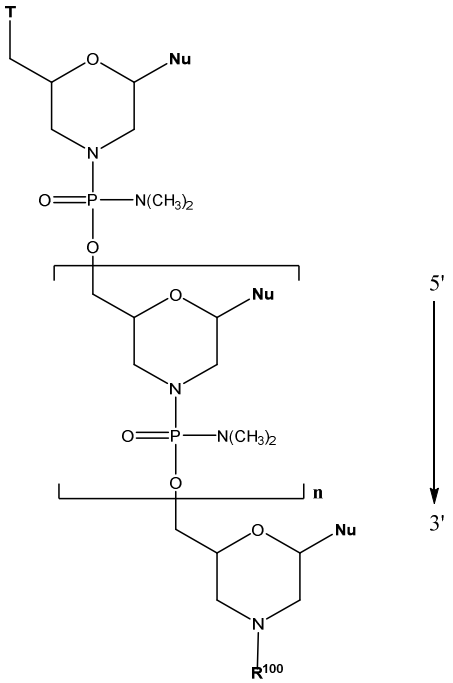
SEQUENCE LISTING*

Description	PMO Identifier	PPMO Identifier	Sequence (5' to 3' or N terminus to C terminus)	SEQ ID NO
H50D(+04-18)	PMO#1	PPMO#1	GGG ATC CAG TAT ACT TAC AGG C	1
H50D(+07-18)	PMO#2	PPMO#2	GGG ATC CAG TAT ACT TAC AGG CTC C	2
H50D(+07-16)	PMO#3	PPMO#3	GAT CCA GTA TAC TTA CAG GCT CC	3
H50D(+07-17)	PMO#4	PPMO#4	GGA TCC AGT ATA CTT ACA GGC TCC	4
H50A(-19+07)	PMO#5	PPMO#5	ACT TCC TCT TTA ACA GAA AAG CAT AC	5
H50D(+07-15)	PMO#6	PPMO#6	ATC CAG TAT ACT TAC AGG CTC C	6
H50A(-02+23)	PMO#7	PPMO#7	GAG CTC AGA TCT TCT AAC TTC CTC T	7
H50D(+06-18)	PMO#8	PPMO#8	GGG ATC CAG TAT ACT TAC AGG CTC	8
H50D(+07-20)	PMO#9	PPMO#9	ATG GGA TCC AGT ATA CTT ACA GGC TCC	9
R_6			RRRRRR	10
R_6G			RRRRRRG	11
Human exon 49 binding forward primer			CCAGCCACTCAGCCAGTGAAG	12
Human exon 52 binding reverse primer			CGATCCGTAATGATTGTTCTAGCC	13
PMO-G	PMO-G	PPMO-G	GTTGCCTCCGGTTCTGAAGGTGTTC	14
$(RXR)_4$			RXRRXRRXRRXR	15
$(RFF)_3R$			RFFRFFRFFR	16
$(RXR)_4XB$			RXRRXRRXRRXRXB	17
$(RFF)_3RXB$			RFFRFFRFFRXB	18
$(RFF)_3RG$			RFFRFFRFFRG	19
R_5G			RRRRRG	20
R_5			RRRRR	21
Intron 49 – EXON 50 – Intron 50	atcttcaaagtgttaatcgaataagtaatgtgtatgcttttctgttaaagAGGAAGTTAGAAGATCTGAGCT CTGAGTGGAAGGCGGTAAACCGTTTACTTCAAGAGCTGAGGGCAAA GCAGCCTGACCTAGCTCCTGGACTGACCACTATTGGAGCCTg taagtatactggatcccattctctttggctctagctatttgttcaaaag			22

* Depending upon the chemistry utilized to link the nucleobases, T can be Thymine or Uracil.

CLAIMS

1. An antisense oligomer according to Formula (I):

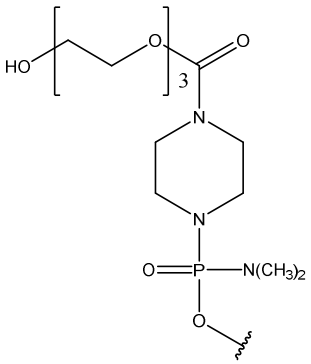


(I)

or a pharmaceutically acceptable salt thereof, wherein:

each **Nu** is a nucleobase which taken together form a targeting sequence;

T is

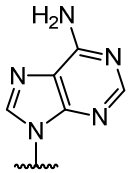
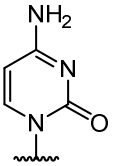
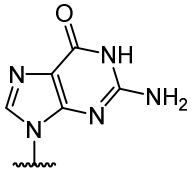
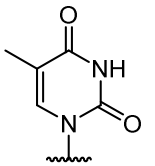


and the distal –OH of the T moiety is optionally linked to a cell-penetrating peptide;

R^{100} is hydrogen or a cell-penetrating peptide;

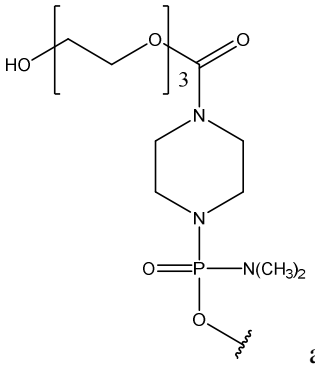
each **Nu** from 5' to 3' corresponds to the nucleobases in:

Annealing Site	Targeting Sequence [5' to 3']	SEQ ID NO:
H50D(+07-16)	GAT CCA GTA TAC TTA CAG GCT CC	SEQ ID NO: 3

wherein A is , C is , G is , and T is .

2. The antisense oligomer of claim 1, wherein the antisense oligomer contains one cell penetrating peptide.

3. The antisense oligomer of claim 1, wherein:

T is ; and

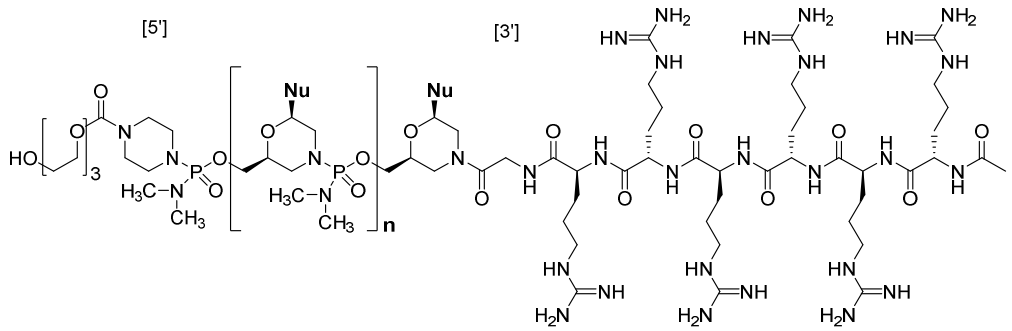
R^{100} is a cell-penetrating peptide.

4. The antisense oligomer of any one of claims 1-3, wherein the cell-penetrating peptide is an arginine-rich peptide, preferably wherein the arginine-rich peptide is selected from the group consisting of $–(RXR)_4-R^a$ (SEQ ID NO: 15), $R-(FFR)_3-R^a$ (SEQ ID NO: 16), -B-X-$(RXR)_4-R^a$ (SEQ ID NO: 17), -B-X-R-$(FFR)_3-R^a$ (SEQ ID NO: 18), -GLY-R-$(FFR)_3-R^a$ (SEQ ID NO: 19), -GLY-R_5-R^a (SEQ ID NO: 20), $–R_5-R^a$ (SEQ ID NO: 21), -GLY-R_6-R^a (SEQ ID NO: 11) and $–R_6-R^a$ (SEQ ID NO: 10), wherein R^a is selected from H, acyl, benzoyl, and stearoyl, and wherein R is arginine, X is 6-aminohexanoic acid, B is β-alanine, F is phenylalanine and GLY (or G) is glycine.

5. The antisense oligomer of any one of claims 1-4, wherein the antisense oligomer is in free base form.

6. The antisense oligomer of any one of claims 1-4, wherein the antisense oligomer is a pharmaceutically acceptable salt thereof.

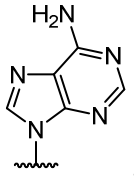
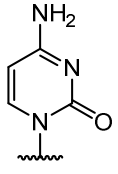
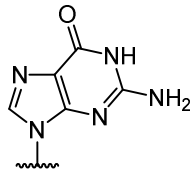
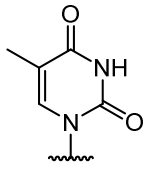
7. The antisense oligomer of claim 1, wherein the antisense oligomer is of Formula (III):



(III)

or a pharmaceutically acceptable salt thereof, where each **Nu** from 5' to 3' corresponds to the nucleobases in one of the following:

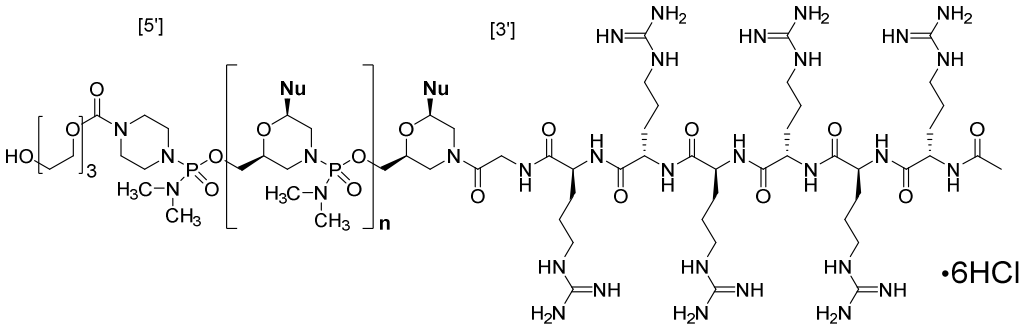
Annealing Site	**Targeting Sequence [5' to 3']**	**SEQ ID NO:**
H50D(+07-16)	GAT CCA GTA TAC TTA CAG GCT CC	SEQ ID NO: 3

wherein A is , C is , G is , and T is ,

and the distal –OH of formula (III) is optionally linked to a cell penetrating peptide.

8. The antisense oligomer of any one of claim 7, wherein the antisense oligomer is in free base form, or wherein the antisense oligomer is a pharmaceutically acceptable salt thereof.

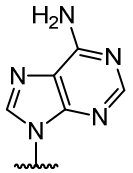
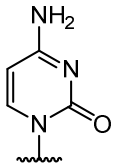
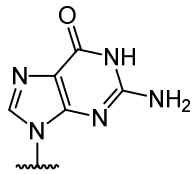
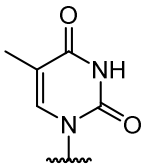
9. The antisense oligomer of claim 1, wherein the antisense oligomer is of Formula (IV):



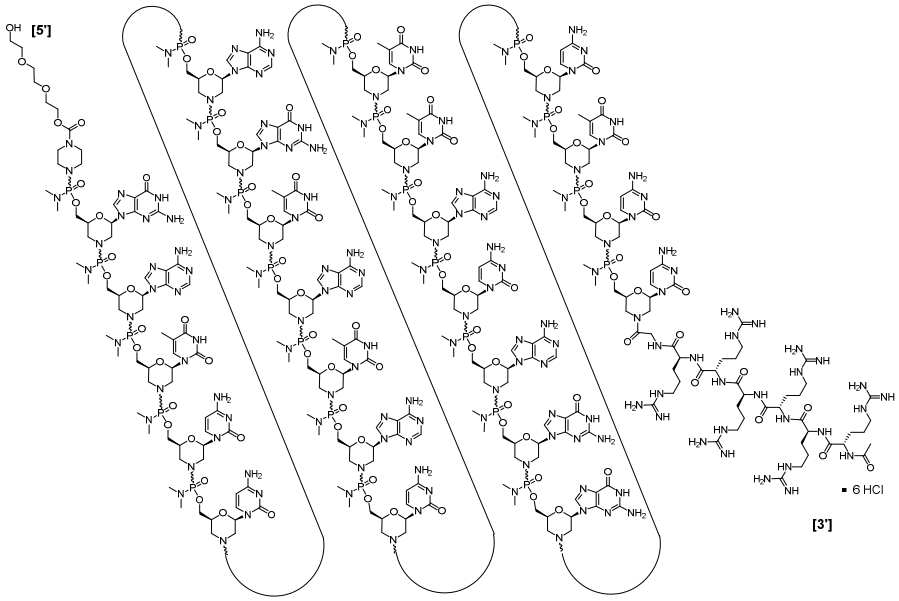
(IV)

where each **Nu** from 5' to 3' corresponds to the nucleobases in one of the following:

Annealing Site	**Targeting Sequence [5' to 3']**	**SEQ ID NO:**
H50D(+07-16)	GAT CCA GTA TAC TTA CAG GCT CC	SEQ ID NO: 3

wherein A is , C is , G is , and T is  and the distal –OH of formula (IV) is optionally linked to a cell penetrating peptide.

10. The antisense oligomer of claim 9, wherein the antisense oligomer is according to the structure of Formula (IVa)



(IVa).

11. A pharmaceutical composition comprising an antisense oligomer of any one of claims 1-10 or a pharmaceutically acceptable salt thereof, and a pharmaceutically acceptable carrier.

12. Use of the antisense oligomer of any one of claims 1 – 10 or the pharmaceutical composition of claim 11 in the manufacture of a medicament for the treatment of Duchenne muscular dystrophy (DMD) in a subject in need thereof.

13. The use of claim 12, wherein the subject has a mutation of the dystrophin gene that is amenable to exon 50 skipping.

14. Use of the antisense oligomer of any one of claims 1 – 10 or the pharmaceutical composition of claim 11 in the manufacture of a medicament for the restoration of an mRNA reading frame to induce dystrophin production in a subject, wherein the subject has a mutation of the dystrophin gene that is amenable to exon 50 skipping.

15. The antisense oligomer of any one of claims 1 – 10 or the pharmaceutical composition of claim 11 for use in therapy.