COMPOUNDS AND METHODS FOR SKIPPING EXON 50 IN DUCHENNE MUSCULAR DYSTROPHY

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims the benefit of priority to the filing date of U.S. Provisional Application Serial No. 63/585,311, filed September 26, 2023; U.S. Provisional Application Serial No. 63/588,115, filed October 5, 2023; U.S. Provisional Application Serial No. 63/557,125, filed February 23, 2024; and U.S. Provisional Application Serial No. 63/640,506, filed April 30, 2024, the disclosure of each of which is specifically incorporated by reference herein in its entirety.

BACKGROUND

[0002] Duchenne Muscular Dystrophy (DMD) is a genetic disorder characterized by progressive muscle degeneration and weakness due to alterations of the protein dystrophin. Genetic modifications in the gene that encodes dystrophin (i.e., the *DMD* gene), cause DMD. These genetic modifications shift the reading frame of the *DMD* gene leading to a nonfunctional truncated DMD protein. One method to treat DMD patients entails delivering to a subject having DMD a compound which restores the reading frame of the *DMD* gene. Antisense oligonucleotides can restore the reading frame of the *DMD* gene by skipping an internal exon associated with the shift of the reading frame of the *DMD* gene leading to the nonfunctional truncated DMD protein. Exon skipping produces dystrophin proteins which retain functionality that is lost in the disease state.

[0003] DMD is caused by mutations in one or more of several exons, for example, in the region of exons 44-55. In 2016, the US Food and Drug Administration (FDA) granted accelerated approval to the first phosphorodiamidate morpholino oligonucleotide (PMO)-based drug developed for DMD exon 51 skipping, Exondys 51 (a.k.a. eteplirsen). About 13 % of DMD patients have a mutation amenable to exon 51 skipping. (Aoki, et al., Proc Natl Acad Sci USA. 109(34):13763-13768). However, there are currently no approved therapy for DMD patients having a mutation amenable to Exon 50 skipping.

SUMMARY

[0004] Disclosed herein are EEV-cargo conjugates for skipping exon 50 in a subject with Duchenne muscular dystrophy (DMD).

[0005] The disclosure relates to an EEV-cargo conjugate comprising:

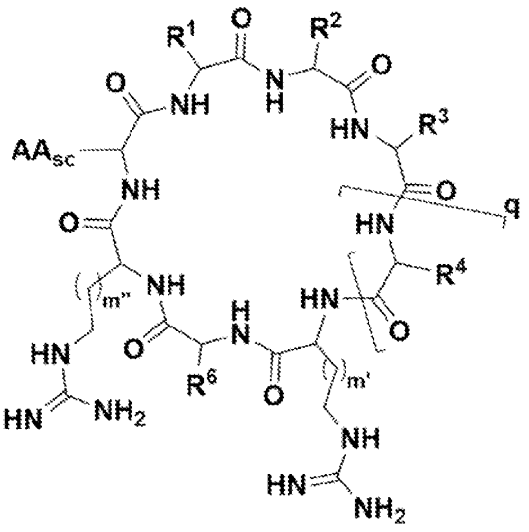
(a) an endosomal escape vehicle (EEV) comprising a cyclic cell penetrating peptide ("cCPP"); and

(b) a cargo comprising an oligonucleotide that is complementary to a target sequence of a pre-mRNA transcript of a *DMD* gene, wherein the target sequence comprises at least a portion of the 5' flanking intron of exon 50, at least a portion of exon 50, at least a portion of the 3' flanking intron of exon 50, or a combination thereof.

[0006] In embodiments, EEV-cargo conjugate can comprise:

(a) a cyclic cell penetrating peptide (cCPP) wherein the cCPP has a structure of Formula (2):

Formula (2):



(2), or a protonated form thereof,

wherein:

R^1, R^2, and R^3 can each independently be H or an amino acid residue having a side chain comprising an aryl or heteroaryl group;

at least two of R^1, R^2, and R^3 are, independently, an aryl or heteroaryl side chain of an amino acid;

R^4 and R^6 are, independently, H or an amino acid side chain;

AAsc is an amino acid side chain;

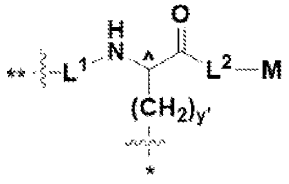
q is 1, 2, 3 or 4; and

m′ and m″ are each, independently, an integer from 0 to 3;

(b) a linear exocyclic peptide (EP), wherein the EP comprises from 2 to 10 amino acid residues;

(c) a linker of Formula (A′):

Formula (A'):



(A′)

wherein:

** is a point of attachment to a linear exocyclic peptide (EP);

* is a point of attachment to a cyclic cell penetrating peptide (cCPP);

L^1 and L^2, are, independently, a linker arm;

^ indicates L- or D-stereochemistry;

y′ is an integer from 1 to 5; and

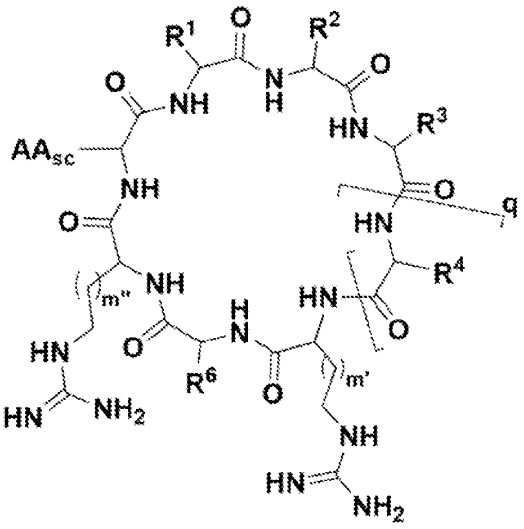
M is a reactive handle comprising a functional group that reacts with a corresponding functional group on the cargo to form a bonding group (M'); and

(d) a cargo that comprises an oligonucleotide that hybridizes to exon 50 in a pre-mRNA transcript of human dystrophin gene (*DMD*) to cause exon skipping, wherein the oligonucleotide hybridizes to or comprises a nucleic acid sequence shown in **Tables 11A-11D**, **12A-12D**, or **13.**

[0007] In embodiments, the EEV-cargo conjugate can comprise:

(a) a cyclic cell penetrating peptide (cCPP) wherein the cCPP has a structure of Formula (2):

Formula (2):



(2), or a protonated form thereof,

wherein:

R^1, R^2, and R^3 can each independently be H or an amino acid residue having a side chain comprising an aryl or heteroaryl group;

at least two of R^1, R^2, and R^3 are, independently, an aryl or heteroaryl side chain of an amino acid;

R^4 and R^6 are, independently, H or an amino acid side chain;

AA_{SC} is an amino acid side chain;

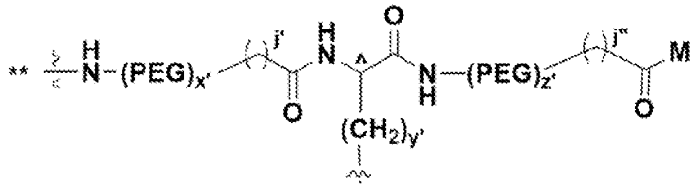
q is 1, 2, 3 or 4; and

m′ and m″ are each, independently, an integer from 0 to 3;

(b) a linear exocyclic peptide (EP), wherein the EP comprises from 2 to 10 amino acid residues;

(c) a linker having the structure of Formula (B′):

Formula (B'):



(B′)

wherein:

** is a point of attachment to the linear exocyclic peptide (EP);

* is a point of attachment to the cyclic cell penetrating peptide (cCPP);

^ indicates L- or D-stereochemistry;

y′ is an integer from 1 to 5;

M is a reactive handle comprising a functional group that reacts with a corresponding functional group on the cargo to form a bonding group (M');

x′ is an integer from 1 to 12;

z′ is an integer from 0 to 12;

j′ is 0, 1, or 2, wherein j′ is 0 when x′ is 0;

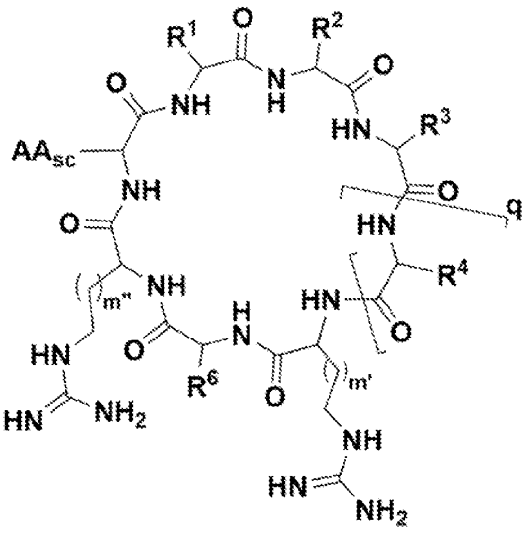
j″ is 0, 1, or 2, wherein j″ is 0 when z′ is 0; and

(d) a cargo that comprises an oligonucleotide that hybridizes to exon 50 in a pre-mRNA transcript of human dystrophin gene (*DMD*) to cause exon skipping, wherein the oligonucleotide hybridizes to or comprises a nucleic acid sequence shown in **Tables 11A-11D**, **12A-12D**, or **13.**

[0008] In embodiments, the EEV-cargo conjugate can comprise:

(a) a cyclic cell penetrating peptide (cCPP) wherein the cCPP has a structure of Formula (2):

Formula (2):



(2), or a protonated form thereof,

wherein:

R^1, R^2, and R^3 can each independently be H or an amino acid residue having a side chain comprising an aryl or heteroaryl group;

at least two of R^1, R^2, and R^3 are, independently, an aryl or heteroaryl side chain of an amino acid;

R^4 and R^6 are independently H or an amino acid side chain;

AAsc is an amino acid side chain;

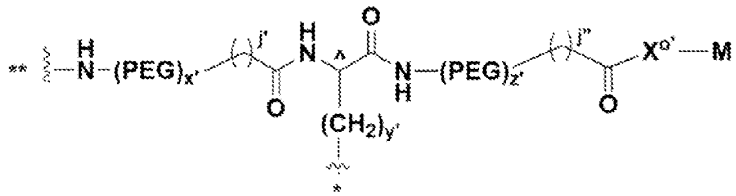
q is 1, 2, 3 or 4; and

m′ and m″ are each, independently, an integer from 0 to 3;

(b) a linear exocyclic peptide (EP) comprising from 2 to 10 amino acid residues;

(c) a linker having the structure of Formula (C′):

Formula (C'):



(C′)

wherein:

** is a point of attachment to the linear exocyclic peptide (EP);

* is a point of attachment to the cyclic cell penetrating peptide (cCPP);

^ indicates L- or D-stereochemistry;

y′ is an integer from 1 to 5;

M is a reactive handle comprising a functional group that reacts with a corresponding functional group on the cargo to form a bonding group (M');

$X^{o\prime}$ is a hydrophobic component;

x′ is an integer from 1 to 12;

z′ is an integer from 0 to 12;

j′ is 0, 1, or 2, wherein j′ is 0 when x′ is 0;

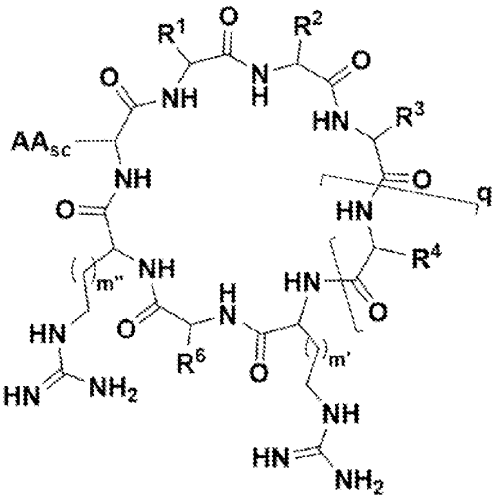
j″ is 0, 1, or 2, wherein j″ is 0 when z′ is 0; and

(d) a cargo that comprises an oligonucleotide that hybridizes to exon 50 in a pre-mRNA transcript of human dystrophin gene (*DMD*) to cause exon skipping, wherein the oligonucleotide hybridizes to or comprises a nucleic acid sequence shown in **Tables 11A-11D**, **12A-12D**, or **13**.

[0009] In embodiments, the EEV-cargo conjugate can comprise:

(a) a cyclic cell penetrating peptide (cCPP) wherein the cCPP has a structure of Formula (2):

Formula (2):



(2), or a protonated form thereof,

wherein:

R^1, R^2, and R^3 can each independently be H or an amino acid residue having a side chain comprising an aryl or heteroaryl group;

at least two of R^1, R^2, and R^3 are, independently, an aryl or heteroaryl side chain of an amino acid;

R^4 and R^6 are independently H or an amino acid side chain;

AA_{SC} is an amino acid side chain;

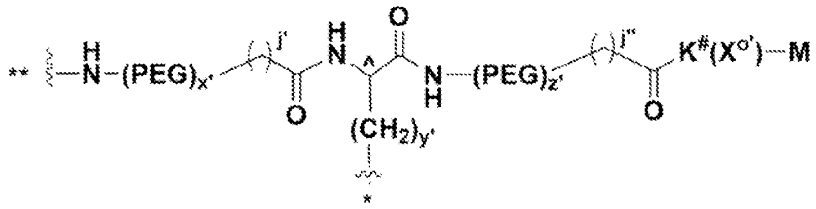
q is 1, 2, 3 or 4; and

m′ and m″ are each, independently, an integer from 0 to 3;

(b) a linear exocyclic peptide (EP) comprising from 2 to 10 amino acid residues;

(c) a linker having the structure of Formula (D′):

Formula (D'):



(D′)

wherein:

** is a point of attachment to the linear exocyclic peptide (EP);

* is a point of attachment to the cyclic cell penetrating peptide (cCPP);

^ indicates L- or D-stereochemistry;

y′ is an integer from 1 to 5;

M is a reactive handle comprising a functional group that reacts with a corresponding functional group on the cargo to form a bonding group (M');

$X^{o\prime}$ is a hydrophobic component;

$K^{\#}$ is D-lysine or L-lysine residue;

x′ is an integer from 1 to 12;

z′ is an integer from 0 to 12;

j′ is 0, 1, or 2, wherein j′ is 0 when x′ is 0;

j″ is 0, 1, or 2, wherein j″ is 0 when z′ is 0; and

(d) a a cargo that comprises an oligonucleotide that hybridizes to exon 50 in a pre-mRNA transcript of human dystrophin gene (*DMD*) to cause exon skipping, wherein the oligonucleotide hybridizes to or comprises a nucleic acid sequence shown in **Tables 11A-11D**, **12A-12D**, or **13**.

[0010] In embodiments, the cargo is an oligonucleotide. In embodiments, the oligonucleotide is a therapeutic oligonucleotide. In embodiments, the cargo is an antisense oligonucleotide. In embodiments, the cargo is an oligonucleotide that comprises at least one modified nucleotide or nucleic acid. In embodiments, the modified nucleotide or nucleic acid is a phosphorothioate (PS) nucleotide, a phosphorodiamidate morpholino oligonucleotide (PMO), a locked nucleic acid (LNA), a peptide nucleic acid (PNA), a nucleotide comprising a 2'-O-methyl (2'-OMe) modified backbone, a 2'O-methoxy-ethyl (2'-MOE) nucleotide, a 2',4' constrained ethyl (cEt) nucleotide, or a 2'-deoxy-2'-fluoro-beta-D-arabinonucleic acid (2'F-ANA). In embodiments, the oligonucleotide comprises at least one phosphorodiamidate morpholino oligonucleotide (PMO). In embodiments, each nucleotide in the oligonucleotide is a phosphorodiamidate morpholino oligonucleotide (PMO).

[0011] In embodiments, the oligonucleotide hybridizes to a nucleic acid sequence of Exon 50 shown in **Tables 11A-11D**, **12A-12D**, or **13.** In embodiments**,** , the oligonucleotide comprises the reverse complement of a nucleic acid sequence in **Tables 11A-11D**, **12A-12D**, or **13.** In embodiments, the oligonucleotide comprises a nucleic acid sequence with at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% nucleic acid sequence identity to a nucleic acid sequence from **Tables 11A-11D**, **12A-12D**, or **13** or a reverse complement thereof.

[0012] In embodiments, the oligonucleotideis complementary to a portion of exon 50. In embodiments, the oligonucleotide is complementary to a portion of the 5' flanking intron of exon 50 and a portion of exon 50. In embodiments, the oligonucleotide is complementary to a portion of the 3' flanking intron of exon 50 and a portion of exon 50.

[0013] In embodiments, the first nucleotide of the oligonucleotide can hybridize to a nucleotide in exon 50 of the *DMD* gene at position -10, -9, -8, -7, -6, -5, -4, -3, -2, -1, 0, +1, +2, +3, +4, +5, +6, +7, +8, +9, +10, +11, +12, +13, +14, +15, +16, +17, +18, +19, +20, +21, +22, +23, +24, +25, +26, +27, +28, +29, +30, +31, +32, +33, +34, +35, +36, +37, +38, +39, +40, +41, +42, +43, +44, +45, +46, +47, +48, +49, +50, +51, +52, +53, +54, +55, +56, +57, +58, +59, +60, +61, +62, +63, +64, +65, +66, +67, +68, +69, +70, +71, +72, +73, +74, +75, +76, +77, +78, +79, +80, +81,

+82, +83, +84, +85, +86, +87, +88, +89, +90, +91, +92, +93, +94, +95, +96, +97, +98, +99, +100, +101, +102, +103, +104, +105, +106, +107, +108, +109 of SEQ ID NO: 1.

[0014] The disclosure relates to a pharmaceutical composition comprising an EEV-cargo conjugate described herein.

[0015] The disclosure relates to a cell comprising an EEV-cargo conjugate described herein.

[0016] The disclosure relates to a method of treating DMD comprising administering an EEV-cargo conjugate described herein to a patient in need thereof.

BRIEF DESCRIPTION OF THE DRAWINGS

[0017] **FIGS. 1A** and **1B** show conjugation chemistries for conjugating an oligonucleotide an endosomal escape vehicle (EEV).

[0018] **FIGS. 2A-2B** show conjugation chemistry for connecting an EEV, comprising a CPP and a linker, to an antisense oligonucleotide, where the antisense oligonucleotide is shown without (**FIG. 2A**) and with (**FIG. 2B**) a component containing PEG_2.

[0019] **FIGS. 3A-3B** shows percent exon 50 skipping in CRL-2061™ cells treated with 5μM 21mer PMO.

[0020] **FIGS. 4A-4B** shows percent exon 50 skipping in CRL-2061™ cells treated with 5 μM 22mer PMO.

[0021] **FIGS. 5A-5B** shows percent exon 50 skipping in CRL-2061™ cells treated with 5 μM 23mer PMO.

[0022] **FIGS. 6A-6B** shows percent exon 50 skipping in CRL-2061™ cells treated with 5 μM 24mer PMO.

[0023] **FIGS. 7A-7D** show the percent of exon 50 skipping in CRL-2061™ cells treated with 1 μM and 3 μM PMO. **FIG. 7A** shows exon 50 skipping in CRL-2061™ cells treated with 1μM and 3 μM of the 21mer PMO listed in **Table 14A**. **FIG. 7B** shows exon 50 skipping in CRL-2061™ cells treated with 1μM and 3μM of the 22mer PMO listed in **Table 14B**. **FIG. 7C** shows exon skipping in CRL-2061™ cells treated with 1 μM and 3 μM of the 23mer PMO listed in **Table 14C. FIG. 7D** shows exon skipping in CRL-2061™ cells treated with 1 μM and 3 μM of the 24mer PMO listed in **Table 14D**.

[0024] **FIGS. 8A-8C** show the percent of exon 50 skipping in gastrocnemius ("gastroc") (**FIG. 8A**), diaphragm (**FIG. 8B**), and heart tissue (**FIG. 8C**) in hDMD mice treated with 15mpk of various EEV-PMOs via IV injection.

[0025] **FIGS. 9A-9C** show the percent of exon 50 skipping in gastrocnemius ("gastroc") (**FIG. 9A**), diaphragm (**FIG. 9B**), and heart tissue (**FIG. 9C**) in hDMD mice treated with 30 mpk of various EEV-PMOs via IV injection.

[0026] **FIG. 10** is a table showing *in silico* off-target analysis of EEV-PMOs for primary and mature RNA. mm = mismatches.

[0027] **FIGS. 11A-11E** show the percent of exon 50 skipping in gastrocnemius (**FIG. 11A**), triceps (**FIG. 11B**), tibialis anterior (**FIG. 11C**), heart (**FIG. 11D**), and diaphragm tissue (**FIG. 11E**) in hDMD mice treated with 15 mpk, 30 mpk, and 60 mpk of EEV-PMO24mer-14 via IV injection.

[0028] **FIGS. 12A-12E** show percent of exon 50 skipping at one week post-injection in the gastrocnemius (**FIG. 12A**), triceps (**FIG. 12B**), tibialis anterior (**FIG. 12C**), heart (**FIG. 12D**), and diaphragm (**FIG. 12E**) in hDMD mice treated with EEV-PMO24mer-14 via IV injection.

[0029] **FIGS. 13A-13E** show percent of exon 50 skipping at six weeks post-injection in the gastrocnemius (**FIG. 13A**), triceps (**FIG. 13B**), tibialis anterior (**FIG. 13C**), heart (**FIG. 13D**), and diaphragm (**FIG. 13E**) in hDMD mice treated with EEV-PMO24mer-14 via IV injection.

[0030] **FIGS. 14A-14E** show percent of exon 50 skipping at 1, 2, 4, 6, 8, and 12 weeks post-injection in the triceps (**FIG. 14A**), tibialis anterior (**FIG. 14B**), gastrocnemius (**FIG. 14C**), diaphragm (**FIG. 14D**), and heart (**FIG. 14E**) in hDMD mice treated with EEV-PMO24mer-14 via IV injection in single dose, duration of effect study.

[0031] **FIGS. 15A-15E** show percent of exon 50 skipping at 4, 8, 16, 24, 48, 96, and 168 hours post-injection in the gastrocnemius (**FIG. 15A**), triceps (**FIG. 15B**), tibialis anterior (**FIG. 15C**), diaphragm (**FIG. 15D**), and heart (**FIG. 15E**) in hDMD mice treated with EEV-PMO24mer-14 via IV injection in single dose, early timepoint study.

[0032] **FIG. 16** is a plot of percent *DMD* Exon 50 skipping in DMDΔ51 patient iPSC-derived cardiomyocites (CMs) treated with EEV-PMO24mer-14 at 0, 0.1, 0.3, 1, 3, and 10 μM as reported by ddPCR.

[0033] **FIG. 17** is a plot of relative *DMD* expression in DMDΔ51 patient iPSC-derived cardiomyocites (CMs) treated with EEV-PMO24mer-14 at 0, 0.1, 0.3, 1, 3, and 10 μM as reported by Simple Western Jess analysis, where dystrophin was normalized to total protein and presented as a percent relative to untreated normal cells.

[0034] **FIG. 18** is a plot of dystrophin fluorescence mean signal intensity in DMDΔ51 patient iPSC-derived cardiomyocites (CMs) treated with EEV-PMO24mer-14 at 0, 0.1, 0.3, 1, 3, and 10 μM as measured by immunofluorescence.

[0035] **FIG. 19** is a plot of dystrophin-positive cardiomyocyte area in DMDΔ51 patient iPSC-derived cardiomyocites (CMs) treated with EEV-PMO24mer-14 at 0, 0.1, 0.3, 1, 3, and 10 μM as measured by immunofluorescence.

[0036] **FIG. 20** is a plot of percent *DMD* Exon 50 skipping in DMDΔ51-55 inducible directly reprogrammable myotubes (iDRMs) treated with EEV-PMO24mer-14 at 0, 1, 3, 10, and 30 μM as reported by ddPCR.

[0037] **FIG. 21** is a plot of relative *DMD* expression in DMDΔ51-55 inducible directly reprogrammable myotubes (iDRMs) treated with EEV-PMO24mer-14 at 0, 1, 3, 10, and 30 μM as analyzed by ddPCR and normalized to HPRT1 expression and as normalized to the control group.

[0038] **FIG. 22** is a plot of relative dystrophin protein expression in DMDΔ51-55 inducible directly reprogrammable myotubes (iDRMs) treated with EEV-PMO24mer-14 at 0, 1, 3, 10, and 30 μM as measured by Simple Western Jess analysis.

[0039] **FIG. 23** is a plot of dystrophin fluorescence mean signal intensity in ind DMDΔ51-55 inducible directly reprogrammable myotubes (iDRMs) treated with EEV-PMO24mer-14 at 0, 1, 3, 10, and 30 μM as measured by immunofluorescence.

[0040] **FIG. 24** is a plot of dystrophin-positive myotube area in DMDΔ51-55 inducible directly reprogrammable myotubes (iDRMs) treated with EEV-PMO24mer-14 at 0, 1, 3, 10, and 30 μM as measured by immunofluorescence.

[0041] **FIG. 25** shows percent of exon 50 skipping in AdMyoD-induced patient-derived DMDΔ51 skeletal myotubes treated with EEV-PMO24mer-14 at 0, 1, 3, 10, and 20 μM.

[0042] **FIG. 26** shows the ratio of dystrophin over total protein in AdMyoD-induced patient-derived DMDΔ51 skeletal myotubes treated with EEV-PMO24mer-14 at 0, 1, 3, 10, and 20 μM.

[0043] **FIG. 27** is a structure of an EEV-cargo conjugate formed via amide chemistry by conjugating an EEV having the sequence Ac-PKKKRKV-PEG_2-K(cyclo[FGFGRGRQ])-PEG_{12}-OH to a 3′ base of a phosphorodiamidate morpholino oligonucleotide (PMO). The # symbol represents the remainder of the PMO.

[0044] **FIG. 28** is a structure of an EEV-cargo conjugate formed via amide chemistry by conjugating an EEV having the sequence Ac-PKKKRKV-PEG_2-K(cyclo[FGFGRGRQ])-PEG_{12}-K(Ac-Bip)-OH to a 3′ base of a phosphorodiamidate morpholino oligonucleotide (PMO). The # symbol represents the remainer of the PMO.

[0045] **FIG. 29** is a structure of an EEV-cargo conjugate formed via amide chemistry by conjugating an EEV having the sequence Ac-PKKKRKV-PEG_2-K(cyclo[FGFGRGRQ])-PEG_{12}-Nal-OH to a 3′ base of a phosphorodiamidate morpholino oligonucleotide (PMO). The # symbol represents the remainer of the PMO.

DETAILED DESCRIPTION

[0046] Disclosed herein are compounds for treating Duchenne Muscular Dystrophy (DMD) in patients with a mutation amendable to exon skipping. In embodiments, DMD is caused by a mutation in the region of exons 44-55 of the *DMD* gene. In embodiments, DMD is caused by a mutation in exon 50. In embodiments, the compound includes an EEV that is designed to deliver an oligonucleotide cargo to a cell, wherein the oligonucleotide cargo is complementary to a target sequence of a pre-mRNA transcript of a *DMD* gene, wherein the target sequence comprises at least a portion of the 5′ flanking intron of exon 50, at least a portion of exon 50, at least a portion of the 3′ flanking intron of exon 50, or a combination thereof. In embodiments, the EEV-cargo conjugate is delivered intracellularly to a subject in need thereof. In embodiments, the EEV delivers an oligonucleotide cargo that is complementary to a target sequence comprising an intron-exon junction of exon 50 of the *DMD* gene. In embodiments, the EEV delivers an oligonucleotide cargo that is complementary to a target sequence comprising an intronic nucleotide sequence upstream (or 5′) of exon 50 of the *DMD* gene. In embodiments, the EEV delivers an oligonucleotide cargo that is complementary to a target sequence comprising an intronic nucleotide sequence downstream (or 3′) of exon 50 of the *DMD* gene.

[0047] In embodiments, the oligonucleotide alters the splicing pattern of the target pre-mRNA to which the oligonucleotide hybridizes, resulting in the formation of re-spliced target protein. In embodiments, the re-spliced target protein has increased function as compared to the target protein produced by the splicing of the target pre-mRNA in the absence of the oligonucleotide. In embodiments, the re-spliced target protein increases target protein function by at least 1%, at least 5%, at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at

least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 99%, at least 100%, at least 150%, at least 200%, at least 250%, at least 300%, at least 350%, at least 400%, at least 450%, at least 500%, or more, compared to the function of the target protein produced by splicing in the absence of the oligonucleotide, inclusive of all values and ranges therebetween. In embodiments, the re-spliced target protein restores function to at least 1%, at least 5%, at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 99%, and up to 100% of the function of a wild-type target protein, inclusive of all values and ranges therebetween.

[0048] In embodiments, the EEV-cargo conjugate comprises an endosomal escape vehicle (EEV) and a cargo. In embodiments, the cargo is an oligonucleotide. In embodiments, the oligonucleotide is a therapeutic oligonucleotide. In embodiments, the oligonucleotide is an antisense oligonucleotide. In embodiments, the EEV comprises a cyclic cell penetrating peptide (cCPP). In embodiments, the EEV-cargo conjugate is able to traverse the cell membrane and bind to target pre-mRNA in vivo. In embodiments, an EEV-cargo conjugate is provided that comprises: a) at least one endosomal escape vehicle (EEV) comprising a cyclic cell penetrating peptide (cCPP); and b) at least one cargo. As used herein, "coupled" can refer to a covalent or non-covalent association between the EEV and the cargo, including chemical conjugation of the EEV to the cargo. Non-limiting examples of conjugation chemistries used for covalent attachment between the EEV and cargo can include, but are not limited to, conjugation using click chemistry or amide formation chemistry. A non-limiting example of non-covalent attachment is through the streptavidin/biotin interaction, wherein the EEV is coupled to the cargo via non-covalent association between biotin and streptavidin.

[0049] In embodiments, the cargo comprises an oligonucleotide Conjugation of the oligonucleotide cargo to the EEV may occur at any appropriate site on the oligonucleotide. In embodiments, the EEV can be conjugated to the 5' end, the 3' end, or the backbone of the oligonucleotide cargo.

Endosomal Escape Vehicles (EEVs)

[0050] An endosomal escape vehicle (EEV) is provided herein that can be used to transport a cargo molecule across a cellular membrane, for example, to deliver the cargo to the cytosol or nucleus of a cell. The cargo can be a therapeutic or diagnostic molecule. The therapeutic molecule can be

an oligonucleotide, peptide, small molecule or a combination thereof. The peptide can be a protein, an antibody, or an enzyme. The cargo can be an oligonucleotide. The oligonucleotide can be an antisense oligonucleotide. The oligonucleotide can be a phosphorodiamidate morpholino oligonucleotide (PMO). The EEV can comprise a cell penetrating peptide (CPP), for example, a cyclic cell penetrating peptide (cCPP), which is conjugated to a linear exocyclic peptide (EP). The EEV can include one or more linkers. The EEV can include a hydrophobic component (X). One or more linker arms of the EEV or the cCPP can include a hydrophobic component (X). In embodiments, when a linker arm of the EEV includes more than one hydrophobic component (X), the hydrophobic components can be indicated using (e.g., $X^{o\prime}$, $X^{o\prime\prime}$, etc.). In embodiments, the EEV can comprise hydrocarbon component, a polyethylene glycol (PEG) component, a hydrophobic component (X), an amino acid component comprising one or more amino acid residues (AA), or a combination thereof. In embodiments, EEV in which the stereochemistry of the amino acids within the peptide sequences of the EEV has been modified are provided.

[0051] In embodiments, the EEV is coupled to a cargo. The cargo can be coupled to the cCPP. The cargo can be coupled to the EP. The cargo can be coupled to a linker. The EP can be coupled to the cCPP. The EP can be coupled to the cargo and the cCPP. Coupling between the EP, cargo, cCPP, or combinations thereof, may be non-covalent or covalent. The EP, cargo, cCPP, or combination thereof, may be coupled via one or more linkers.

Cell Penetrating Peptides (CPP)

[0052] An endosomal escape vehicle (EEV) is provided herein that comprises at least one cell penetrating peptide (CPP). The cell penetrating peptide can be a cyclic cell penetrating peptide (cCPP). In embodiments, the cCPP can penetrate a cell membrane. In embodiments, the cCPP can deliver the cargo to the cytosol of the cell. The cCPP can deliver the cargo to a cellular location where a target gene, target transcript, and/or target protein is located. To conjugate the cCPP to a cargo, an EP, and/or a linker, at least one bond or lone pair of electrons on the cCPP can be replaced.

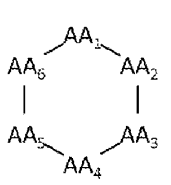
[0053] In embodiments, a hydrophobic component (X) is appended to one or more amino acid residues of the cCPP. In embodiments, a hydrophobic component (X) is appended to a side chain of a lysine amino acid residue of the cCPP.

[0054] X can be a D or L amino acid residue with a hydrophobic side chain. X can be a naturally occurring or a non-naturally occurring amino acid residue with a hydrophobic side chain. X can

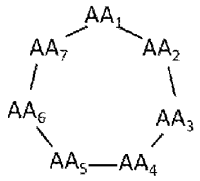
be an amino acid residue with an aromatic side chain. X can be an amino acid residue with a heteroaromatic side chain. X can be selected from phenylalanine, 3-(4',4-biphenyl)-L-alanine, tryptophan, tyrosine, valine, isoleucine, leucine, or histidine, or a combination thereof. X can be 2-naphtylalanine. X can be Nal. X can be d-Nal (nal). X can be 3-(4',4-biphenyl)-L-alanine. X can be Bip. X can be D-Bip (bip). X can be a C_4-C_8 alkyl hydrocarbon. X can be a C_6 alkyl hydrocarbon.

[0055] The total number of amino acid residues in the cCPP is from 6 to 20 amino acid residues, e.g., 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 amino acid residues, inclusive of all ranges and subranges therebetween. The cCPP can comprise from 6 to 13 amino acid residues. The cCPP can comprise from 6 to 10 amino acids. By way of example, cCPP comprising 6-10 amino acid residues can have a structure according to any of Formula I-A to I-E:

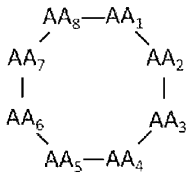
[0056] Formula I-A to 1-E:



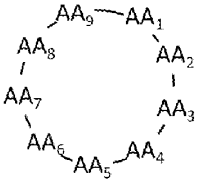
I-A



I-B

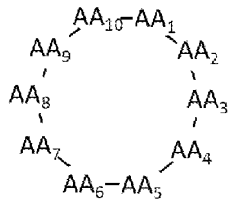


I-C



I-D

, , , , or



I-E

, wherein AA_1, AA_2, AA_3, AA_4, AA_5, AA_6, AA_7, AA_8, AA_9, and AA_{10} are amino acid residues.

[0057] The cCPP can comprise from 6 to 8 amino acids. The cCPP can comprise 8 amino acids.

[0058] Each amino acid in the cCPP may be a natural or non-natural amino acid. Abbreviations used herein for some natural and non-natural amino acids are shown in **Table 1**.

[0059] As used herein, the term "amino acid" refers to compounds having an amino group and a carboxylic acid group. Most amino acids (except for glycine) also have a side chain. As used

herein, “amino acid side chain” or "side chain" refers to the characterizing substituent bound to the α-carbon of the amino acid.

[0060] An “α-amino acid” is an amino acid in which the amino group is attached to the first (alpha) carbon adjacent to the carboxylic acid group, such that the carbon atom of the carbonyl is separated from the nitrogen atom of the amino group by one carbon atom. A “b-amino acid” (also called “beta-amino acid,” and “β-amino acid”) is an analog of an α -amino acid in which the amino group is attached to the second (beta) carbon, rather than the alpha-carbon, such that the carbon atom of the carbonyl is separated from the nitrogen atom of the amino group by two carbon atoms. Examples of b-amino acids include but are not limited to b-alanine and b-homophenylalanine.

[0061] An “uncharged” amino acid is an amino acid that does not have a charge at a physiological pH (for example, from 6.5 to 8.0 or from 6.8 to 7.6). It is noted that histidine can exist in neutral or positively charged forms at physiological pH.

[0062] A side chain that does not comprise an aryl or heteroaryl group, can be referred to herein as a “non-aryl” side chain. In embodiments, the side chain that does not comprise an aryl or heteroaryl group can be uncharged and is referred to herein as an uncharged, non-aryl side chain. Amino acids with uncharged non-aryl amino side chains include, but are not limited to, histidine, threonine, serine, leucine, isoleucine, valine, neopentylglycine, alanine, homoalanine, homoserine, 3-(4-thiazolyl)-alanine, 3-(4-furanyl)-alanine, 3-(4-thienyl)-alanine, and b-amino acid derivatives thereof.

[0063] The term “non-natural amino acid” refers to an organic compound that is a congener of a natural amino acid in that it has a structure similar to a natural amino acid so that it mimics the structure and reactivity of a natural amino acid. The non-natural amino acid can be a modified amino acid, and/or amino acid analog, that is not one of the 20 common naturally occurring amino acids or the rare natural amino acids selenocysteine or pyrrolysine. Non-natural amino acids can also be a D-isomer of a natural amino acid. Examples of suitable amino acids include, but are not limited to, alanine, allosoleucine, arginine, citrulline, asparagine, aspartic acid, cysteine, glutamine, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, napthylalanine, phenylalanine, proline, pyroglutamic acid, serine, threonine, tryptophan, tyrosine, valine, a derivative thereof, or combinations thereof.

***Table 1**: Amino Acid Abbreviations*

Amino Acid	**Abbreviations* L-amino acid**	**Abbreviations* D-amino acid**
Alanine	Ala (A)	ala (a)
Allo-isoleucine	Aile	aile
Arginine	Arg (R)	arg (r)
Asparagine	Asn (N)	asn (n)
Aspartic acid	Asp (D)	asp (d)
Cysteine	Cys (C)	cys (c)
Citrulline	Cit	cit
Cyclohexylalanine	Cha	cha
2,3-diaminopropionic acid	Dap	dap
4-fluorophenylalanine	Fpa (Σ)	pfa
Glutamic acid	Glu (E)	glu (e)
Glutamine	Gln (Q)	gln (q)
Glycine	Gly (G)	gly (g)
Histidine	His (H)	his (h)
Homoproline (aka pipecolic acid)	Pip (Θ)	pip (ɵ)
Isoleucine	Ile (I)	ile (i)
Leucine	Leu (L)	leu (l)
Lysine	Lys (K)	lys (k)
Methionine	Met (M)	met (m)
3-(2-naphthyl)-alanine	Nal (Φ) (L-Nal or 2-Nal)	nal (ϕ), (dNal, dnal, D-Nal, or 2-nal)
3-(1-naphthyl)-alanine	1-Nal	1-nal
Norleucine	Nle (Ω)	nle
Phenylalanine	Phe (F)	phe (f)
Phenylglycine	Phg (Ψ)	phg
4-(phosphonodifluoromethyl)phenylalanine	F_2Pmp (Λ)	$f_2$pmp
Proline	Pro (P)	pro (p)
Sarcosine	Sar (Ξ)	sar
Selenocysteine	Sec (U)	sec (u)
Serine	Ser (S)	ser (s)
Threonine	Thr (T)	thr (y)
Tyrosine	Tyr (Y)	tyr (y)
Tryptophan	Trp (W)	trp (w)
Valine	Val (V)	val (v)
Tert-butyl-alanine	Tle	tle
Penicillamine	Pen	pen
Homoarginine	HomoArg	homoarg
Nicotinyl-lysine	Lys (NIC)	lys (NIC)
Triflouroacetyl-lysine	Lys (TFA)	lys (TFA)
Methyl-leucine	MeLeu	meleu

Amino Acid	Abbreviations* L-amino acid	Abbreviations* D-amino acid
3-(3-benzothienyl)-alanine	Bta	bta
* single letter abbreviations: capital letters indicate the L-amino acid form, lower case letter indicate the D-amino acid form.		

[0064] In embodiments, the cCPP can comprise 2 contiguous amino acids with hydrophobic side chains. In embodiments, the cCPP can comprise 3 contiguous amino acids with hydrophobic side chains. In embodiments, the amino acids with hydrophobic side chain are selected from phenylalanine and naphthylalanine. In embodiments, the contiguous amino acids with hydrophobic side chains can have D- or L-stereochemistry. In embodiments, the contiguous amino acids with hydrophobic side chains can have the same stereochemistry. In embodiments, the contiguous amino acids with hydrophobic side chains can have alternating stereochemistry. In embodiments, the contiguous amino acids with hydrophobic side chains can have all L-stereochemistry. In embodiments, the contiguous amino acids with hydrophobic side chains can have all D-stereochemistry.

[0065] In embodiments, one or two amino acids in the cCPP can have no side chain. In embodiments, all amino acids in the cCPP have a side chain. As used herein, when no side chain is present, the amino acid has two hydrogen atoms on the carbon atom(s) (e.g., $-CH_2$) linking the amine and carboxylic acid of the amino acid residue. The amino acid having no side chain can be glycine. The amino acid having no side chain can be β-alanine.

[0066] In embodiments, the cCPP can comprise 2 contiguous amino acids either hydrophobic side chains, no side chain, or a combination thereof. In embodiments, the cCPP can comprise 3 contiguous amino acids with hydrophobic side chains, no side chain, or a combination thereof. In embodiments, the contiguous amino acids with hydrophobic side chains, no side chains, or combination thereof, can have alternating stereochemistry. In embodiments, the contiguous amino acids with hydrophobic side chains, no side chains, or combination thereof, can have all L-stereochemistry. In embodiments, the contiguous amino acids with hydrophobic side chains, no side chains, or combination thereof, can have all D-stereochemistry.

[0067] The cCPP can comprise from 6 to 20, from 6 to 10, or from 6 to 8 amino acid residues, wherein: (i) at least two amino acids can, independently, be glycine, β-alanine, serine, histidine, citrulline, or 4-aminobutyric acid; (ii) at least two amino acids can have a side chain comprising

an aryl or heteroaryl group; and (iii) at least two amino acids, independently, have a side chain comprising a guanidine group, or a protonated form thereof. In embodiments, (i) two amino acids can, independently, be glycine, β-alanine, serine, histidine, citrulline, or 4-aminobutyric acid; (ii) two or three amino acids can have a side chain comprising an aryl or heteroaryl group; and (iii) two amino acids, independently, have a side chain comprising a guanidine group, or a protonated form thereof.

[0068] In embodiments, one amino acid of the cCPP can be glycine, β-alanine, serine, histidine, citrulline, or 4-aminobutyric acid. In embodiments, two amino acids can be, independently, glycine, β-alanine, serine, histidine, citrulline, or 4-aminobutyric acid. In embodiments, three amino acids can be glycine, β-alanine, serine, histidine, citrulline, or 4-aminobutyric acid.

[0069] In embodiments, one amino acid of the cCPP can have a side chain comprising an aryl or heteroaryl group. In embodiments, two amino acids of the cCPP can have a side chain comprising an aryl or heteroaryl group. In embodiments, three amino acids of the cCPP can have a side chain comprising an aryl or heteroaryl group. In embodiments, none of the amino acids having the side chain comprising the aryl or heteroaryl group are contiguous. In embodiments, two amino acids having the side chain comprising the aryl or heteroaryl group can be contiguous. In embodiments, two contiguous amino acids can have opposite stereochemistry. In embodiments, the two contiguous amino acids can have the same stereochemistry. In embodiments, three amino acids having the side chain comprising the aryl or heteroaryl group can be contiguous. In embodiments, three contiguous amino acids can have the same stereochemistry. In embodiments, three contiguous amino acids can have alternating stereochemistry. The amino acid residue having a side chain comprising an aryl or heteroaryl group can each be independently a residue of phenylalanine, naphthylalanine, or β-homophenylalanine, each of which is optionally substituted with one or more substituents. At least one amino acid residue having a side chain comprising an aryl or heteroaryl group can be a residue of phenylalanine. One amino acid residue having a side chain comprising an aryl or heteroaryl group can be a residue of phenylalanine. At least two amino acid residues having a side chain comprising an aryl or heteroaryl group can be residues of phenylalanine. Two amino acid residues having a side chain comprising an aryl or heteroaryl group can be residues of phenylalanine. Each amino acid residue having a side chain comprising an aryl or heteroaryl group can be a residue of phenylalanine. At least one amino acid residue having a side chain comprising an aryl or heteroaryl group can be a residue of naphthylalanine. One amino

acid residue having a side chain comprising an aryl or heteroaryl group can be a residue of naphthylalanine. At least one amino acid residue having a side chain comprising an aryl or heteroaryl group can be a residue of β-homophenylalanine. One amino acid residue having a side chain comprising an aryl or heteroaryl group can be a residue of b-homophenylalanine.

[0070] In embodiments, one amino acid of the cCPP can have a side chain that does not comprise an aryl or heteroaryl group, referred to herein as a "non-aryl" side chain. In embodiments, the side chain that does not comprise an aryl or heteroaryl group can be uncharged and is referred to herein as an uncharged, non-aryl side chain. In embodiments, two amino acids of the cCPP can have an uncharged, non-aryl side chain. In embodiments, three amino acids of the cCPP can have an uncharged, non-aryl side chain. Amino acids with uncharged non-aryl amino side chains include, but are not limited to, histidine, threonine, serine, leucine, isoleucine, valine, neopentylglycine, alanine, homoalanine, homoserine, 3-(4-thiazolyl)-alanine, 3-(4-furanyl)-alanine, and 3-(4-thienyl)-alanine.

[0071] In embodiments, one amino acid of the cCPP has a side chain comprising a guanidine group, or a protonated form thereof. In embodiments, two amino acids of the cCPP can have a side chain comprising a guanidine group, or a protonated form thereof. In embodiments, three amino acids of the cCPP can have a side chain comprising a guanidine group, or a protonated form thereof. In embodiments, four amino acids of the cCPP can have a side chain comprising a guanidine group, or a protonated form thereof. In embodiments, the amino acids of the cCPP having a side chain comprising a guanidine group, or a protonated form thereof, are contiguous. In embodiments, the amino acids of the cCPP having a side chain comprising a guanidine group, or a protonated form thereof, are not contiguous. In embodiments, the amino acid comprising a guanidine group, or protonated form thereof, is arginine. In embodiments, the amino acids of the cCPP having a side chain comprising a guanidine group, or a protonated form thereof, have D- or L- stereochemistry. In embodiments, the amino acids of the cCPP having a side chain comprising a guanidine group, or a protonated form thereof, have alternating D- and L- stereochemistry. In embodiments, the amino acids of the cCPP having a side chain comprising a guanidine group, or a protonated form thereof, have the same stereochemistry. In embodiments, the amino acids of the cCPP having a side chain comprising a guanidine group, or a protonated form thereof, have D-stereochemistry. In embodiments, the amino acids of the cCPP having a side chain comprising a guanidine group, or a protonated form thereof, have L- stereochemistry.

[0072] In embodiments, the cCPP can comprise from 6 to 10 amino acid residues, wherein: (i) at least two amino acids can, independently, be glycine, β-alanine, or 4-aminobutyric acid residues; (ii) at least two amino acids can have a side chain comprising an aryl or heteroaryl group; and (iii) at least two amino acids can, independently, have a side chain comprising a guanidine group, or a protonated form thereof.

[0073] In embodiments, the cCPP can comprise from 6 to 10 amino acid residues, wherein: (i) two amino acid can independently be glycine, β-alanine, or 4-aminobutyric acid residues; (ii) two or three amino acids can have a side chain comprising an aryl or heteroaryl group; and (iii) two amino acid can, independently, have a side chain comprising a guanidine group, or a protonated form thereof.

[0074] In embodiments, the cCPP can comprise from 6 to 10 amino acid residues, wherein: (i) at least two amino acids can independently be glycine, β-alanine, or 4-aminobutyric acid residues; (ii) at least three amino acids can have a side chain comprising an aryl or heteroaryl group; and (iii) at least two amino acids comprise arginine.

Glycine and Related Amino Acid Residues

[0075] In embodiments, the cCPP can comprise 1, 2, 3, 4, 5, or 6 glycine, β-alanine, 4-aminobutyric acid residues, or combinations thereof. In embodiments, the cCPP can comprise 2 glycine, β-alanine, 4-aminobutyric acid residues, or combinations thereof. In embodiments, the cCPP can comprise 3 glycine, β-alanine, 4-aminobutyric acid residues, or combinations thereof. In embodiments, the cCPP can comprise 4 glycine, β-alanine, 4-aminobutyric acid residues, or combinations thereof. In embodiments, the cCPP can comprise 5 glycine, β-alanine, 4-aminobutyric acid residues, or combinations thereof. In embodiments, the cCPP can comprise 6 glycine, β-alanine, 4-aminobutyric acid residues, or combinations thereof. In embodiments, the cCPP can comprise 3, 4, 5, or 6 glycine, β-alanine, 4-aminobutyric acid residues, or combinations thereof. In embodiments, the cCPP can comprise 3, 4, or 5 glycine, β-alanine, 4-aminobutyric acid residues, or combinations thereof. In embodiments, the cCPP can comprise 3 or 4 glycine, β-alanine, 4-aminobutyric acid residues, or combinations thereof.

[0076] In embodiments, the cCPP can comprise 1, 2, 3, 4, 5, or 6 glycine residues. The cCPP can comprise 2 glycine residues. In embodiments, the cCPP can comprise 3 glycine residues. In embodiments, the cCPP can comprise 4 glycine residues. In embodiments, the cCPP can comprise

5 glycine residues. In embodiments, the cCPP can comprise 6 glycine residues. In embodiments, the cCPP can comprise 3, 4, or 5 glycine residues. In embodiments, the cCPP can comprise 3 or 4 glycine residues. In embodiments, the cCPP can comprise 2 or 3 glycine residues. In embodiments, the cCPP can comprise 1 or 2 glycine residues.

[0077] In embodiments, the cCPP can comprise at least three glycine residues. In embodiments, the cCPP can comprise 3, 4, 5, or 6 glycine residues. In embodiments, the cCPP can comprise 3 glycine residues. In embodiments, the cCPP can comprise 4 glycine residues. In embodiments, the cCPP can comprise 5 glycine residues. In embodiments, the cCPP can comprise 6 glycine residues. In embodiments, the cCPP can comprise 3, 4, or 5 glycine residues. The cCPP can comprise 3 or 4 glycine residues.

[0078] In embodiments, none of the glycine, β-alanine, or 4-aminobutyric acid residues in the cCPP are contiguous. In embodiments, two or three glycine, β-alanine, 4-or aminobutyric acid residues can be contiguous. In embodiments, two glycine, β-alanine, or 4-aminobutyric acid residues can be contiguous.

[0079] In embodiments, none of the glycine residues in the cCPP are contiguous. For example, each glycine residues in the cCPP can be separated by an amino acid residue that is not glycine.

[0080] In embodiments, two or more of the glycine residues in the cCPP are contiguous. In embodiments, two or three glycine residues can be contiguous. In embodiments, two glycine residues are contiguous.

Amino Acid Side Chains with an Aryl or heteroaryl Group

[0081] In embodiments, the cCPP can comprise 2, 3, 4, 5, or 6 amino acid residues independently having a side chain comprising an aryl or heteroaryl group. In embodiments, the cCPP can comprise 2 amino acid residues independently having a side chain comprising an aryl or heteroaryl group. In embodiments, the cCPP can comprise 3 amino acid residues independently having a side chain comprising an aryl or heteroaryl group. In embodiments, the cCPP can comprise 2, 3, or 4 amino acid residues independently having a side chain comprising an aryl or heteroaryl group. In embodiments, the cCPP can comprise 2 or 3 amino acid residues independently having a side chain comprising an aryl or heteroaryl group.

[0082] The aryl group can be a 6- to 14-membered aryl. Aryl can be phenyl, naphthyl or anthracenyl, each of which is optionally substituted. Aryl can be phenyl or naphthyl, each of which

is optionally substituted. The heteroaryl group can be a 6- to 14-membered heteroaryl having 1, 2, or 3 heteroatoms selected from N, O, and S. Heteroaryl can be pyridyl, quinolyl, or isoquinolyl.

[0083] The amino acid residue having a side chain comprising an aryl or heteroaryl group can each be independently a residue of phenylalanine, naphthylalanine, phenylglycine, β-homophenylalanine, homonaphthylalanine, bis(homophenylalanine), bis-(homonaphthylalanine), tryptophan, or tyrosine, each of which is optionally substituted with one or more substituents. The amino acid residue having a side chain comprising an aryl or heteroaryl group can each independently be a residue of tyrosine, phenylalanine, 1-naphthylalanine, 2-naphthylalanine, tryptophan, 3-benzothienylalanine, 4-phenylphenylalanine, 3,4-difluorophenylalanine, 4-trifluoromethylphenylalanine, 2,3,4,5,6-pentafluorophenylalanine, homophenylalanine, β-homophenylalanine, 4-tert-butyl-phenylalanine, 4-pyridinylalanine, 3-pyridinylalanine, 4-methylphenylalanine, 4-fluorophenylalanine, 4-chlorophenylalanine, 3-(9-anthryl)-alanine. The amino acid residue having a side chain comprising an aryl or heteroaryl group can each independently be a residue of 3-(3-benzothienyl)-alanine, 3-(2-quinolyl)-alanine, O-benzylserine, 3-(4-(benzyloxy)phenyl)-alanine, S-(4-methylbenzyl)cysteine, *N*-(naphthalen-2-yl)glutamine, 3-(1,1'-biphenyl-4-yl)-alanine, 3-(3-benzothienyl)-alanine. The amino acid residue having a side chain comprising an aryl or heteroaryl group can each independently be a residue of phenylalanine, 1-naphthylalanine, 2-naphthylalanine, phenylglycine, β-homophenylalanine, or homonaphthylalanine, each of which is optionally substituted with one or more substituents. The amino acid residue having a side chain comprising an aryl or heteroaryl group can each be independently a residue of phenylalanine, 2-naphthylalanine, or β-homophenylalanine, each of which is optionally substituted with one or more substituents. The amino acid residue having a side chain comprising an aryl or heteroaryl group can each be independently a residue of phenylalanine or 2-naphthylalanine, each of which is optionally substituted with one or more substituents. At least one amino acid residue having a side chain comprising an aryl or heteroaryl group can be a residue of phenylalanine. At least two amino acid residues having a side chain comprising an aryl or heteroaryl group can be residues of phenylalanine. Each amino acid residue having a side chain comprising an aryl or heteroaryl group can be a residue of phenylalanine. One amino acid residue having a side chain comprising an aryl or heteroaryl group can be a residue of 2-naphthylalanine. One amino acid residue having a side chain comprising an aryl or heteroaryl group can be a residue of β-homophenylalanine.

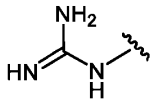
[0084] In embodiments, none of the amino acids having the side chain comprising the aryl or heteroaryl group are contiguous. In embodiments, two amino acids having the side chain comprising the aryl or heteroaryl group can be contiguous. In embodiments, two contiguous amino acids can have opposite stereochemistry. In embodiments, the two contiguous amino acids have the same stereochemistry. In embodiments, three amino acids having the side chain comprising the aryl or heteroaryl group can be contiguous. In embodiments, three contiguous amino acids having a side chain comprising an aryl or heteroaryl group have the same stereochemistry. In embodiments, three contiguous amino acids have alternating stereochemistry.

[0085] The amino acid residues comprising aryl or heteroaryl groups can be L-amino acids. The amino acid residues comprising aryl or heteroaryl groups can be D-amino acids. The amino acid residues comprising aryl or heteroaryl groups can be a mixture of D- and L-amino acids.

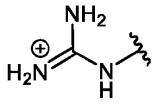
[0086] The hydrophobicity of amino acid residues can be measured and/or calculated using a variety of techniques. In embodiments, the hydrophobicity of an amino acid residue can be determined by calculating its consensus value on the consensus scale of D. Eisenberg et al., using the method described in D. Eisenberg et al., "Hydrophobic Moments and Protein Structure," Faraday Symp. Chem. Soc. 1982, 17, 109-120 (e.g., D. Eisenberg et al.). A hydrophobic amino acid is an amino acid that has a hydrophobic side chain.

Amino Acid Residues Having a Side Chain Comprising a Guanidine Group, Guanidine Replacement Group, or Protonated Form Thereof

[0087] As used herein, guanidine refers to the structure:



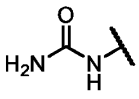
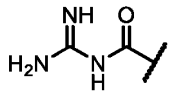
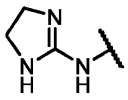
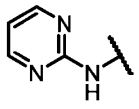
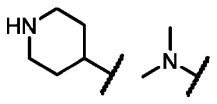
[0088] As used herein, a protonated form of guanidine refers to the structure:



[0089] Guanidine replacement groups refer to functional groups on the side chain of amino acids that will be positively charged at or above physiological pH or those that can recapitulate the hydrogen bond donating and accepting activity of guanidinium groups.

[0090] While not wishing to be bound by theory, it is believed that guanidine replacement groups may facilitate cell penetration and delivery of a therapeutic agent while reducing toxicity associated with guanidine groups or protonated forms thereof. The cCPP can comprise at least one amino acid having a side chain comprising a guanidine or guanidinium replacement group. The cCPP can comprise two amino acids having a side chain comprising a guanidine or guanidinium replacement group. The cCPP can comprise three amino acids having a side chain comprising a guanidine or guanidinium replacement group. The cCPP can comprise four amino acids having a side chain comprising a guanidine or guanidinium replacement group. The cCPP can comprise five amino acids having a side chain comprising a guanidine or guanidinium replacement group. The cCPP can comprise six amino acids having a side chain comprising a guanidine or guanidinium replacement group.

[0091] The guanidine or guanidinium group can be an isostere of guanidine or guanidinium. The guanidine or guanidinium replacement group can be less basic than guanidine.

[0092] As used herein, a guanidine replacement group refers to  ,  ,  ,  ,  , or a protonated form thereof.

[0093] In embodiments, a cCPP comprising from 6 to 10 amino acids residues is provided, wherein: (i) at least two amino acid have a side chain comprising a guanidine group, or a protonated form thereof; (ii) at least one amino acid residue has no side chain or a side chain of a guanidine replacement group, or a protonated form thereof; and (iii) at least two amino acids residues independently have a side chain comprising an aryl or heteroaryl group. In embodiments, two amino acids independently have a side chain comprising an aryl or heteroaryl group. In embodiments, three amino acids independently have a side chain comprising an aryl or heteroaryl group. In embodiments, at least two amino acids have no side chain or a side chain comprising a guanidine replacement group or a protonated form thereof. As used herein, when no side chain is present, the amino acid has two hydrogen atoms on the carbon atom(s) (e.g., $-CH_2-$) linking the amine and carboxylic acid. In embodiments, the amino acid having no side chain can be glycine.

[0094] In embodiments, the cCPP can comprise 2, 3, 4, 5, or 6 amino acid residues independently having a side chain comprising a guanidine group, guanidine replacement group, or a protonated

form thereof. In embodiments, the cCPP can comprise 2 amino acid residues independently having a side chain comprising a guanidine group, guanidine replacement group, or a protonated form thereof. In embodiments, the cCPP can comprise 3 amino acid residues independently having a side chain comprising a guanidine group, guanidine replacement group, or a protonated form thereof. In embodiments, the cCPP can comprise 4 amino acid residues independently having a side chain comprising a guanidine group, guanidine replacement group, or a protonated form thereof. In embodiments, the cCPP can comprise 5 amino acid residues independently having a side chain comprising a guanidine group, guanidine replacement group, or a protonated form thereof. In embodiments, the cCPP can comprise 6 amino acid residues independently having a side chain comprising a guanidine group, guanidine replacement group, or a protonated form thereof. In embodiments, the cCPP can comprise 2, 3, 4, or 5 amino acid residues independently having a side chain comprising a guanidine group, guanidine replacement group, or a protonated form thereof. In embodiments, the cCPP can comprise 2, 3, or 4 amino acid residues independently having a side chain comprising a guanidine group, guanidine replacement group, or a protonated form thereof. In embodiments, the cCPP can comprise 2 or 3 amino acid residues independently having a side chain comprising a guanidine group, guanidine replacement group, or a protonated form thereof.

[0095] In embodiments, the amino acid residues independently having the side chain comprising the guanidine group, guanidine replacement group, or the protonated form thereof, can be L-amino acids. In embodiments, the amino acid residues independently having the side chain comprising the guanidine group, guanidine replacement group, or the protonated form thereof, can be D-amino acids. In embodiments, the amino acid residues independently having the side chain comprising the guanidine group, guanidine replacement group, or the protonated form thereof, can be a mixture of L- or D-amino acids.

[0096] In embodiments, each amino acid residue having the side chain comprising the guanidine group, or the protonated form thereof, can independently be a residue of arginine, homoarginine, 2-amino-3-propionic acid, 2-amino-4-guanidinobutyric acid or a protonated form thereof. In embodiments, each amino acid residue having the side chain comprising the guanidine group, or the protonated form thereof, can independently be a residue of arginine or a protonated form thereof. In embodiments, the amino acid residue having the side chain comprising the guanidine replacement group, or the protonated form thereof, can independently be a residue of citrulline.

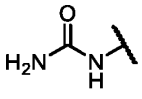
[0097] In embodiments, the cCPP can comprise a residue of asparagine, aspartic acid, glutamine, glutamic acid, or homoglutamine. In embodiments, the cCPP can comprise a residue of asparagine. In embodiments, the cCPP can comprise a residue of glutamine.

Chirality

[0098] While not wishing to be bound by theory, it is believed that the chirality of the amino acids in the cCPPs may impact cytosolic uptake efficiency. In embodiments, the cCPP can comprise at least one D amino acid. In embodiments, the cCPP can comprise 1 to 20 D amino acids. In embodiments, the cCPP can comprise 1 to 15 D amino acids. In embodiments, the cCPP can comprise 1 to 10 D amino acids. In embodiments, the cCPP can comprise 1 to 8 D amino acids. In embodiments, the cCPP can comprise 1, 2, 3, 4, 5, 6, 7, or 8 D amino acids. In embodiments, the cCPP can comprise all D amino acids. In embodiments, the cCPP can comprise at least one L amino acid. In embodiments, the cCPP can comprise 1 to 20 L amino acids. In embodiments, the cCPP can comprise 1 to 15 L amino acids. In embodiments, the cCPP can comprise 1 to 10 L amino acids. In embodiments, the cCPP can comprise 1 to 8 L amino acids. In embodiments, the cCPP can comprise 1, 2, 3, 4, 5, 6, 7, or 8 L amino acids. In embodiments, the cCPP can comprise all L amino acids. In embodiments, the cCPP can comprise 2, 3, 4, 5, 6, 7, or 8 contiguous amino acids having alternating D and L chirality. In embodiments, the cCPP can comprise three contiguous amino acids having the same chirality. In embodiments, the cCPP can comprise two contiguous amino acids having the same chirality. In embodiments, at least two of the amino acids can have the opposite chirality. In embodiments, at least two amino acids having opposite chirality can be adjacent to each other. In embodiments, at least three amino acids can have alternating stereochemistry relative to each other. In embodiments, the at least three amino acids having the alternating chirality relative to each other can be adjacent to each other. In embodiments, at least four amino acids have alternating stereochemistry relative to each other. In embodiments, the at least four amino acids having the alternating chirality relative to each other can be adjacent to each other. In embodiments, at least two of the amino acids can have the same chirality. In embodiments, at least two amino acids having the same chirality can be adjacent to each other. In embodiments, at least two amino acids have the same chirality and at least two amino acids have the opposite chirality. In embodiments, the at least two amino acids having the opposite chirality can be adjacent to the at least two amino acids having the same chirality. Accordingly, adjacent amino acids in the cCPP can have any of the following sequences: D-L; L-D; D-L-D, L-D-L, D-

L-L-D; L-D-D-L; L-D-L-L-D; D-L-D-D-L; D-L-L-D-L; or L-D-D-L-D. In embodiments, the amino acid residues that form the cCPP can all be L-amino acids. In embodiments, the amino acid residues that form the cCPP can all be D-amino acids.

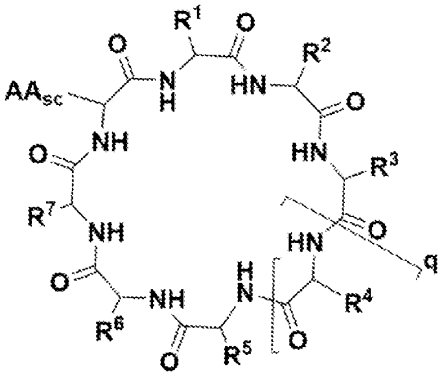
[0099] In embodiments, one or more amino acid residues that form the cCPP can be achiral. In embodiments, the cCPP can comprise a motif of 3, 4, or 5 amino acids, wherein two amino acids having the same chirality can be separated by an achiral amino acid. The cCPPs can comprise the following sequences: D/L-X-D/L; D/L-X-D/L-X; D/L-X-D/L-X-D/L; D-X-D; D-X-D-X; D-X-D-X-D; L-X-L; L-X-L-X; or L-X-L-X-L; wherein D/L indicates that the amino acid can be a D or an L amino acid and X is an achiral amino acid. The achiral amino acid can be glycine.

[0100] In embodiments, an amino acid having a side chain comprising a guanidine replacement group, or a protonated form thereof, can be adjacent to an amino acid having a side chain comprising an aryl or heteroaryl group. In embodiments, an amino acid having a side chain comprising a guanidine replacement group, or a protonated form thereof, can be adjacent to at least one amino acid having a side chain comprising a guanidine or protonated form thereof. In embodiments, an amino acid having a side chain comprising a guanidine or protonated form thereof can be adjacent to an amino acid having a side chain comprising an aryl or heteroaryl group. In embodiments, two amino acids having a side chain comprising a guanidine replacement group or protonated forms thereof can be adjacent to each other. In embodiments, two amino acids having a side chain comprising a guanidine or protonated form thereof are adjacent to each other. In embodiments, a cCPP can comprise at least two contiguous amino acids having a side chain can comprise an aryl or heteroaryl group and at least two non-adjacent amino acids having a side chain comprising a guanidine replacement group or a protonated form thereof. In embodiments, a cCPP can comprise at least two contiguous amino acids having a side chain comprising an aryl or heteroaryl group and at least two non-adjacent amino acids having a side chain comprising  , or a protonated form thereof. In embodiments, the adjacent amino acids can have the same chirality. In embodiments, the adjacent amino acids can have the opposite chirality. Other combinations of amino acids can have any arrangement of D and L amino acids, e.g., any of the sequences described in the preceding paragraph.

[0101] In embodiments, at least two amino acids having a side chain comprising a guanidine replacement group or a protonated form thereof, are alternating with at least two amino acids having a side chain comprising a guanidine group or protonated form thereof.

[0102] In embodiments, the cCPP can comprise the structure of Formula (1):

Formula (1):



(1), or a protonated form thereof,

wherein:

R^1, R^2, R^3, R^4, R^5, R^6, and R^7 are independently H or an amino acid side chain;

AA_{SC} is an amino acid side chain; and

q is 1, 2, 3, or 4.

[0103] The cCPP of Formula (1) can have any configuration and/or amino acid side chain, including, but not limited to those described in PCT Publication Nos: WO 2015/179691, filed May 21, 2015, entitled "CELL PENETRATING PEPTIDES AND METHODS OF MAKING AND USING THEREOF"; WO 2021/127650, filed December 21, 2020, entitled "COMPOSITIONS FOR DELIVERY OF ANTISENSE COMPOUNDS"; WO 2022/213118, filed March 31, 2022, entitled "CYCLIC CELL PENETRATING PEPTIDES"; WO 2022/241408, filed May 9, 2022, entitled "COMPOSITIONS AND METHODS FOR MODULATING TISSUE DISTRIBUTION OF INTRACELLULAR THERAPEUTICS", U.S. Patent Publication No. 2023/0312653, filed March 30, 2023, entitled "CYCLIC CELL PENETRATING PEPTIDES"; and US Patent No. 11,225,506, filed April 20, 2020, entitled "CELL PENETRATING PEPTIDES AND METHODS OF MAKING AND USING THEREOF", the disclosures of each of which are hereby incorporated by reference herein in their entireties.

[0104] In embodiments, the cCPP are of Formula (1) or a protonated form thereof, wherein:

R^1, R^2, and R^3 are each independently H or an aryl or heteroaryl side chain of an amino acid;

at least two of R^1, R^2, and R^3 is an aryl or heteroaryl side chain of an amino acid;

R^4, R^5, R^6, R^7 are independently H or an amino acid side chain;

AA_{SC} is an amino acid side chain; and

q is 1, 2, 3, or 4.

[0105] In embodiments, the cCPP are of Formula (1) or a protonated form thereof, wherein:

R^1, R^2, and R^3 are each independently H or an aryl or heteroaryl side chain of an amino acid;

at least two of R^1, R^2, and R^3 is an aryl or heteroaryl side chain of an amino acid;

R^4, R^5, R^6, R^7 are independently H or an amino acid side chain;

at least two of R^4, R^5, R^6, R^7 are, independently, a side chain of arginine;

AA_{SC} is an amino acid side chain; and

q is 1, 2, 3, or 4.

[0106] In embodiments of Formula (1),

R^1, R^2, and R^3 are each independently H or a side chain comprising an aryl or heteroaryl group;

at least two of R^1, R^2, and R^3 are a side chain of phenylalanine;

R^4, R^5, R^6, and R^7 are independently H or an amino acid side chain;

AA_{SC} is an amino acid side chain; and

q is 1, 2, 3, or 4.

[0107] In embodiments of Formula (1),

one of R^1, R^2, and R^3 is H;

two of R^1, R^2, and R^3 are CH_2Ph, where Ph is a phenyl group; and

R^4, R^5, R^6, and R^7 are, independently, H or an amino acid side chain.

[0108] In embodiments of Formula (1),

one of R^1, R^2, and R^3 is H;

two of R^1, R^2, and R^3 are CH_2Ph; and

R^4, R^5, R^6, and R^7 are, independently, H or an amino acid side chain of arginine.

[0109] In embodiments of Formula (1),

R^1, R^2, and R^3 are $-CH_2Ph$;

R^4, R^5, R^6, and R^7 are, independently, H or an amino acid side chain.

[0110] In embodiments of Formula (1),

R^1, R^2, and R^3 are each independently a side chain comprising an aryl or heteroaryl group;

at least two of R^1, R^2, and R^3 are a side chain of phenylalanine;

R^4, R^5, R^6, and R^7 are, independently, H or an amino acid side chain; and

q is 1, 2, 3 or 4.

[0111] In embodiments of Formula (1),

R^1, R^2, and R^3 are each independently H or an amino acid side chain;

at least one of R^1, R^2, and R^3 is an aryl or heteroaryl side chain of an amino acid;

at least one of R^1, R^2, and R^3 is a side chain of arginine;

R^4, R^5, R^6, and R^7 are, independently, H or an amino acid side chain; and

q is 1, 2, 3 or 4.

[0112] In embodiments of Formula (1),

R^1, R^2, and R^3 are each independently H or an amino acid side chain;

at least two of R^1, R^2, and R^3 are a side chain of arginine;

R^4, R^5, R^6, and R^7 are, independently, H or an amino acid side chain; and

q is 1, 2, 3 or 4.

[0113] In embodiments of Formula (I),

R^1, R^2, and R^3 are each independently a side chain comprising an aryl or heteroaryl group;

at least one of R^1, R^2, and R^3 is a side chain of naphthylalanine;

R^4, R^5, R^6, and R^7 are, independently, H or an amino acid side chain; and

q is 1, 2, 3, or 4.

[0114] In embodiments of Formula (I),

R^1, R^2, and R^3 are each independently a side chain comprising an aryl or heteroaryl group;

at least two of R^1, R^2, and R^3 are a side chain of naphthylalanine;

R^4, R^5, R^6, and R^7 are, independently, H or an amino acid side chain; and

q is 1, 2, 3, or 4.

[0115] In embodiments of Formula (I),

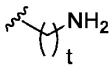
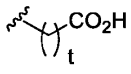
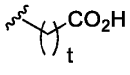
R^1, R^2, and R^3 are each independently a side chain comprising an aryl or heteroaryl group;

at least two of R^1, R^2, and R^3 are a side chain of phenylalanine;

at least one of R^1, R^2, and R^3 is a side chain of naphthylalanine;

R^4, R^5, R^6, and R^7 are, independently, H or an amino acid side chain; and

q is 1, 2, 3, or 4.

[0116] AAsc can be a side chain or terminus of an amino acid residue on the cCPP. Non-limiting examples of AAsc include aspartic acid, glutamic acid, glutamine, asparagine, or lysine, or a modified side chain of glutamine or asparagine (e.g., a reduced side chain having an amino group). In embodiments, AAsc can be  or  , wherein t can be an integer from 0 to 5. AAsc can be  , wherein t can be 0 or an integer from 1 to 5. In embodiments, t can be 1 to 5. In embodiments, t is 2 or 3. In embodiments, t can be 2. In embodiments, t can be 3. In embodiments, AAsc can be conjugated to a linker. In embodiments, the AAsc is the side chain of a glutamine residue. In embodiments, the AAsc is the side chain of a glutamic acid residue. In embodiments when the AAsc is the side chain of glutamine, the carboxamide amide nitrogen of the glutamine side chain forms a bond with the -$(CH_2)_y$- group.

[0117] In embodiments, the cCPP is of Formula (1), where at least one of R^4, R^5, R^6, R^7 is independently an uncharged, non-aromatic side chain of an amino acid. In embodiments, at least one of R^4, R^5, R^6, and R^7 is independently H or a side chain of serine, histidine or citrulline. In embodiments, one of R^4, R^5, R^6, and R^7 is independently H or a side chain of serine, histidine or citrulline. In embodiments, at least two of R^4, R^5, R^6, and R^7 are independently H or a side chain of serine, histidine or citrulline. In embodiments, two of R^4, R^5, R^6, and R^7 are independently H or a side chain of serine, histidine or citrulline.

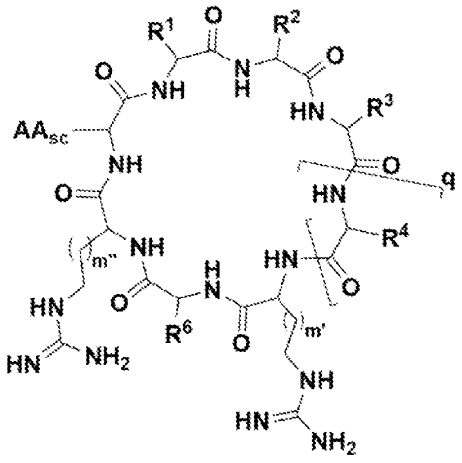
[0118] In embodiments, EEVs are provided that include a cCPP having from 6 to 10 amino acids, wherein at least two amino acids of the cCPP are charged amino acids, at least two amino acids of the cCPP are aromatic hydrophobic amino acids and at least two amino acids of the cCPP are uncharged, non-aromatic amino acids. In embodiments, at least two charged amino acids of the cCPP are arginine. In embodiments, two charged amino acids of the cCPP are arginine. In

embodiments, at least two aromatic, hydrophobic amino acids of the cCPP are, independently, phenylalanine, 2-naphthylalanine, or a combination thereof. In embodiments, two or three aromatic, hydrophobic amino acids of the cCPP are, independently, phenylalanine, 2-naphthylalanine, or a combination thereof. In embodiments, at least two uncharged, non-aromatic amino acids of the cyclic peptide are citrulline, histidine, serine, glycine, or a combination thereof. In embodiments, two amino acids of the cCPP are citrulline, histidine, serine, glycine, or a combination thereof. In embodiments, the cCPP has from 6 to 10 amino acids wherein two amino acids of the cCPP are arginine, two or three amino acids are, independently, aromatic, hydrophobic amino acids selected from phenylalanine, 2-naphthylalanine, β-homophenylalanine, and two amino acids are independently, uncharged, non-aromatic amino acids selected from citrulline, serine, histidine, and glycine.

[0119] In embodiments the cCPP is of Formula (1), where at least two of R^4, R^5, R^6, and R^7 are a positively charged side chain of an amino acid residue. In embodiments, two of R^4, R^5, R^6, and R^7 are a positively charged side chain of an amino acid residue. In embodiments, at least three of R^4, R^5, R^6, and R^7 are a positively charged side chain of an amino acid residue. In embodiments, three of R^4, R^5, R^6, R^7 are a positively charged side chain of an amino acid residue. In embodiments, R^4, R^5, R^6, and R^7 are a positively charged side chain of an amino acid residue.

[0120] The cCPP can comprise the structure of Formula (2):

Formula (2):



(2), or a protonated form thereof,

wherein:

R^1, R^2, and R^3 can each independently be H or an amino acid residue having a side chain comprising an aryl or heteroaryl group;

at least two of R^1, R^2, and R^3 are an aryl or heteroaryl side chain of an amino acid;

R^4 and R^6 are independently H or an amino acid side chain;

AA_{SC} is an amino acid side chain;

m′ is an integer from 0 to 3;

m″ is an integer from 0 to 3; and

q is 1, 2, 3, or 4.

[0121] In embodiments, the cCPP are of Formula (1) or (2), where R^1, R^2, and R^3 can each independently be H, -alkylene-aryl, -alkylene-heteroaryl, or an amino acid side chain comprising a guanidine group, a guanidine replacement group, or a protonated form thereof. R^1, R^2, and R^3 can each independently be H, -C_{1-3}alkylene-aryl, -C_{1-3}alkylene-heteroaryl or an amino acid side chain comprising a guanidine group, or a protonated form thereof. In embodiments, the cCPP are of Formula (1) or (2), where R^1, R^2, and R^3 can each independently be H, -alkylene-aryl, or -alkylene-heteroaryl. R^1, R^2, and R^3 can each independently be H, -C_{1-3}alkylene-aryl, or -C_{1-3}alkylene-heteroaryl. R^1, R^2, and R^3 can each independently be H or -alkylene-aryl. R^1, R^2, and R^3 can each independently be H or -C_{1-3}alkylene-aryl. C_{1-3}alkylene can be methylene. Aryl can be a 6- to 14-membered aryl. Heteroaryl can be a 6- to 14-membered heteroaryl having one or more heteroatoms selected from N, O, and S. Aryl can be selected from phenyl, naphthyl, or anthracenyl. Aryl can be phenyl or naphthyl. Aryl can be phenyl. Heteroaryl can be pyridyl, quinolyl, and isoquinolyl. The amino acid side chain comprising a guanidine group can be arginine. R^1, R^2, and R^3 can each independently be H, -C_{1-3}alkylene-Ph or -C_{1-3}alkylene-naphthyl. R^1, R^2, and R^3 can each independently be H, -CH_2Ph, or -CH_2-naphthyl. R^1, R^2, and R^3 can each independently be H or -CH_2Ph. One of R^1, R^2, and R^3 can be arginine.

[0122] In embodiments, the cCPP are of Formula (1) or (2), where R^1, R^2, and R^3 can each independently be the side chain of tyrosine, phenylalanine, 1-naphthylalanine, 2-naphthylalanine, tryptophan, 3-benzothienylalanine, 4-phenylphenylalanine, 3,4-difluorophenylalanine, 4-trifluoromethylphenylalanine, 2,3,4,5,6-pentafluorophenylalanine, homophenylalanine, β-homophenylalanine, 4-tert-butyl-phenylalanine, 4-pyridinylalanine, 3-pyridinylalanine, 4-methylphenylalanine, 4-fluorophenylalanine, 4-chlorophenylalanine, 3-(9-anthryl)-alanine or

arginine. R^1, R^2, and R^3 can each independently be the side chain of tyrosine, phenylalanine, 1-naphthylalanine, 2-naphthylalanine, arginine or tryptophan.

[0123] In embodiments, the cCPP are of Formula (1) or (2), where R^1 can be the side chain of tyrosine. R^1 can be the side chain of phenylalanine. R^1 can be the side chain of 1-naphthylalanine. R^1 can be the side chain of 2-naphthylalanine. R^1 can be the side chain of tryptophan. R^1 can be the side chain of 3-benzothienylalanine. R^1 can be the side chain of 4-phenylphenylalanine. R^1 can be the side chain of 3,4-difluorophenylalanine. R^1 can be the side chain of 4-trifluoromethylphenylalanine. R^1 can be the side chain of 2,3,4,5,6-pentafluorophenylalanine. R^1 can be the side chain of homophenylalanine. R^1 can be the side chain of β-homophenylalanine. R^1 can be the side chain of 4-tert-butyl-phenylalanine. R^1 can be the side chain of 4-pyridinylalanine. R^1 can be the side chain of 3-pyridinylalanine. R^1 can be the side chain of 4-methylphenylalanine. R^1 can be the side chain of 4-fluorophenylalanine. R^1 can be the side chain of 4-chlorophenylalanine. R^1 can be the side chain of 3-(9-anthryl)-alanine. R^1 can be H. R^1 can be a side chain of arginine.

[0124] In embodiments, the cCPP are of Formula (1) or (2), where R^2 can be the side chain of tyrosine. R^2 can be the side chain of phenylalanine. R^2 can be the side chain of 1-naphthylalanine. R^2 can be the side chain of 2-naphthylalanine. R^2 can be the side chain of tryptophan. R^2 can be the side chain of 3-benzothienylalanine. R^2 can be the side chain of 4-phenylphenylalanine. R^2 can be the side chain of 3,4-difluorophenylalanine. R^2 can be the side chain of 4-trifluoromethylphenylalanine. R^2 can be the side chain of 2,3,4,5,6-pentafluorophenylalanine. R^2 can be the side chain of homophenylalanine. R^2 can be the side chain of β-homophenylalanine. R^2 can be the side chain of 4-tert-butyl-phenylalanine. R^2 can be the side chain of 4-pyridinylalanine. R^2 can be the side chain of 3-pyridinylalanine. R^2 can be the side chain of 4-methylphenylalanine. R^2 can be the side chain of 4-fluorophenylalanine. R^2 can be the side chain of 4-chlorophenylalanine. R^2 can be the side chain of 3-(9-anthryl)-alanine. R^2 can be H. R^2 can be a side chain of arginine.

[0125] In embodiments, the cCPP are of Formula (1) or (2), where R^3 can be the side chain of tyrosine. R^3 can be the side chain of phenylalanine. R^3 can be the side chain of 1-naphthylalanine. R^3 can be the side chain of 2-naphthylalanine. R^3 can be the side chain of tryptophan. R^3 can be the side chain of 3-benzothienylalanine. R^3 can be the side chain of 4-phenylphenylalanine. R^3 can be the side chain of 3,4-difluorophenylalanine. R^3 can be the side chain of 4-

trifluoromethylphenylalanine. R^3 can be the side chain of 2,3,4,5,6-pentafluorophenylalanine. R^3 can be the side chain of homophenylalanine. R^3 can be the side chain of β-homophenylalanine. R^3 can be the side chain of 4-tert-butyl-phenylalanine. R^3 can be the side chain of 4-pyridinylalanine. R^3 can be the side chain of 3-pyridinylalanine. R^3 can be the side chain of 4-methylphenylalanine. R^3 can be the side chain of 4-fluorophenylalanine. R^3 can be the side chain of 4-chlorophenylalanine. R^3 can be the side chain of 3-(9-anthryl)-alanine. R^3 can be H. R^3 can be a side chain of arginine.

[0126] In embodiments, the cCPP are of Formula (1) or (2), where R^4 can be H, or a side chain of arginine, citrulline, serine, or histidine. R^4 can be H or a side chain of arginine. R^4 can be H. R^4 can be a side chain of arginine. R^4 can be a side chain of citrulline. R^4 can be a side chain of serine. R^4 can be a side chain of histidine. R^4 can be a side chain of valine. R^4 can be a side chain of leucine.

[0127] In embodiments, the cCPP are of Formula (1), where R^5 can be H, or a side chain of arginine, citrulline, serine, or histidine. R^5 can be H or a side chain of arginine. R^5 can be H. R^5 can be a side chain of arginine. R^5 can be a side chain of citrulline. R^5 can be a side chain of serine. R^5 can be a side chain of histidine.

[0128] In embodiments, the cCPP are of Formula (1) or (2), where R^6 can be H, or a side chain of arginine, citrulline, serine, or histidine. R^6 can be H or a side chain of arginine. R^6 can be H. R^6 can be a side chain of arginine. R^6 can be a side chain of citrulline. R^6 can be a side chain of serine. R^6 can be a side chain of histidine. R^6 can be a side chain of valine. R^6 can be a side chain of leucine.

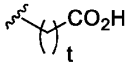
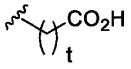
[0129] In embodiments, the cCPP are of Formula (2), where R^7 can be H, or a side chain of arginine, citrulline, serine, or histidine. R^7 can be H or a side chain of arginine. R^7 can be H. R^7 can be a side chain of arginine. R^7 can be a side chain of citrulline. R^7 can be a side chain of serine. R^7 can be a side chain of histidine.

[0130] In embodiments, the cCPP are of Formula (1) or (2), where one, two, or three of R^1, R^2, R^3, R^4, R^5, R^6, and R^7 can be H. At least one of R^1, R^2, R^3, R^4, R^5, R^6, and R^7 can be H. One of R^1, R^2, R^3, R^4, R^5, R^6, and R^7 can be H. Two of R^1, R^2, R^3, R^4, R^5, R^6, and R^7 can be H. Three of R^1, R^2, R^3, R^5, R^6, and R^7 can be H. One of R^1, R^2, and R^3 can be H. At least one of R^4, R^5, R^6, and R^7 can be H. One of R^4, R^5, R^6, and R^7 can be H. Two of R^4, R^5, R^6, and R^7 can be H. Three of R^4, R^5, R^6, and R^7 can be H. Four of R^4, R^5, R^6, and R^7 can be H.

[0131] In embodiments, the cCPP are of Formula (1) or (2), where at least one of R^4, R^5, R^6, and R^7 can be H or a side chain of arginine, citrulline, serine, or histidine. At least one of R^4, R^5, R^6, and R^7 is H. At least one of R^4, R^5, R^6, and R^7 can be side chain of arginine. At least one of R^4, R^5, R^6, and R^7 can be side chain of citrulline. At least one of R^4, R^5, R^6, and R^7 can be side chain of serine. At least one of R^4, R^5, R^6, and R^7 can be side chain of histidine. One of R^4, R^5, R^6, and R^7 can be H or a side chain of arginine, citrulline, serine, or histidine. One of R^4, R^5, R^6, and R^7 is H. One of R^4, R^5, R^6, and R^7 can be side chain of arginine. One of R^4, R^5, R^6, and R^7 can be side chain of citrulline. One of R^4, R^5, R^6, and R^7 can be side chain of serine. One of R^4, R^5, R^6, and R^7 can be side chain of histidine.

[0132] In embodiments, the cCPP are of Formula (1) or (2), where two of R^4, R^5, R^6, and R^7 can be H or a side chain of arginine, citrulline, serine, or histidine. Two of R^4, R^5, R^6, and R^7 can be H. Two of R^4, R^5, R^6, and R^7 can be side chain of arginine. Two of R^4, R^5, R^6, and R^7 can be side chain of citrulline. Two of R^4, R^5, R^6, and R^7 can be side chain of serine. Two of R^4, R^5, R^6, and R^7 can be side chain of histidine.

[0133] In embodiments, the cCPP are of Formula (1) or (2), where three of R^4, R^5, R^6, and R^7 can be H or a side chain arginine, citrulline, serine, or histidine. Three of R^4, R^5, R^6, and R^7 can be H. Three of R^4, R^5, R^6, and R^7 can be side chain of arginine. Three of R^4, R^5, R^6, and R^7 can be side chain of citrulline. Three of R^4, R^5, R^6, and R^7 can be side chain of serine. Three of R^4, R^5, R^6, and R^7 can be side chain of histidine.

[0134] In embodiments, the cCPP are of Formula (1) or (2), where AA_{SC} can be a side chain of a residue of asparagine, glutamine, or homoglutamine. AA_{SC} can be a side chain of a residue of glutamine. In embodiments, AA_{SC} can be  or  , wherein t can be an integer from 0 to 5. AA_{SC} can be  , wherein t can be 0 or an integer from 1 to 5. In embodiments, t can be 1 to 5. In embodiments, t is 2 or 3. In embodiments, t can be 2. In embodiments, t can be 3. In embodiments, the AAsc is the side chain of a glutamine residue. In embodiments, the AAsc is the side chain of a glutamic acid residue. In embodiments when the AA_{SC} is the side chain of glutamine, the carboxamide amide nitrogen of the glutamine side chain forms a bond with the -$(CH_2)_y$- group.

[0135] In embodiments, the cCPP are of Formula (1) or (2), where q can be 1, 2, or 3. q can 1 or 2. q can be 1. q can be 2. q can be 3. q can be 4.

[0136] In embodiments, the cCPP are of Formula (1) or (2), where m′ can be 1 to 3. m′ can be 1 or 2. m′ can be 0. m′ can be 1. m′ can be 2. m′ can be 3.

[0137] In embodiments, the cCPP are of Formula (1) or (2), where m″ can be 1 to 3. m″ can be 1 or 2. m″ can be 0. m″ can be 1. m″ can be 2. m″ can be 3.

[0138] In embodiments, the cCPP comprises the structure of Formula (2) or a protonated form thereof; wherein:

one of R^1, R^2, and R^3 is H;

two of R^1, R^2, and R^3 are -CH_2Ph;

R^4 and R^6 are independently H or an amino acid side chain;

AA_{SC} is an amino acid side chain;

q is 1, 2, 3 or 4;

m′ is an integer from 0 to 3; and

m″ is an integer from 0 to 3.

[0139] In embodiments, the cCPP comprises the structure of Formula (2) or a protonated form thereof, wherein:

R^1, R^2, and R^3 are -CH_2Ph;

R^4 and R^6 are independently H or an amino acid side chain;

AA_{SC} is an amino acid side chain;

q is 1, 2, 3 or 4;

m′ is an integer from 0 to 3; and

m″ is an integer from 0 to 3.

[0140] In embodiments, the cCPP comprises the structure of Formula (2) or a protonated form thereof, wherein:

R^1, R^2, and R^3 are each independently a side chain of an amino acid comprising an aryl or heteroaryl group;

at least one of R^1, R^2, and R^3 is a side chain of naphthylalanine;

AA_{SC} is an amino acid side chain;

R^4 and R^6 are independently H or an amino acid side chain of arginine, serine, histidine or citrulline;

q is 1, 2, 3 or 4;

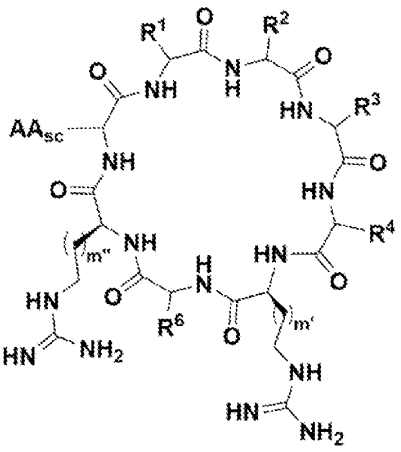
m′ is an integer from 0 to 3; and

m″ is an integer from 0 to 3.

[0141] In embodiments, the cCPP comprises the structure of Formula (2) or a protonated form thereof, and R^4 and R^6 are independently H, or a side chain of arginine, histidine, serine or citrulline. In embodiments, R^4 and R^6 are in independently H, or a side chain of arginine, histidine, or serine. In embodiments, R^4 is H. In embodiments, R^4 is a side chain of arginine. In embodiments, R^4 is a side chain of histidine. In embodiments, R^4 is a side chain of serine. In embodiments, R^4 is a side chain of citrulline. In embodiments, R^6 is H. In embodiments, R^6 is a side chain of arginine. In embodiments, R^6 is a side chain of histidine. In embodiments, R^6 is a side chain of serine. In embodiments, R^6 is a side chain of citrulline.

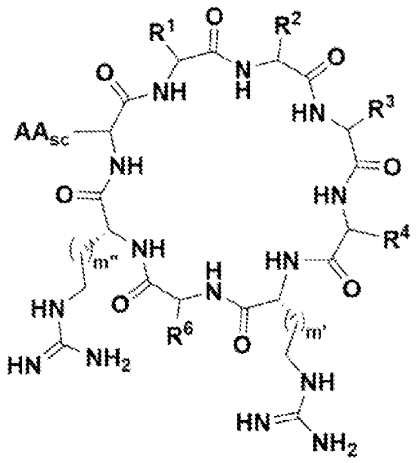
[0142] The cCPP of Formula (2) can comprise the structure of Formula (2-a) or Formula (II-b):

Formula (2-a):



(2-a);

Formula (2-b):

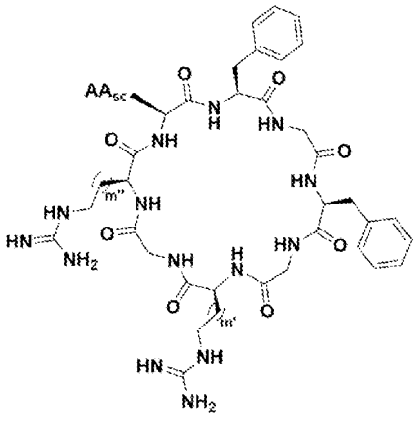


(2-b), or a protonated form thereof,

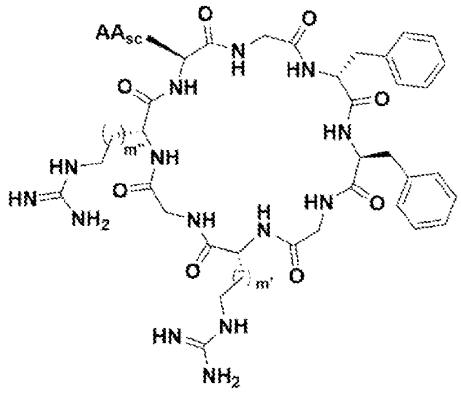
wherein AAsc, R^1, R^2, R^3, R^4, R^6, m′, and m″ are as defined herein relative to Formula (2).

[0143] The cCPP of Formula (2) can comprise the structures of Formulae (2-a1), (2-b1), (2-a2), (2-b2), (2-a3), or (2-b3):

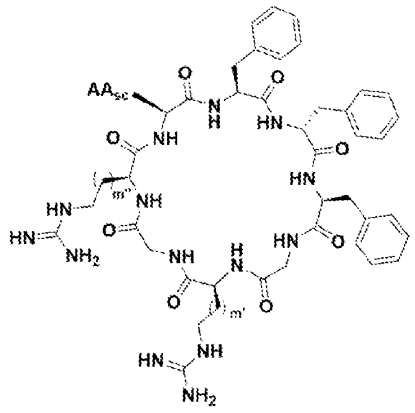
Formulae (2-a1), (2-b1), (2-a2), (2-b2), (2-a3), or (2-b3):



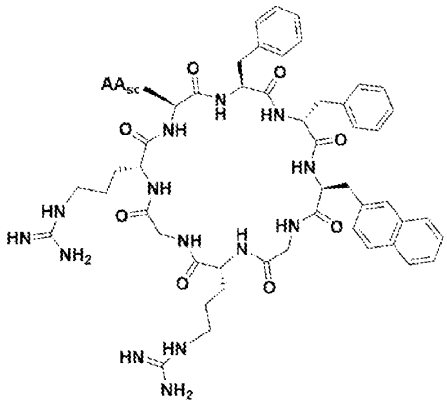
(2-a1);



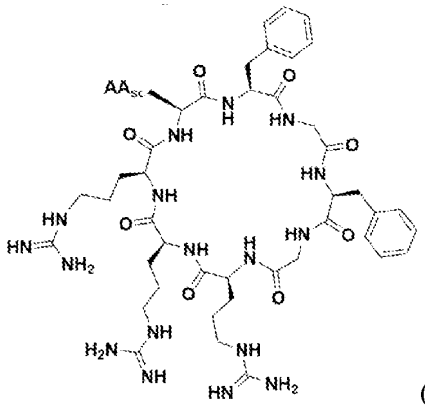
(2-b1);



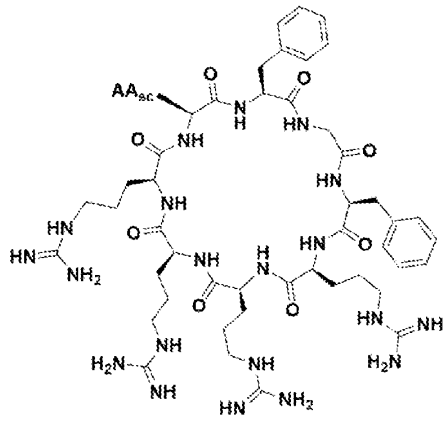
(2-a2);



(2-b2);



(2-a3);



(2-b3); or

protonated form thereof, wherein AAsc, m′, and m″ are as defined herein relative to Formula (2).

[0144] In embodiments, the cCPP of Formula (1) or (2) can include those having one of the following sequences: FGFGRGR; GfFGrGr; FfFGRGR; FGFGRRR; or FGFRRRR.

[0145] In embodiments, the cCPP of Formula (1) or (2) can include those having one of the following sequences: FGFGHGH or FGFSHSH.

[0146] In embodiments, the cCPP of Formula (1) or (2) can include those having one of the following sequences: FfFSRSR or FGFSRSR.

[0147] In embodiments, the cCPP of Formula (1) or (2) can include those having one of the following sequences: Ff-Nal-RrRr; Ff-Nal-GrGr; Ff-Nal-GRGR; Ff-Nal-HrHr; or Ff-Nal-SrSr.

[0148] In embodiments, the cCPP of Formula (1) or (2) can comprise one of the following sequences: Ff-Nal-Cit-r-Cit-r; Ff-Nal-Rr-Cit-r; Ff-Nal-Cit-rRr; or Ff-Nal-R-cit-R-cit.

[0149] In embodiments, the cCPP of Formula (1) or (2) can include those having one of the following sequences: FGFGRGRQ; GfFGrGrQ; FfFGRGRQ; FGFGRRRQ; or FGFRRRRQ.

[0150] In embodiments, the cCPP of Formula (1) or (2) can include those having one of the following sequences: FGFGHGHQ or FGFSHSHQ.

[0151] In embodiments, the cCPP of Formula (1) or (2) can include those having one of the following sequences: FfFSRSRQ or FGRSRSRQ.

[0152] In embodiments, the cCPP of Formula (1) or (2) can include those having one of the following sequences: Ff-Nal-RrRrQ; Ff-Nal-GrGrQ; Ff-Nal-GRGRQ; Ff-Nal-HrHrQ; or Ff-Nal-SrSrQ.

[0153] In embodiments, the cCPP of Formula (1) or (2) can comprise one of the following sequences: Ff-Nal-Cit-r-Cit-rQ; Ff-Nal-Rr-Cit-rQ; Ff-Nal-Cit-rRrQ; or Ff-Nal-R-cit-R-cit-Q.

cCPP sequence		
F-Nal-RRRQ	FRRRR-Nal-Q	F-Nal-RRRRQK
F-Nal-RRRC	rRFR-Nal-RQ	F-Nal-RRRRQC
F-Nal-RRRU	RR-Nal-FRRQ	f-Nal-RrRrRQ
RRR-Nal-FQ	CRRRRFWQ	F-Nal-RRRRRQ
RRRR-Nal-F	Ff-Nal-RrRrQ	RRRR-Nal-FDΩC
F-Nal-RRRR	FF-Nal-RRRRQ	F-Nal-RRR
F-nal-rRrRq	RFRFR-Nal-RQ	FWRRR
F-nal-rRrRQ	URRRRFWQ	RRR-Nal-F
F-Nal-RRRRQ		RRRWF
f-Nal-RrRrQ		
RRFR-Nal-RQ		

where Nal = L-naphthylalanine; nal = D-naphthylalanine; Ω = L-norleucine.

[0154] In embodiments, the cCPP of Formula (1) or (2) can be selected from Ff-Nal-GrGrQ; FfFGRGRQ; FGFGRGRQ; GfFGrGrQ; FfFGRGRQ; FGFGRRRQ; and FGFRRRRQ.

[0155] In embodiments, the cCPP of Formula (1) or (2) can be selected from fNalRrRrQ, Ff-Nal-Cit-r-Cit-r-Q, and Ff-Nal-GrGrQ.

[0156] In embodiments, the cCPP of Formula (1) or (2) has a sequence of FfFGRGRQ.

[0157] In embodiments, the cCPP of Formula (1) or (2) has a sequence of FGFGRGRQ or GfFGrGrQ.

[0158] In embodiments, the cCPP of Formula (1) or (2) can be FAFARARQ.

[0159] In embodiments, the cCPP of Formula (1) or (2) can be Ff-Nal-GrGrQ.

[0160] In embodiments, the cCPP of Formula (1) or (2) can be selected from FfFGRGRQ, FfFRRRRQ, FfFRrRrQ, fffrrrrQ or FfFRrRrQ.

[0161] In embodiments, the cCPP of Formula (1) or (2) can be FG-Nal-GRGRQ.

[0162] In embodiments, the cCPP of Formula (1) or (2) can be selected from FGFGRGRQ, fGfGrGrQ, fGfGrGrQ, FGFGRGRQ, FGFGRQ, fGfrrrrQ, FGFRRRRQ, or FGFRRRRQ.

[0163] In embodiments, the cCPP of Formula (I) or (II) can be selected from FFFRRRRQ, FFFGRRRQ, FFFRGRRQ; FFFRRGRQ, FFFRRRGQ, GFFRRRRQ, or FFGRRRRQ.

[0164] In embodiments, the cCPP of Formula (I) or (II) can be FFFRRRRQ.

[0165] In embodiments, the cCPP of Formula (I) or (II) can be FFFGRRRQ.

[0166] In embodiments, the cCPP of Formula (I) or (II) can be FFFRGRRQ.

[0167] In embodiments, the cCPP of Formula (I) or (II) can be FFFRRGRQ.

[0168] In embodiments, the cCPP of Formula (I) or (II) can be FFFRRRGQ.

[0169] In embodiments, the cCPP of Formula (I) or (II) can be GFFRRRRQ.

[0170] In embodiments, the cCPP of Formula (I) or (II) can be FFGRRRRQ.

[0171] In embodiments, the cCPP of Formula (I) or (II) can be selected from Nal-G-Nal-GRGRQ; FGFKRKRQ; FGFRRRRQ; FfGFRRRRQ; or FGFRRGRRQ.

[0172] In embodiments, the cCPP of Formula (I) or (II) can be Nal-G-Nal-GRGRQ.

[0173] In embodiments, the cCPP of Formula (I) or (II) can be FGFKRKRQ.

[0174] In embodiments, the cCPP of Formula (I) or (II) can be FGFRRRRQ.

[0175] In embodiments, the cCPP of Formula (I) or (II) can be FfGFRRRRQ.

[0176] In embodiments, the cCPP of Formula (I) or (II) can be FGFRRGRRQ.

[0177] In embodiments, the cCPP of Formula (I) or (II) can be selected from RGRGRGRQ; FFFGRGRQ; FGFRRRRQ; FGFRGRGQ; FRGRGRGQ; FRFGRGRQ; FRFRFRFQ; FGFLRLRQ; or FGFVRVRQ.

[0178] In embodiments, the cCPP of Formula (I) or (II) can be RGRGRGRQ.

[0179] In embodiments, the cCPP of Formula (I) or (II) can be FFFGRGRQ.

[0180] In embodiments, the cCPP of Formula (I) or (II) can be FGFRRRRQ.

[0181] In embodiments, the cCPP of Formula (I) or (II) can be FGFRGRGQ.

[0182] In embodiments, the cCPP of Formula (I) or (II) can be FRGRGRGQ.

[0183] In embodiments, the cCPP of Formula (I) or (II) can be FRFGRGRQ.

[0184] In embodiments, the cCPP of Formula (I) or (II) can be FRFRFRFQ.

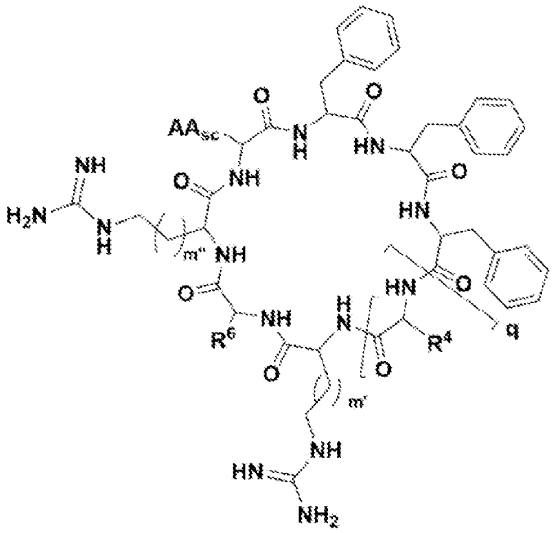
[0185] In embodiments, the cCPP of Formula (I) or (II) can be FGFLRLRQ.

[0186] In embodiments, the cCPP of Formula (I) or (II) can be FGFVRVRQ. In embodiments, the cCPP of Formula (1) or (2) can be FGWRGRQ.

[0187] In embodiments, the cCPP of Formula (1) or (2) can be fFRGRQ.

[0188] The cCPP of Formula (2) can have the structure of Formula (II-c):

Formula (2-c):

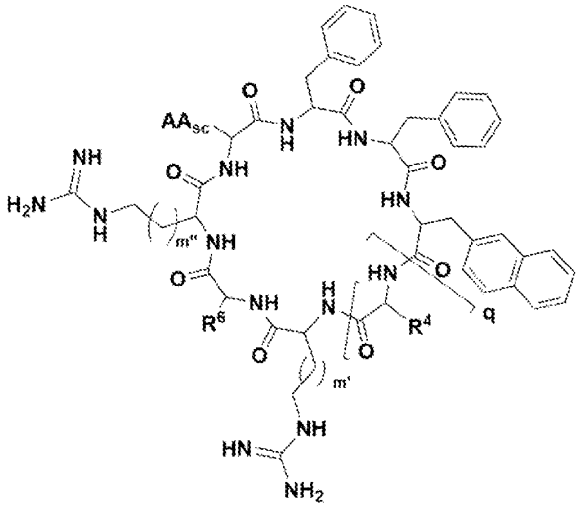


(2-c), or a protonated form thereof,

wherein R^4, R^6, q, m′, m″, and AAsc are as defined herein.

[0189] The cCPP of Formula (2) can have the structure of Formula (2-d):

Formula (2-d):

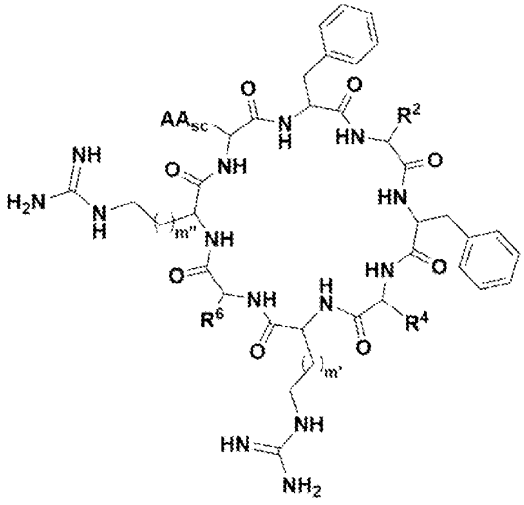


(2-d), or a protonated form thereof,

wherein R^4, R^6, q, m′, m″, and AAsc are as defined herein.

[0190] The cCPP of Formula (2) can have the structure of Formula (2-e):

Formula (2-e):

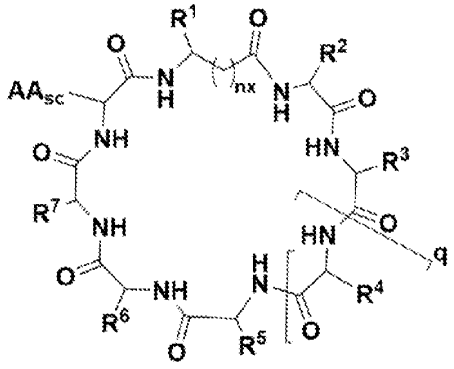


(2-e), or a protonated form thereof,

wherein: R^2, R^4, R^6, m′, m″, and AAsc are as defined herein. In embodiments, R_2 is H.

[0191] In embodiments, the cCPP can be of the Formula (3):

Formula (3):



(3), or a protonated form thereof,

wherein:

R^1, R^2, and R^3 can each independently be H or an amino acid residue having a side chain comprising an aryl or heteroaryl group;

at least two of R^1, R^2, and R^3 is an aryl or heteroaryl side chain of an amino acid;

R^4, R^5, R^6, and R^7 are independently H or an amino acid side chain;

at least two of R^4, R^5, R^6, and R^7 are independently a side chain of arginine;

AA_{SC} is an amino acid side chain;

n^X is 0 or 1; and

q is 1, 2, 3, or 4.

[0192] In embodiments, the cCPP is of Formula (3) where at least one of R^1, R^2, or R^3 is H. In embodiments, the amino acid residue having a side chain comprising an aryl or heteroaryl group is phenylalanine, homophenylalanine, or 2-naphthylalanine. In embodiments, the cCPP is of Formula (3) where at least two of R^4, R^5, R^6, or R^7 are each independently an amino acid residue having a side chain comprising a charged group. In embodiments, the amino acid residue having a side chain comprising a charged group is arginine. In embodiments, the cCPP is of Formula (3) where q is 1.

[0193] In embodiments, the cCPP is of Formula (3) where n^X is 1, where R^5 and R^7 are a side chain of arginine. In embodiments, the cCPP is of Formula (3) where n^X is 1, wherein the aryl or heteroaryl group is phenylalanine, beta-homophenylalanine, or 2-naphthylalanine, and where at least two of R^4, R^5, R^6, and R^7 are the side chain of arginine. In embodiments, the cCPP is of Formula (3) where n^X is 1, where R^4, R^5, R^6, and R^7 are the side chain of arginine. In

embodiments, the cCPP is of Formula (3) where n^X is 1, where R^5 and R^7 are the side chain of arginine, and R^4 and R^6 are H.

[0194] In embodiments, the cCPP is of Formula (3) where at least one of R^4, R^5, R^6, or R^7 is the amino acid side chain of serine or histidine. In embodiments the cCPP is of Formula (3) where at least two of R^4, R^5, R^6, or R^7 are, independently, the amino acid side chain of serine, citrulline, or histidine. In embodiments the cCPP is of Formula (3) where at least three of R^4, R^5, R^6, or R^7 are, independently, the amino acid side chain of serine or histidine. In embodiments the cCPP is of Formula (3) where at least four of R^4, R^5, R^6, or R^7 are, independently, the amino acid side chain of serine or histidine.

[0195] In embodiments of the cCPP of Formula (3):

at least two of R^1, R^2, and R^3 are independently a side chain of phenylalanine, or 2-naphthylalanine;

at least two of R^4, R^5, R^6, or R^7 are independently a side chain of arginine;

at least two of R^4, R^5, R^6, or R^7 are independently H, or a side chain of arginine, serine, citrulline, or histidine;

AA_{SC} is an amino acid side chain;

n^X is 0 or 1; and

q is 1.

[0196] In embodiments, the cCPP is of Formula (3), where two of R^4, R^5, R^6, or R^7 are a side chain of serine. In embodiments, the cCPP is of Formula (3), where two of R^4, R^5, R^6, or R^7 are a side chain of histidine. In embodiments, the cCPP is of Formula (3), where two of R^4, R^5, R^6, or R^7 are a side chain of citrulline. In embodiments, the cCPP is of Formula (3), where two of R^4, R^5, R^6, or R^7 are independently, H.

[0197] In embodiments, the cCPP is of Formula (3) wherein:

at least two of R^1, R^2, and R^3 are a side chain of phenylalanine or naphthylalanine;

at least two of R^4, R^5, R^6, or R^7 are a side chain of arginine;

at least two of R^4, R^5, R^6, or R^7 are independently H, or a side chain of an uncharged non-aryl amino acid selected from histidine, threonine, serine, leucine, isoleucine, valine, neopentylglycine, alanine, homoalanine, homoserine, 3-(4-thiazolyl)-alanine, 3-(4-furanyl)-alanine, citrulline, and 3-(4-thienyl)-alanine;

AA_{SC} is an amino acid side chain;

n^X is 0 or 1; and

q is 1.

[0198] In embodiments, the cCPP is of Formula (3) wherein:

at least two of R^1, R^2, and R^3 are independently a side chain of phenylalanine or naphthylalanine;

at least two of R^4, R^5, R^6, or R^7 are a side chain of arginine;

at least two of R^4, R^5, R^6, or R^7 are independently a side chain of serine, citrulline, or histidine;

AA_{SC} is an amino acid side chain;

n^X is 0 or 1; and

q is 1.

[0199] In embodiments, the cCPP is of Formula (3), wherein at least one of R^1, R^2, or R^3 is H. In embodiments, the cCPP is of Formula (3), wherein at least one of R^1, R^2, or R^3 is a side chain of phenylalanine. In embodiments, the cCPP is of Formula (3), wherein at least two of R^1, R^2, or R^3 are a side chain of phenylalanine. In embodiments, the cCPP is of Formula (3), wherein at least one of R^1, R^2, or R^3 is a side chain of 2-naphthylalanine.

[0200] In embodiments, the cCPP is of Formula (3), wherein at least two of R^4, R^5, R^6, or R^7 are independently a side chain of serine, citrulline, or histidine.

[0201] In embodiments, the cCPP is of Formula (3), wherein at least one of R^4, R^5, R^6, or R^7 is independently an uncharged, non-aryl side chain of an amino acid. In embodiments, at least two of R^4, R^5, R^6, or R^7 are independently side chains of an uncharged non-aryl amino acid (e.g., histidine, citrulline, threonine, serine, leucine, isoleucine, valine, neopentylglycine, alanine, homoalanine, homoserine, 3-(4-thiazolyl)-alanine, 3-(4-furanyl)-alanine, and 3-(4-thienyl)-alanine). In embodiments, the cCPP is of Formula (3), wherein at least two of R^4, R^5, R^6, or R^7 are independently side chains of an uncharged non-aryl amino acid selected from histidine, citrulline, threonine, serine, leucine, isoleucine, valine, neopentylglycine, alanine, homoalanine, homoserine, 3-(4-thiazolyl)-alanine, 3-(4-furanyl)-alanine, and 3-(4-thienyl)-alanine. In embodiments, the cCPP is of Formula (3), wherein at least two of R^4, R^5, R^6, or R^7 are independently side chains of an uncharged non-aryl amino acid selected from histidine, citrulline, and serine.

[0202] In embodiments, the cCPP is of Formula (3), wherein at least one of R^4, R^5, R^6, or R^7 is, independently, H. In embodiments, the cCPP is of Formula (3), wherein two of R^4, R^5, R^6, or R^7 are, independently, H.

[0203] In embodiments, the cCPP can be of Formula (3), wherein:

R^1, R^2, and R^3 can each independently be H or an amino acid residue having a side chain comprising an aryl or heteroaryl group;

at least two of R^1, R^2, and R^3 are phenylalanine;

R^4, R^5, R^6, R^7 are independently H or an amino acid side chain;

two of R^4, R^5, R^6, R^7 are independently a side chain of arginine;

AA_{SC} is an amino acid side chain;

n^X is 0 or 1; and

q is 1, 2, 3, or 4.

[0204] In embodiments, the cCPP is of Formula (3) where at least one of R^1, R^2, or R^3 is, independently, H. In embodiments, the cCPP is of Formula (3) where at least one of R^1, R^2, and R^3 is, independently, an amino acid residue having a side chain comprising an aryl or heteroaryl group. In embodiments, the amino acid residue having a side chain comprising an aryl or heteroaryl group is phenylalanine, β-homophenylalanine, or 2-naphthylalanine. In embodiments, the cCPP is of Formula (3) where at least two of R^4, R^5, R^6, and R^7 are each independently an amino acid residue having a side chain comprising a charged group. In embodiments, the amino acid residue having a side chain comprising a charged group is arginine. In embodiments, the cCPP is of Formula (3) where q is 1.

[0205] In embodiments, the cCPP is of Formula (3) where n^X is 1 and where R^5 and R^7 are a side chain of arginine. In embodiments, the cCPP is of Formula (3) where n^X is 1, wherein the aryl or heteroaryl group is phenylalanine, b-homophenylalanine, or 2-naphthylalanine, and where at least two of R^4, R^5, R^6, and R^7 are the side chain of arginine. In embodiments, the cCPP is of Formula (3) where n^X is 1, where R^4, R^5, R^6, and R^7 are the side chain of arginine. In embodiments, the cCPP is of Formula (3) where n^X is 1, where R^5 and R^7 are the side chain of arginine, and R^4 and R^6 are H. In embodiments, the cCPP is of Formula (3) where at least one of R^4, R^5 (if present), R^6, or R^7 (if present) is H or the amino acid side chain of serine, citrulline, or histidine. In embodiments the cCPP is of Formula (3) where at least two of R^4, R^5 (if present), R^6, or R^7 (if present) are, independently, H or the amino acid side chain of serine or histidine. In embodiments the cCPP is

of Formula (3) where at least three of R^4, R^5, R^6, or R^7 are, independently, H or the amino acid side chain of serine, citrulline, or histidine. In embodiments the cCPP is of Formula (3) where at least four of R^4, R^5, R^6, or R^7 are, independently, H or the amino acid side chain of serine, citrulline, or histidine.

[0206] In embodiments of the cCPP of Formula (3): at least two of R^1, R^2, and R^3 are independently a side chain of phenylalanine, b-homophenylalanine, or 2-naphthylalanine; at least two of R^4, R^5, R^6, or R^7 are independently a side chain of arginine; at least two of R^4, R^5, R^6, or R^7 are independently H or a side chain of arginine, serine, citrulline, or histidine; AA_{SC} is an amino acid side chain; n^X is 0 or 1; and q is 1. It is understood that n^X is 1 when R^1 is a side chain of b-homophenylalanine.

[0207] In embodiments of the cCPP of Formula (3): at least two of R^1, R^2, and R^3 are independently a side chain of phenylalanine, or 2-naphthylalanine; R^4 and R^6 are each independently H or a side chain of arginine, serine, citrulline, or histidine; R^5 and R^7 are a side chain of arginine; AA_{SC} is an amino acid side chain; n^X is 0 or 1; and q is 1.

[0208] In embodiments, the cCPP is of Formula (3), where two of R^4, R^5 (if present), R^6, or R^7 (if present) are independently a side chain of serine. In embodiments, the cCPP is of Formula (3), where two of R^4, R^5 (if present), R^6, or R^7 (if present) are independently a side chain of histidine. In embodiments, the cCPP is of Formula (3), where two of R^4, R^5 (if present), R^6, or R^7 (if present) are independently, H.

[0209] In embodiments, the cCPP is of Formula (3) wherein: at least two of R^1, R^2, and R^3 are independently a side chain of phenylalanine, beta-homophenylalanine, or naphthylalanine; at least two of R^4, R^5, R^6, or R^7 are independently a side chain of arginine; at least two of R^4, R^5, R^6, or R^7 are independently H or a side chain of an uncharged non-aryl amino acid selected from histidine, threonine, serine, citrulline, leucine, isoleucine, valine, neopentylglycine, alanine, homoalanine, homoserine, 3-(4-thiazolyl)-alanine, 3-(4-furanyl)-alanine, and 3-(4-thienyl)-alanine; AA_{SC} is an amino acid side chain; n^X is 0 or 1; and q is 1. It is understood that n^X is 1 when R^1 is a side chain of b-homophenylalanine.

[0210] In embodiments, the cCPP is of Formula (3) wherein: at least two of R^1, R^2, and R^3 are independently a side chain of phenylalanine, 2-naphthylalanine, or b-homophenylalanine; R^4 and R^6 are independently H or a side chain of an uncharged non-aryl amino acid selected from histidine, threonine, serine, citrulline, leucine, isoleucine, valine, neopentylglycine, alanine,

homoalanine, homoserine, 3-(4-thiazolyl)-alanine, 3-(4-furanyl)-alanine, and 3-(4-thienyl)-alanine; R^5 and R^7 are a side chain of arginine; AA_{SC} is an amino acid side chain; n^X is 0 or 1; and q is 1. It is understood that n^X is 1 when R^1 is a side chain of b-homophenylalanine.

[0211] In embodiments, the cCPP is of Formula (3) wherein: at least two of R^1, R^2, and R^3 are independently a side chain of phenylalanine, b-homophenylalanine, or 2-naphthylalanine; at least two of R^4, R^5, R^6, or R^7 are independently a side chain of arginine; at least two of R^4, R^5, R^6, or R^7 are independently a side chain of serine, citrulline, or histidine; AA_{SC} is an amino acid side chain; n^X is 0 or 1; and q is 1. It is understood that n^X is 1 when R^1 is a side chain of b-homophenylalanine.

[0212] In embodiments, the cCPP is of Formula (3) wherein: at least two of R^1, R^2, and R^3 are independently a side chain of phenylalanine, beta-homophenylalanine, or naphthylalanine; R^4 and R^6 are independently a side chain of serine, citrulline, or histidine; R^5 and R^7 are a side chain of arginine; AA_{SC} is an amino acid side chain; n^X is 0 or 1; and q is 1. It is understood that n^X is 1 when R^1 is a side chain of b-homophenylalanine.

[0213] In embodiments, the cCPP is of Formula (3), wherein at least one of R^1, R^2, and R^3 is H. In embodiments, the cCPP is of Formula (3), wherein at least one of R^1, R^2, and R^3 is a side chain of phenylalanine. In embodiments, the CPP is of Formula (3), wherein at least two of R^1, R^2, and R^3 are a side chain of phenylalanine. In embodiments, the CPP is of Formula (3), wherein two of R^1, R^2, and R^3 are a side chain of phenylalanine. In embodiments, the CPP is of Formula (3), wherein three of R^1, R^2, and R^3 are a side chain of phenylalanine. In embodiments, the cCPP is of the general Formula (3), wherein one of R^1, R^2, and R^3 is a side chain of naphthylalanine.

[0214] In embodiments, the cCPP is of Formula (3), wherein at least two of R^4, R^5, R^6, or R^7 are independently a side chain of serine or histidine. In embodiments, the cCPP is of the general Formula (3), wherein R^4 and R^6 are independently H, or a side chain of serine, citrulline, or histidine. In embodiments, the cCPP is of Formula (3), wherein R^4 and R^6 are the side chain of serine. In embodiments, the cCPP is of Formula (3), wherein R^4 and R^6 are the side chain of histidine. In embodiments, the cCPP is of Formula (3), wherein R^4 and R^6 are the side chain of citrulline. In embodiments, the cCPP is of Formula (3), wherein R^4 and R^6 are H. In embodiments, the cCPP is of Formula (3), wherein R^5 and R^7 are a side chain of arginine.

[0215] In embodiments, the cCPP is of Formula (3), wherein at least one of R^4, R^5 (if present), R^6, R^7 (if present) is independently an uncharged, non-aryl side chain of an amino acid. In embodiments, the cCPP is of Formula (3), wherein at least two of R^4, R^5 (if present), R^6, R^7 (if

present) are independently side chains of an uncharged non-aryl amino acid (e.g., histidine, threonine, serine, citrulline, leucine, isoleucine, valine, neopentylglycine, alanine, homoalanine, homoserine, 3-(4-thiazolyl)-alanine, 3-(4-furanyl)-alanine, and 3-(4-thienyl)-alanine). In embodiments, the cCPP is of Formula (3), wherein at least two of R^4, R^5 (if present), R^6, R^7 (if present) are independently side chains of an uncharged non-aryl amino acid selected from histidine, threonine, serine, leucine, isoleucine, valine, neopentylglycine, alanine, homoalanine, homoserine, 3-(4-thiazolyl)-alanine, 3-(4-furanyl)-alanine, and 3-(4-thienyl)-alanine. In embodiments, the CPP is of Formula (3), wherein at least two of R^4, R^5 (if present), R^6, R^7 (if present) are independently side chains of an uncharged non-aryl amino acid selected from histidine and serine. In embodiments, the cCPP is of Formula (3), wherein R^5 and R^7 are a side chain of arginine.

[0216] In embodiments, the cCPP is of Formula (3), wherein at least one of R^4, R^5, R^6, or R^7 is, independently, H. In embodiments, the cCPP is of Formula (3), wherein two of R^4, R^5, R^6, or R^7 are, independently, H. In embodiments, the cCPP is of Formula (3), wherein at least one of R^4 or R^6 is, independently, H. In embodiments, the cCPP is of Formula (3), wherein R^4 and R^6 are H. In embodiments, the cCPP is of Formula (3), wherein R^5 and R^7 are a side chain of arginine.

[0217] In embodiments, EEVs are provided that include a cCPP having from 6 to 10 amino acids, wherein at least two amino acids of the cCPP are charged amino acids, at least two amino acids of the cCPP are aryl or heteroaryl hydrophobic amino acids, and at least two amino acids of the cCPP are uncharged, non-aryl amino acids. In embodiments, at least two charged amino acids of the cCPP are arginine. In embodiments, at least two aryl or heteroaryl, hydrophobic amino acids of the cCPP are phenylalanine, naphthylalanine (3-naphth-2-yl-alanine), b-homophenylalanine, or a combination thereof. In embodiments, at least two uncharged, non-aryl amino acids of the cCPP are glycine. In embodiments, two of the uncharged amino acids are serine, citrulline, histidine, or a combination thereof.

[0218] In embodiments, the cCPP of Formula (3) can include those having one of the following sequences: hFf-Nal-GrGr; bhF-F-Nal-SRSR; bhF-F-Nal-GRGR; bhF-F-Nal-HRHR; bhF-f-Nal-GrGr; bhF-f-Nal-SRSR; bhF-f-Nal-SrSr; or bhFf-Nal-HrHr (where bhF – b-homophenylalanine; hFf – homophenylalanine).

[0219] In embodiments, the cCPP of Formula (3) can include those having one of the following sequences: hFf-Nal-GrGrQ; bhF-F-Nal-SRSRQ; bhF-F-Nal-GRGRQ; bhF-F-Nal-HRHRQ; bhF-

f-Nal-GrGrQ; bhF-f-Nal-SRSRQ; bhF-f-Nal-SrSrQ; or bhFf-Nal-HrHrQ (where bhF – b-homophenylalanine; hFf – homophenylalanine).

[0220] In embodiments, the cCPP of Formula (3) can include those having one of the following sequences: hFf-Nal-GrGr; bhF-F-Nal-SRSR; bhF-F-Nal-GRGR; bhF-F-Nal-HRHR; bhF-f-Nal-GrGr; bhF-f-Nal-SRSR; bhF-f-Nal-SrSr; or bhFf-Nal-HrHr (where bhF – beta-homophenylalanine; hFf – homophenylalanine).

[0221] In embodiments, the cCPP of Formula (3) can include those having one of the following sequences: hFf-Nal-GrGrQ; βhF-F-Nal-SRSRQ; βhF-F-Nal-GRGRQ; βhF-F-Nal-HRHRQ; βhF-f-Nal-GrGrQ; βhF-f-Nal-SRSRQ; βhF-f-Nal-SrSrQ; or βhFf-Nal-HrHrQ.

Linker

[0222] The EEV can include one or more linker arms. The linker can link a cargo to the cCPP. The linker can link an EP to the cCPP. The linker can be attached to the side chain of an amino acid of the cCPP, and the cargo can be attached at a suitable position on the linker.

[0223] The linker can be any appropriate moiety which can conjugate a cCPP to one or more additional moieties, e.g., an exocyclic peptide (EP) and/or a cargo. Prior to conjugation (e.g., to the cCPP and/or one or more additional moieties), the linker has two or more functional groups, each of which is independently capable of forming a covalent bond (e.g., to the cCPP and/or one or more additional moieties). If the cargo is an oligonucleotide, the linker can be covalently bound to the 5' end of the cargo or the 3' end of the oligonucleotide cargo. The linker can be covalently bound to the 5' end of the oligonucleotide cargo. The linker can be covalently bound to the 3' end of the oligonucleotide cargo. If the cargo is a peptide, the linker can be covalently bound to the N-terminus or the C-terminus of the peptide cargo. The linker can be covalently bound to the backbone (e.g., somewhere in the middle and not at a terminus or termini) of the oligonucleotide or peptide cargo. The linker can be any appropriate moiety that conjugates a cCPP described herein to a therapeutic moiety such as an oligonucleotide, peptide or small molecule.

[0224] The linker can be covalently bound to cargo at any suitable location on the cargo. The linker can be covalently bound to the 3' end of oligonucleotide cargo or the 5' end of an oligonucleotide cargo. The linker can be covalently bound to the N-terminus or the C-terminus

of a peptide cargo. The linker can be covalently bound to the backbone of an oligonucleotide or a peptide cargo. The linker can be covalently bound to the cargo via a bonding group M′.

[0225] The linker can be bound to the side chain of an amino acid (AA_{SC}) on the cCPP, including for example, aspartic acid, glutamic acid, glutamine, asparagine, or lysine, or a modified side chain of glutamine or asparagine (e.g., a reduced side chain having an amino group). The linker can be bound to the side chain of lysine on the cCPP.

[0226] In embodiments, the linker can include one or more polyethylene glycol (PEG) components. Each (PEG) component can have, for example, from 0 to 12 repeat (PEG) units. A (PEG) unit refers to the group $-(CH_2CH_2O)-$ which can be repeated any number of times.

[0227] While not wishing to be bound by theory, it is believed that the linkers can influence efficacy and tolerability of the EEV-cargo conjugate, for example to increase efficacy. In embodiments, efficacy is increased without decreasing tolerability. In embodiments, one or more hydrophobic components (X) are added to a linker of the EEV. In embodiments, the length of the linker can be varied. The linker length can influence efficacy and tolerability. In some instances, as linker length decreases, efficacy increases and tolerability decreases; and as linker length increases, efficacy decreases and tolerability increases. In embodiments, increasing hydrophobicity of the linker can increase efficacy.

[0228] A hydrophobic component X can be added at one or more positions on the linker. For example, X can be present in a linker between the CPP and EP; or in a linker between the CPP and the cargo. In embodiments, X can be the bonding group between the CPP and EP. In embodiments, X is not the bonding group between the CPP and EP. In embodiments, X can be the bonding group between the CPP and cargo. In embodiments, X is not the bonding group between the CPP and cargo. X can form part of the backbone of the linker between the CPP and EP. X can form part of the backbone of the linker between the CPP and cargo. X can be appended to the linker. X can be appended to the side chain of a lysine residue in the linker. X can be appended to the linker between the CPP and EP. X can be appended to the linker between the CPP and cargo.

[0229] In embodiments, the hydrophobic component (X) in the linker can be aliphatic, alkene, alkyne, aromatic (e.g., carbocyclic or heteroaromatic), or a combination of aliphatic and aromatic.

[0230] X can be a D or L amino acid residue with a hydrophobic side chain. X can be a naturally occurring or a non-naturally occurring amino acid residue with a hydrophobic side chain. X can be an amino acid residue with an aromatic side chain. X can be an amino acid residue with a heteroaromatics side chain. X can be selected from phenylalanine, 3-(4',4-biphenyl)-L-alanine, tryptophan, tyrosine, valine, isoleucine, leucine, or histidine, or a combination thereof. X can be 2-naphtylalanine. X can be Nal. X can be d-Nal (nal). X can be 3-(4',4-biphenyl)-L-alanine. X can be Bip. X can be D-Bip (bip). X can be a C_4-C_8 alkyl hydrocarbon. X can be a C_6 alkyl hydrocarbon.

[0231] In embodiments, the hydrophobic component X comprises an optionally substituted alkyl group. The alkyl group can comprise a branched alkyl group. In embodiments, the alkyl group comprises a double bond. In embodiments, the alkyl group comprises an ethyl or propyl group. In embodiment, the alkyl group is a butyl, pentyl, or hexyl group. In embodiment, the alkyl group is -$CH_2(CH_2)_nCH_2$-, $CH_3(CH_2)_nCH_2$-, $C(H)C(CH_2)_nCH_2$-, $CH_3(CH_2)_nCH_2NH$-, $C(H)C(CH_2)_nCH_2NH$-, $CH_3(CH_2)_nCO$-, $CH_3(CH_2)_nCH_2O$-, $CH_3(CH_2)_nCH_2S$-, -$CH_2(CH_2)_nCH_2SH$-, -$OC(CH_2)_nCH_2$-, -$OC(CH_2)_nCH_2NH$-, -$OC(CH_2)_nCO$-, -$OC(CH_2)_nCH_2O$-, -$OC(CH_2)_nCH_2S$-, -$HNC(CH_2)_nCH_2$-, -$HNC(CH_2)_nCH_2NH$-, -$HNC(CH)_nCO$-, -$HNC(CH_2)_nCH_2O$-, -$HNC(CH_2)_nCH_2S$-, -$OCH_2(CH_2)_nCH_2$-, -$OCH_2(CH_2)_nCH_2NH$-, -$OCH_2(CH_2)_nCO$-, -$OCH_2(CH_2)_nCH_2O$-, -$OCH_2(CH_2)_nCH_2S$-, $NH_2(CH_2)_nCH_2$-, $SH(CH_2)_nCH_2$-, or N_3-$C(O)CH_2(CH_2)_nCH_2$-, group, wherein "n" is 4-34, inclusive.

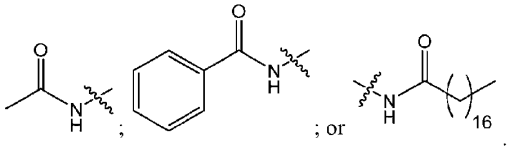
[0232] In embodiments, the hydrophobic component X can be an optionally substituted aromatic group. Aromatic groups can be carbocyclic heteroaromatic, monocyclic, bicyclic, or polycyclic. Carbocyclic aryl groups include benzene, naphthalene, phenanthrene, phenol, aniline, anthracene, and the like. Heteroaryl groups include, for example, pyrrole, furan, thiophene, imidazole, oxazole, thiazole, triazole, pyrazole, pyridine, pyrazine, pyridazine and pyrimidine, and the like.

[0233] In embodiments, the hydrophobic component X may comprise one or more hydrophobic amino acid residues. The amino acid residues can be D or L. The amino acid residues can be natural hydrophobic amino acid or non-natural hydrophobic amino acid. In embodiments, the hydrophobic amino acid residues comprise a substituted or unsubstituted alkyl, alkenyl, alkynyl, aryl, or aralkyl side chain wherein the alkyl, alkenyl, and alkynyl side chain includes at most one heteroatom for every six carbon atoms. X can be a D or L amino acid residue with a hydrophobic side chain. X

can be a naturally occurring or a non-naturally occurring amino acid residue with a hydrophobic side chain. X can be an amino acid residue with an aromatic side chain. X can be an amino acid residue with a heteroaromatic side chain. The hydrophobic component X can comprise an amino acid residue with a hydrophobic side chain that can be selected from valine, proline, alanine, leucine, isoleucine, phenylalanine, cysteine, glycine, histidine, methionine, and tryptophan. The amino acid residue with a hydrophobic side chain can be selected from the group consisting of glycine, phenylglycine, alanine, valine, leucine, isoleucine, norleucine, phenylalanine, tryptophan, naphthylalanine, proline, and combinations thereof, wherein the aromatic side chains on phenylglycine, phenylalanine, tryptophan, or naphthylalanine are each optionally substituted. The amino acid residue with a hydrophobic side chain can be naphthylalanine, lysine-naphtylalanine, biphenylalanine, or lysine-biphenylalanine. X can be an aliphatic hydrocarbon. X can be a C_4-C_{12} aliphatic hydrocarbon. X can be a C_4-C_8 aliphatic hydrocarbon. X can be a C_6 aliphatic hydrocarbon. X can be selected from phenylalanine, 3-(4',4-biphenyl)-L-alanine, tryptophan, tyrosine, valine, isoleucine, leucine, or histidine, or a combination thereof. X can be amino acid selected from tryptophan, tyrosine, isoleucine, leucine, histidine, phenylalanine, or a combination thereof.

[0234] X can be 2-naphthylalanine. X can be Nal. X can be d-Nal (nal). X can be 3-(4',4-biphenyl)-L-alanine (Bip). X can be 3-(4',4-biphenyl)-D-alanine (d-Bip or bip).

[0235] In embodiments, the hydrophobic component X can include amino acid subunits that have been modified. For example, the amino terminal or carboxy terminal amino acid subunit may be modified. Such modifications include caping the amino terminus or the carboxy terminus with a group making the amino acid subunit more hydrophobic. For example, the amino terminus may be capped with an acyl group (e.g., acetyl, benzoyl, or stearoyl moiety). For example, the amino terminus of the of the modified amino acid subunit can be depicted as follows:



[0236] In embodiments, X can be appended to the backbone of the linker via lysine or a $SH(CH_2)_nCH_2$- , or N_3-$C(O)CH_2(CH_2)_nCH_2$-.

[0237] In embodiments, the size of an aromatic or heteroaromatic group present in the hydrophobic component X may be selected to improve cytosolic delivery efficiency of the

Cargo. While not wishing to be bound by theory, it is believed that the presence of a hydrophobic component X in the linker of the EEV may improve cytosolic delivery efficiency of the Cargo as compared to an otherwise identical EEV that does not include the hydrophobic component X.

[0238] The linker can comprise hydrocarbon linker.

[0239] The linker can comprise a cleavage site. The cleavage site can be a disulfide, or caspase-cleavage site (e.g., Val-Cit-*p*-aminobenzyloxycarbonlyl, also referred to as Val-Cit-PABC).

[0240] The linker can comprise: (i) one or more D or L amino acids, each of which is optionally substituted; (ii) one or more -(U^1-J-U^2)i′- subunits, wherein each of U^1 and U^2, at each instance, are independently selected from alkylene, each J is independently C, NU^3, -NU^3C(O)-, S, and O, wherein U^3 is independently selected from H, alkyl, alkenyl, alkynyl, carbocyclyl, and heterocyclyl, each of which is optionally substituted, and i′ is an integer from 1 to 50.

[0241] The linker can comprise one or more D or L amino acids and/or -$(OCH_2CH_2)_i$- i is an integer from 0 to 60; or combinations thereof. i can be an integer from 0 to 12. i can be 0, 2, 4, 8, or 12. i can be 0. i can be 2. i can be 4. i can be 8. i can be 12. "-$(OCH_2CH_2)_i$" can also be referred to as polyethylene glycol (PEG).

[0242] The linker can comprise one or more PEG components -$(OCH_2CH_2)_i$-, wherein i is an integer from 0 to 12. The PEG components can be represented as (PEG)x′, (PEG)z′, and/or (PEG)z″. The linker can comprise one or more hydrophobic components (X^o′) and (X^o″). The hydrophobic component can be appended to a side chain of an amino acid in the linker. The hydrophobic component (X^o′) can be appended to a side chain of lysine in linker (K(X^o′). The linker can comprise a bonding group (M).

[0243] The linker can comprise (i) a β alanine residue and lysine residue; (ii) -(J-U^1)$_{i'}$; or (iii) a combination thereof. Each U^1 can independently be alkylene, alkenylene, alkynylene, carbocyclyl, or heterocyclyl, each J is independently C, NU^3, -NU^3C(O)-, S, or O, wherein U^3 is H, alkyl, alkenyl, alkynyl, carbocyclyl, or heterocyclyl, each of which is optionally substituted, and z″ can be an integer from 1 to 50. Each U^1 can be alkylene and each J can be O.

[0244] The linker can comprise (i) residues of β-alanine, glycine, lysine, 4-aminobutyric acid, 5-aminopentanoic acid, 6-aminohexanoic acid or combinations thereof; and (ii) -(U^1-J)$_{i'}$ or -(J-U^1)$_{i'}$. Each U^1 can independently be alkylene, alkenylene, alkynylene, carbocyclyl, or heterocyclyl, each J is independently C, NU^3, -NU^3C(O)-, S, or O, wherein U^3 is H, alkyl, alkenyl, alkynyl,

carbocyclyl, or heterocyclyl, each of which is optionally substituted, and i′ can be an integer from 1 to 50. Each U^1 can be alkylene and each J can be O. The linker can comprise glycine, beta-alanine, 4-aminobutyric acid, 5-aminopentanoic acid, 6-aminohexanoic acid, or a combination thereof.

[0245] The linker can also incorporate a cleavage site, including a disulfide [NH_2-$(CH_2O)_n$-S-S-$(CH_2O)_n$-COOH], or caspase-cleavage site (Val-Cit-PABC).

[0246] The linker can include a residue of glycine or β-alanine.

[0247] The linker can be bivalent and link the cCPP to a cargo. The linker can be bivalent and link the cCPP to an exocyclic peptide (EP).

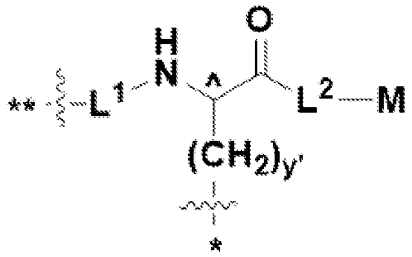
[0248] The linker can be trivalent and link the cCPP to a cargo and to an EP.

[0249] The linker can be a bivalent or trivalent C_1-C_{50} alkylene, wherein 1-25 methylene groups are optionally and independently replaced by -N(H)-, -N(C_1-C_4 alkyl)-, -N(cycloalkyl)-, -O-, -C(O)-, -C(O)O-, -S-, -S(O)-, -$S(O)_2$-, -$S(O)_2$N(C_1-C_4 alkyl)-, -$S(O)_2$N(cycloalkyl)-, -N(H)C(O)-, -N(C_1-C_4 alkyl)C(O)-, -N(cycloalkyl)C(O)-, -C(O)N(H)-, -C(O)N(C_1-C_4 alkyl), -C(O)N(cycloalkyl), aryl, heterocyclyl, heteroaryl, cycloalkyl, or cycloalkenyl. The linker can be a bivalent or trivalent C_1-C_{50} alkylene, wherein 1-25 methylene groups are optionally and independently replaced by -N(H)-, -O-, -C(O)N(H)-, or a combination thereof.

[0250] The cargo can be coupled to the glutamic acid of the cyclic peptide, which converts the glutamic acid to glutamine. The linker (L) can couple the cargo to the glutamine/glutamic acid of the cyclic peptide. In embodiments, a linker (L) is covalently bound to the backbone of the cargo.

[0251] The linker can be a trivalent linker with the structure shown in Formula (A′):

Formula (A′)



(A′);

wherein:

** is a point of attachment to a linear exocyclic peptide (EP) as defined herein;

* is a point of attachment to a cell penetrating peptide (CPP) as defined herein;

L^1 and L^2, are, independently, a linker arm;

^ indicates L- or D-stereochemistry;

y′ is an integer from 1 to 5; and

M comprises a reactive handle.

[0252] In embodiments, L^1 and L^2, are each, independently, absent, or comprise hydrocarbon component, a polyethylene glycol (PEG) component, a hydrophobic component (X), an amino acid (AA) component comprising one or more amino acid residues, or a combination thereof. In embodiments, L^1 comprises a polyethylene glycol (PEG)x′ component, wherein x′ is an integer from 1-12. In embodiments, L^2 comprises a polyethylene glycol (PEG)z′ component, wherein z′ is an integer from 1-12. In embodiments, L^2 comprises a hydrophobic component ($X^{o\prime}$).

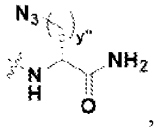
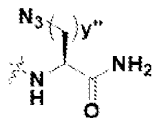
[0253] In embodiments, L^1, L^2 or cCPP comprise one or more hydrophobic component(s) (X).

[0254] In embodiments, L^1 comprises a PEG component, and L^2 comprises a PEG component, a hydrophobic component (X), or a combination thereof.

[0255] In embodiments, L^1 is absent. In embodiments, when x′ is 0, L^1 is absent. In embodiments, when L^1 is absent, the lysine linked to the cyclic peptide is acylated (Ac). In embodiments, L^1 is absent, and L^2 comprises a PEG component (PEG)z′, a hydrophobic component ($X^{o\prime}$), or a combination thereof.

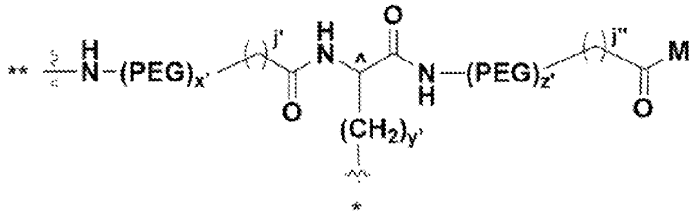
[0256] In embodiments, L^2 comprises (PEG)z′, a hydrophobic component ($X^{o\prime}$), or a combination thereof. In embodiments, L^2 comprises a PEG component (PEG)z′. In embodiments, L^2 comprises a hydrophobic component ($X^{o\prime}$). In embodiments, L^2 comprises (PEG)z′ and a hydrophobic component ($X^{o\prime}$). In embodiments, the hydrophobic component ($X^{o\prime}$) is appended from a side chain of a lysine amino acid residue.

[0257] M comprises a functional group (sometimes referred to herein as a reactive handle) that can react with a corresponding functional group on a cargo to form an EEV-cargo conjugate or a linker-cargo conjugate. Following conjugation, the EEV-cargo or linker-cargo conjugate includes a bonding group M′, which is the reaction product of the two functional groups.

[0258] In embodiments, M can be -OH (e.g., as part of the C terminus of an amino acid). In embodiments, M comprises -OH,  , or  , herein y″ is an integer from 1-4. y″ can be 1. y″ can be 2. y″ can be 3. y″ can be 4.

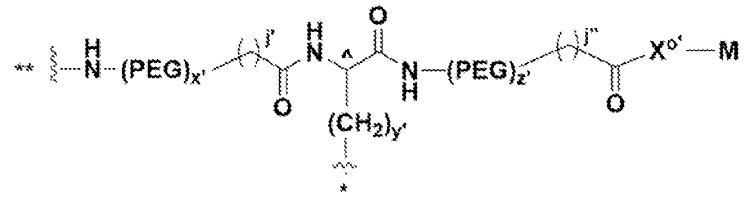
[0259] In embodiments, the linker Formula (A′) can have a Formula selected from Formula (B′), (C′), or (D′):

Formula (B′):



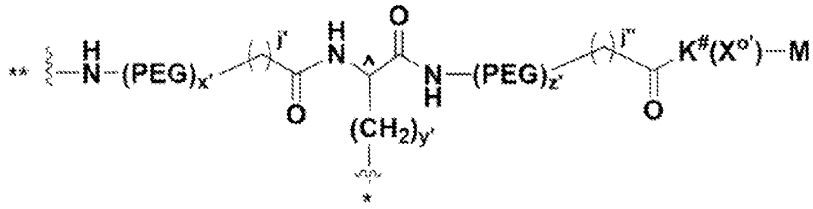
(B′);

Formula (C′):



(C′); or

Formula (D′):



(D′);

x′ is an integer from 1 to 12;

j′ is 0, 1, or 2, wherein j′ is 0 when x′ is 0;

z′ is an integer from 0 to 12;

j″ is 0, 1, or 2, wherein j″ is 0 when z′ is 0;

$X^{o\prime}$ is a hydrophobic component; and

$K^{\#}$ is D-lysine or L-lysine residue.

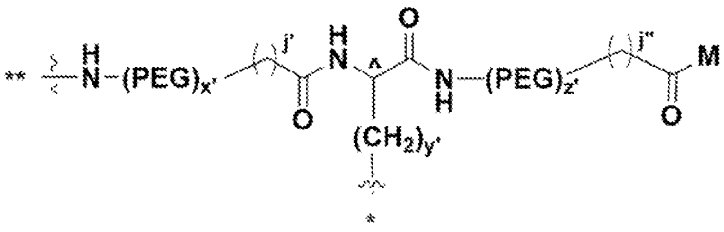
[0260] With the exception of glycine (G), which has H as a side chain, amino acids each have an asymmetric or chiral α-carbon atom and exists in two enantiomeric forms, designated "D-" and "L-". The "L-" isomers are most commonly found in naturally occurring proteins. While not wishing to be bound by theory, it is believed that the chirality of the amino acids in a cell-penetrating peptide (CPP) or endosomal escape vehicle (EEV) can impact cytosolic delivery efficiency, as well as toxicity. Disclosed herein are EEV in which the stereochemistry of the amino acids within the peptide sequences of the EEV has been modified to influence cytosolic delivery efficiency and/or reduce toxicity.

[0261] In embodiments, the linker comprises a structure selected from Formula (B′), (C′), or (D′), wherein EP comprises all D-amino acids; and cCPP comprises D-amino acids, achiral amino acids and AAsc, wherein AAsc is an amino acid side chain and AA_{SC} conjugates cCPP to the linker.

[0262] In embodiments, the cCPP comprises at least 2 D-amino acid residues with a hydrophobic side chain. In embodiments, the cCPP comprises 2 D-amino acid residues with a hydrophobic side chain. In embodiments, the cCPP comprises 3 D-amino acid residues with a hydrophobic side chain. In embodiments, the cCPP comprises at least 2 D-arginine amino acid residues. In embodiments, the cCPP comprises 2 D-arginine amino acid residues. In embodiments, the cCPP comprises 3 D-arginine amino acid residues. In embodiments, the cCPP comprises 4 D-arginine amino acid residues. In embodiments, the cCPP comprises 5 D-arginine amino acid residues. In embodiments, the cCPP comprises 6 D-arginine amino acid residues. In embodiments, the cCPP comprises at least 2 D-phenylalanine residues. In embodiments, the cCPP comprises 2 D-phenylalanine residues. In embodiments, the cCPP comprises 3 D-phenylalanine residues. In embodiments, the cCPP comprises at least 2 glycine residues. In embodiments, the cCPP comprises 2 glycine residues. In embodiments, the cCPP comprises 3 glycine residues. In embodiments, the cCPP comprises 4 glycine residues. In embodiments, the cCPP comprises 5 glycine residues. In embodiments, the cCPP comprises 6 glycine residues. In embodiments, the cCPP comprises at least 2 D-amino acid residues with a hydrophobic side chain and at least 2 D-arginine residues. In embodiments, the cCPP comprises at least 2 D-phenylalanine residues and at least 2 D-arginine residues. In embodiments, AA_{SC} is a side chain of L-glutamine.

[0263] The linker can be of Formula (B′):

Formula (B'):

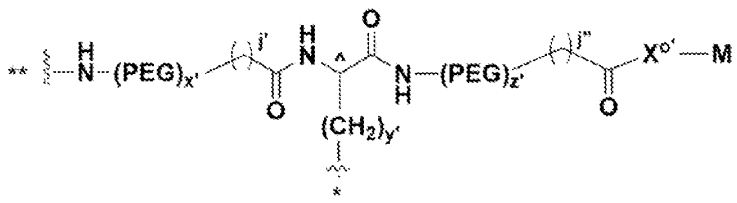


(B′);

wherein **, *, x′, j′, ^, y′, z′, j″, and M are as defined herein.

[0264] The linker can be of Formula (C′):

Formula (C'):

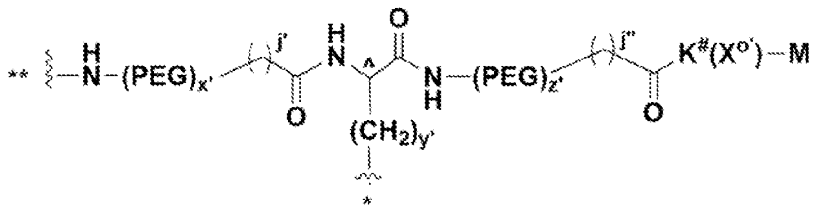


(C′);

wherein **, *, x′, j′, ^, y′, z′, j″, $X^{o'}$, and M are as defined herein.

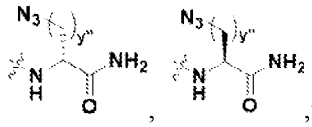
[0265] The linker can be of Formula (D′):

Formula (D'):



(D′);

wherein **, *, x′, j′, ^, y′, z′, j″, $X^{o'}$, $K^{\#}$, and M are as defined herein.

[0266] In embodiments, M comprises -OH,  ,wherein y″ is an integer from 1-4. y″ can be 1. y″ can be 2. y″ can be 3. y″ can be 4.

[0267] x′ can be an integer from 0 to 12. x′ can be an integer from 1 to 12. x′ can be an integer from 2 to 12. x′ can be 0, 2, 4, 8, or 12. x′ can be 0 or 2. x′ can be 0, 2, or 12. x′ can be 0 or 12. x′ can be 2 or 12. x′ can be 0. x′ can be 1. x′ can be 2. x′ can be 3. x′ can be 4. x′ can be 5. x′ can be 6. x′ can be 7. x′ can be 8. x′ can be 9. x′ can be 10. x′ can be 11. x′ can be 12.

[0268] j′ can be 0, 1, or 2. j′ can be 1. j′ can be 2. It is understood that j′ is 0 when x′ is 0.

[0269] ^ can indicate L-stereochemistry. ^ can indicate D-stereochemistry.

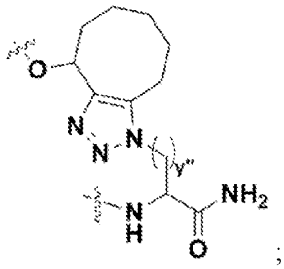
[0270] y′ can be an integer from 1 to 5, e.g., 1, 2, 3, 4, or 5, inclusive of all ranges and subranges therebetween. y′ can be an integer from 2 to 5. y′ can be an integer from 3 to 5. y′ can be 3 or 4. y′ can be 4 or 5. y′ can be 1. y′ can be 2. y′ can be 3. y can be 4. y′ can be 5.

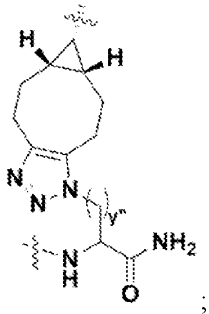
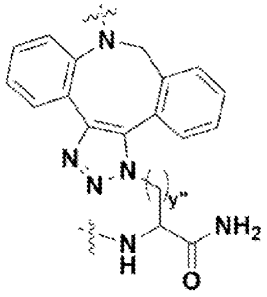
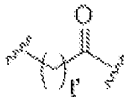
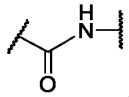
[0271] z′ can be an integer from 0 to 12. z′ can be an integer from 1 to 12. z′ can be an integer from 2 to 12. z′ can be 0, 2, 4, 8, or 12. z′ can be 2 or 12. z′ can be 0, 2, or 12. z′ can be 0 or 12. z′ can be 2 or 12. z′ can be 0. z′ can be 1. z′ can be 2. z′ can be 3. z′ can be 4. z′ can be 5. z′ can be 6. z′ can be 7. z′ can be 8. z′ can be 9. z′ can be 10. z′ can be 11. z′ can be 12.

[0272] j″ can be 0, 1, or 2. j″ can be 0. j″ can be 1. j″ can be 2. It is understood that when z′ is 0, j″ is 0.

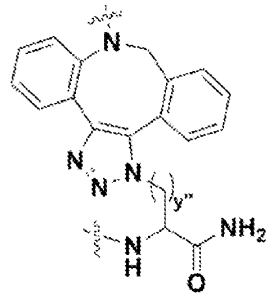
[0273] $K^{\#}$ indicates an L-lysine residue. $K^{\#}$ indicates a D-lysine residue.

[0274] In embodiments, ^ indicates L-stereochemistry. In embodiments, ^ indicates D-stereochemistry.

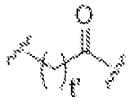
[0275] In embodiments, M′ comprises -NH-, -C(O)-, -O-,  ;

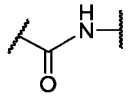
 ; or  ,  , or  , wherein y″ is an

integer from 1 to 4 and t′ is an integer from 0 to 10. In embodiments, M′ comprises -C(O)-.



, wherein y″ is an integer from 1 to 4. In embodiments, M′ comprises -



, wherein t′ is an integer from 0 to 10. In embodiments, M′ comprises  .

[0276] AAsc can be a side chain or terminus of an amino acid residue on the cCPP. Non-limiting examples of AAsc include aspartic acid, glutamic acid, glutamine, asparagine, or lysine, or a modified side chain of glutamine or asparagine (e.g., a reduced side chain having an amino group). In embodiments, the AAsc is the side chain of a glutamine residue. In embodiments, the AAsc is the side chain of a glutamic acid residue. In embodiments when the AA_{SC} is the side chain of glutamine, the carboxamide amide nitrogen of the glutamine side chain forms a bond with the $-(CH_2)_{y'}-$ group.

[0277] y″ can be an integer from 0 to 10, e.g., 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, and 10. y″ can be 0, 1, 2, 3, or 4. y″ can be 0. y″ can be 1. y″ can be 2. y″ can be 3. y″ can be 4.

[0278] $X^{o\prime}$ can be an aliphatic hydrocarbon. $X^{o\prime}$ can be a C_4-C_{12} aliphatic hydrocarbon. $X^{o\prime}$ can be a C_4-C_8 aliphatic hydrocarbon. $X^{o\prime}$ can be a C_6 aliphatic hydrocarbon. $X^{o\prime}$ can be selected from phenylalanine, 3-(4′,4-biphenyl)-L-alanine, tryptophan, tyrosine, valine, isoleucine, leucine, or histidine, or any combinations thereof. $X^{o\prime}$ can be amino acid residue selected from tryptophan, tyrosine, isoleucine, leucine, histidine, phenylalanine, or any combinations thereof.

[0279] $X^{o\prime}$ can be a D- or L-amino acid residue with a hydrophobic side chain. $X^{o\prime}$ can be a naturally occurring or a non-naturally occurring amino acid residue with a hydrophobic side chain. $X^{o\prime}$ can be an amino acid residue with an aromatic side chain. $X^{o\prime}$ can be an amino acid residue with a heteroaromatic side chain. $X^{o\prime}$ can be selected from phenylalanine; 2-naphtylalanine (Nal or nal); 3-(4′,4-biphenyl)-L-alanine (Bip or bip); tryptophan; tyrosine; valine; isoleucine; leucine; or histidine, or any combinations thereof. $X^{o\prime}$ can be 2-naphtylalanine

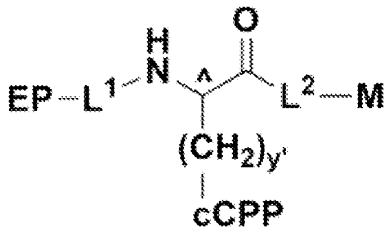
(Nal or nal). $X^{o\prime}$ can be L-2-naphtylalanine (L-Nal or Nal). $X^{o\prime}$ can be D-2-naphtylalanine (d-nal or nal). $X^{o\prime}$ can be 3-(4′,4-biphenyl)-L-alanine. $X^{o\prime}$ can be 3-(4′,4-biphenyl)-L-alanine (L-Bip or Bip). $X^{o\prime}$ can be 3-(4′,4-biphenyl)-D-alanine (d-bip or bip). $X^{o\prime}$ can be a C_4-C_8 alkyl or dialkyl hydrocarbon. $X^{o\prime}$ can be a C_6 alkyl or dialkyl hydrocarbon.

Endosomal Escape Vehicles (EEVs)

[0280] EEVs comprising a cyclic cell penetrating peptide (cCPP) and one or more linkers are provided. In embodiments, the EEV includes an exocyclic peptide (EP). In embodiments, the EP is absent.

[0281] An EEV can comprise the structure of Formula (A):

Formula (A):



(A);

wherein:

EP is a linear exocyclic peptide;

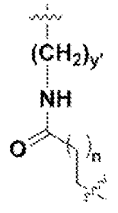
cCPP is a cell penetrating peptide;

L^1 and L^2, are, independently, a linker arm;

^ indicates L- or D-stereochemistry;

y′ is an integer from 1 to 5; and

M comprises a reactive handle.

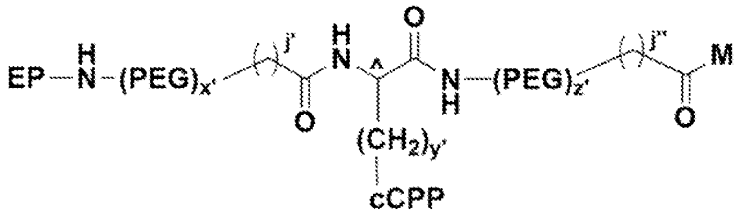
[0282] In embodiments, the cCPP is linked to the -$(CH_2)_{y'}$- group through the AAsc of the cCPP. In embodiments, the cCPP is linked to the -$(CH_2)_{y'}$- group through the AAsc of the cCPP such that the connection is of the structure 

wherein n is an integer from 1 to 5. In embodiments, n is 1. In embodiments, n is 2. In embodiments, n is 3. In embodiment, n is 4. In

embodiments, n is 5. In embodiments when n is 1, the AAsc is the side chain of a glutamine residue. In embodiments when n is 1, the AAsc is the side chain of a glutamic acid residue. In embodiments when the AA_{SC} is the side chain of glutamine, the carboxamide amide nitrogen of the glutamine side chain forms a bond with the $-(CH_2)_{y'}-$ group.

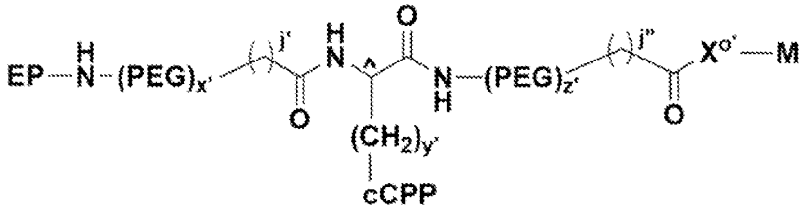
[0283] In embodiments, the EEV of Formula (A) comprises a structure selected from Formula (B), (C), or (D):

Formula (B):



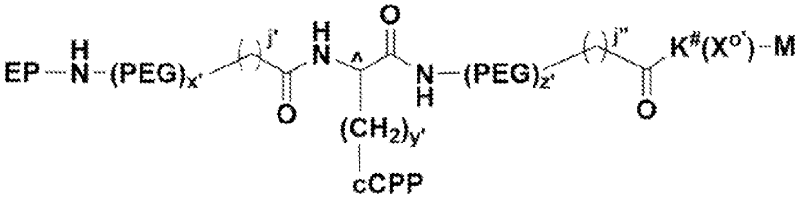
(B);

Formula (C):



(C); or

Formula (D):



(D);

wherein:

EP is a linear exocyclic peptide;

cCPP is a cyclic cell penetrating peptide;

x′ is an integer from 0 to 12;

j′ is 0, 1, or 2, wherein j′ is 0 when x′ is 0;

^ indicates L- or D-stereochemistry;

y′ is an integer from 1 to 5;

z′ is an integer from 0 to 12;

j″ is 0, 1, or 2, wherein j″ is 0 when z′ is 0;

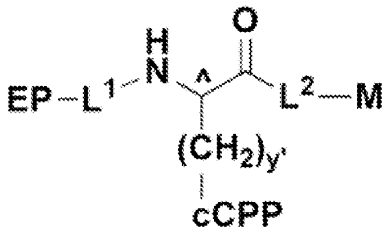
$X^{o\prime}$ comprises a hydrophobic component;

$K^{\#}$ is D-lysine or L-lysine residue; and

M comprises a reactive handle.

[0284] An EEV can comprise the structure of Formula (A):

Formula (A):



(A);

wherein

EP is a linear exocyclic peptide;

cCPP is a cell penetrating peptide;

L^1 and L^2, are, independently, a linker arm;

^ indicates D-stereochemistry;

y′ is an integer from 1 to 5;

M comprises a reactive handle; and

wherein EP comprises all D-amino acids; and cCPP comprises D-amino acids, achiral amino acids and AAsc, wherein AAsc is an amino acid side chain and AAsc conjugates cCPP to the linker.

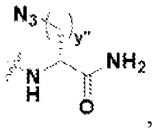
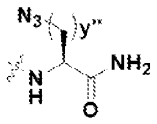
[0285] In embodiments, the EEV of Formula (A) comprises a structure selected from Formula (B), Formula (C), or Formula (D), wherein wherein EP comprises all D-amino acids; and cCPP comprises D-amino acids, achiral amino acids and AAsc, wherein AAsc is an amino acid side chain and AAsc conjugates cCPP to the linker.

[0286] X can be a D or L amino acid residue with a hydrophobic side chain. X can be a naturally occurring or a non-naturally occurring amino acid residue with a hydrophobic side chain. X can be an amino acid residue with an aromatic side chain. X can be an amino acid residue with a

heteroaromatic side chain. X can be 2-naphtylalanine. X can be Nal. X can be d-Nal (nal). X can be 3-(4-biphenyl)-D-alanine. X can be Bip. X can be bip. X can be a C_4-C_8 alkyl hydrocarbon. X can be a C_6 alkyl hydrocarbon. X can be amino acid selected from tryptophan, tyrosine, isoleucine, leucine, histidine, phenylalanine, or a combination thereof.

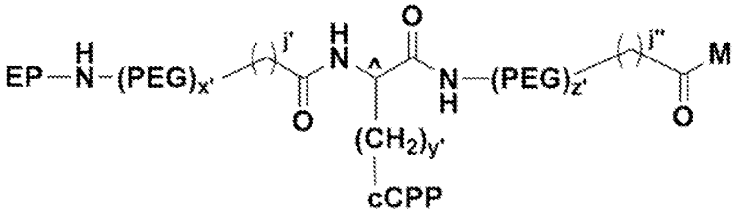
[0287] In embodiments, L^1 and L^2, when present, are each, independently, a hydrocarbon linker (e.g., NR_hH-(CH_2)n-COOH), a PEG component (e.g., NR_hH-$(CH_2O)_n$-COOH, wherein R_h is H, methyl or ethyl), a linker comprising a hydrophobic component, one or more amino acid residue, or a combination thereof.

[0288] In embodiments, AAsc is a side chain of glutamine. In embodiments, AAsc is a side chain of L-glutamine. In embodiments, AAsc is a side chain of D-glutamine.

[0289] In embodiments, M comprises -OH or  , or  , wherein y″ is an integer from 1 to 4.

[0290] In embodiments, the EEV comprises Formula (B):

Formula (B):

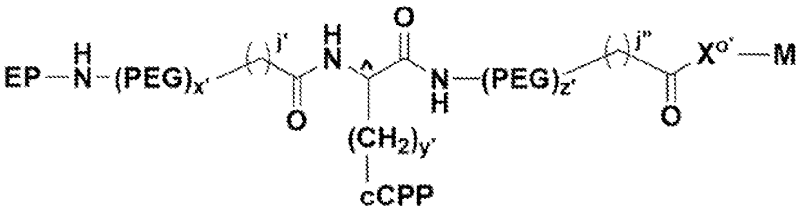


(B);

wherein EP, cCPP, x′, j′, ^, y′, z′, j″, and M are as defined herein.

[0291] In embodiments, the EEV comprises Formula (C):

Formula (C):

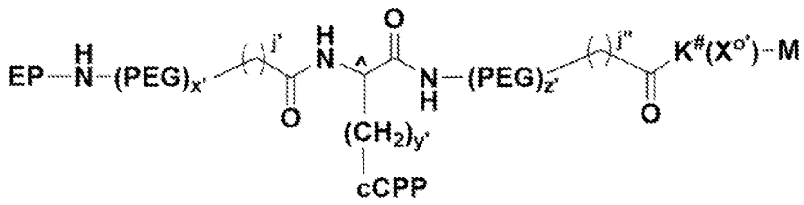


(C);

wherein EP, cCPP, x′, j′, ^, y′, z′, j″, $X^{o'}$, and M are as defined herein.

[0292] In embodiments, the EEV comprises Formula (D):

Formula (D):

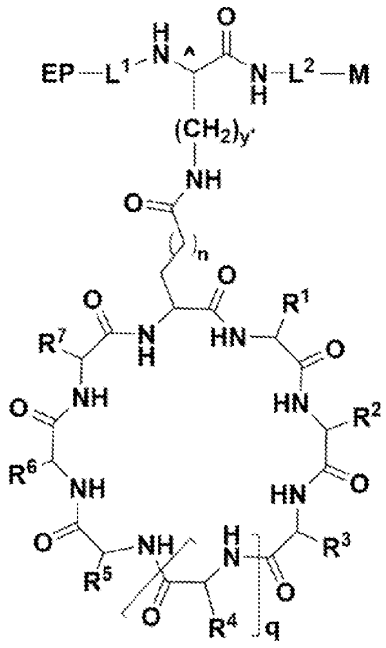


(D);

wherein EP, cCPP, x′, j′, ^, y′, z′, j″, X^{o}′, $K^{\#}$, and M are as defined herein.

[0293] In embodiments, the EEV can comprise Formula (A-2):

Formula (A-2):



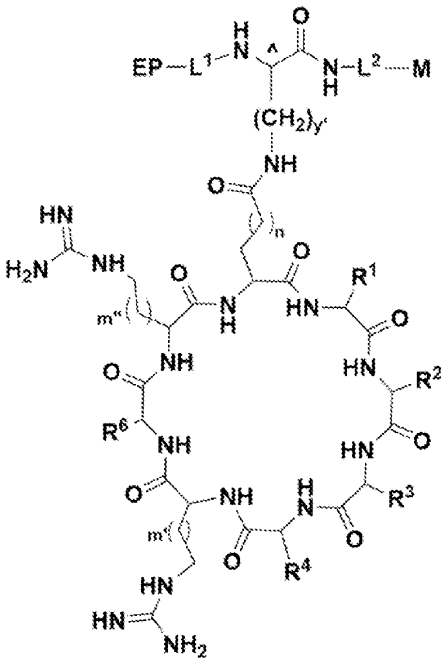
(A-2)

wherein EP, L^1, L^2, M, y′, ^, n, q, R_1, R_2, R_3, R_4, R_5, R_6, and R_7 are as defined herein.

[0294] In embodiments, the EEV of Formula (A-2) can comprise a linker of Formula (B′). In embodiments, the EEV of Formula (A-2) can comprise a linker of Formula (C′). In embodiments, the EEV of Formula (A-2) can comprise a linker of Formula (D′).

[0295] In embodiments, the EEV can comprise Formula (A-2a):

Formula (A-2a):



(A-2a)

wherein EP, L^1, L^2, M, y ′, ^, n, m′, m″, R^1, R^2, R^3, R^4, and R^6, are as defined herein.

[0296] In embodiments, the EEV of Formula (A-2a) can comprise a linker of Formula (B′). In embodiments, the EEV of Formula (A-2a) can comprise a linker of Formula (C′). In embodiments, the EEV of Formula (A-2a) can comprise a linker of Formula (D′).

[0297] In embodiments, the EEV can have a structure shown in Formula (B), wherein x′ is 0 and z′ is 12. In embodiments, the EEV can have a sequnce shown in **Table 2a**:

***Table 2a**: EEV Sequences*

Ac-PKKKRKV-K(cyclo[Ff-Nal-RrRrQ])-PEG_{12}-K(N_3)-NH_2
Ac-PKKKRKV-K(cyclo[Ff-Nal-GrGrQ])-PEG_{12}-K(N_3)-NH_2

[0298] In embodiments, the EEV can have a structure shown in Formula (B), wherein x′ is 2 and z′ is 12. In embodiments, the EEV can have a sequnce shown in **Table 2b**:

***Table 2b**: EEV Sequences*

Ac-PKKKRKV-PEG_2-K(cyclo[Ff-Nal-Cit-r-Cit-r-Q])-PEG_{12}-K(N_3)-NH_2
Ac-PKKKRKV-PEG_2-K(cyclo[Ff-Nal-GrGrQ])-PEG_{12}-OH
Ac-PKKKRKV-PEG_2-K(cyclo[FGFGRGRQ])-PEG_{12}-OH
Ac-PKKKRKV-PEG_2-K(cyclo[GfFGrGrQ])-PEG_{12}-OH

[0299] In embodiments, the EEV can have a structure shown in Formula (B), wherein x′ is 2 and z′ is 2. In embodiments, the EEV can have a sequnce shown in **Table 2c**:

***Table 2c**: EEV Sequences*

Ac-PKKKRKV-PEG_2-K(cyclo[FGFGRGRQ])-PEG_2-K(N_3)-NH_2
Ac-PKKKRKV-PEG_2-K(cyclo[GfFGrGrQ])-PEG_2-K(N_3)-NH_2
Ac-PKKKRKV-PEG_2-K(cyclo[FfFGRGRQ])-PEG_2-K(N_3)-NH_2

[0300] In embodiments, the EEV can have a structure shown in Formula (B), wherein x′ is 2 and z′ is 12. In embodiments, the EEV can have a sequnce shown in **Table 3a**:

***Table 3a**: EEV Sequences*

Ac-PKKKRKV-PEG_2-K(cyclo[βhF-F-Nal-SRSRQ])-PEG_{12}-OH

[0301] In embodiments, the EEV can have a structure shown in Formula (D), wherein x′ is 2 and z′ is 12. In embodiments, the EEV can have a sequnce shown in **Table 3b**:

***Table 3b**: EEV Sequences*

Ac-PKKKRKV-PEG_2-K(cyclo[FGFGRGRQ])-PEG_{12}-K(Bip)-OH
Ac-PKKKRKV-PEG_2-K(cyclo[FGFGRGRQ])-PEG_{12}-K(Nal)-OH

[0302] In embodiments, the EEV can have a structure shown in Formula (C), wherein x′ is 2 and z′ is 12. In embodiments, the EEV can have a sequnce shown in **Table 3c**:

***Table 3c**: EEV Sequences*

Ac-PKKKRKV-PEG_2-K(cyclo[FGFGRGRQ])-PEG_{12}-Nal-OH
Ac-PKKKRKV-PEG_2-K(cyclo[FGFGRGRQ])-PEG_{12}-Bip-OH

[0303] In embodiments, the EEV can be selected from **Table 4**, where the cCPP has the formula: FGFGRGRQ.

***Table 4**: Endosomal Escape Vehicle with cCPP of Formula FGFGRGRQ*

Ac-PKKKRKV-PEG_2-K(cyclo[FGFGRGRQ])-PEG_2-K(N_3)-NH_2
Ac-PKKKRKV-PEG_2-K(cyclo[FGFGRGRQ])-PEG_{12}-K(N_3)-NH_2
Ac-PKKKRKV-PEG_2-K(cyclo[FGFGRGRQ])-PEG_2-OH
Ac-PKKKRKV-PEG_2-K(cyclo[FGFGRGRQ])-PEG_{12}-OH

[0304] In embodiments, the EEV can be selected from **Table 5**, where the cCPP has the formula: GfFGrGrQ.

***Table 5**: Endosomal Escape Vehicle with cCPP of Formula GfFGrGrQ*

Ac-PKKKRKV-PEG_2-K(cyclo[GfFGrGrQ])-PEG_2-K(N_3)-NH_2
Ac-PKKKRKV-PEG_2-K(cyclo[GfFGrGrQ])-PEG_{12}-K(N_3)-NH_2
Ac-PKKKRKV-PEG_2-K(cyclo[GfFGrGrQ])-PEG_2-OH
Ac-PKKKRKV-PEG_2-K(cyclo[GfFGrGrQ])-PEG_{12}-OH

[0305] In embodiments, the EEV construct can be selected from **Table 6**, where the cCPP has the formula: FfFGRGRQ.

***Table 6**: Endosomal Escape Vehicle with cCPP of Formula FfFGRGRQ*

Ac-PKKKRKV-PEG_2-K(cyclo[FfFGRGRQ])-PEG_2-K(N_3)-NH_2
Ac-PKKKRKV-PEG_2-K(cyclo[FfFGRGRQ])-PEG_{12}-K(N_3)-NH_2
Ac-PKKKRKV-PEG_2-K(cyclo[FfFGRGRQ])-PEG_2-OH
Ac-PKKKRKV-PEG_2-K(cyclo[FfFGRGRQ])-PEG_{12}-OH

[0306] In embodiments, the EEV can be selected from **Table 7**, where the cCPP has the formula: Ff-Nal-GrGrQ.

***Table 7**: Endosomal Escape Vehicle with cCPP of Formula Ff-Nal-GrGrQ*

Ac-PKKKRKV-PEG_2-K(cyclo[Ff-Nal-GrGrQ])-PEG_2-K(N_3)-NH_2
Ac-PKKKRKV-PEG_2-K(cyclo[Ff-Nal-GrGrQ])-PEG_{12}-K(N_3)-NH_2
Ac-PKKKRKV-PEG_2-K(cyclo[Ff-Nal-GrGrQ])-PEG_2-OH
Ac-PKKKRKV-PEG_2-K(cyclo[Ff-Nal-GrGrQ])-PEG_{12}-OH

[0307] In embodiments, the EEV can be selected from **Table 8A**, where the linker includes a hydrophobic group.

***Table 8A**: Endosomal Escape Vehicle with cCPP Formulas Containing a Hydrophobic Group*

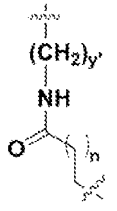
Ac-PKKKRKV-PEG_2-K(cyclo[FGFGRGRQ])-PEG_{12}-K(Bip)-OH
Ac-PKKKRKV-PEG_2-K(cyclo[FGFGRGRQ])-PEG_{12}-K(Nal)-OH
Ac-PKKKRKV-PEG_2-K(cyclo[FGFGRGRQ])-PEG_{12}-Nal-OH
Ac-PKKKRKV-PEG_2-K(cyclo[FGFGRGRQ])-PEG_{12}-Bip-OH

[0308] In embodiments, the EEV can be selected from **Table 8B**, where the cCPP includes citrulline (where Cit = L-citrulline and cit = D-citrulline).

***Table 8B**: Endosomal Escape Vehicle with cCPP Formulas Containing Citrulline*

Ac-PKKKRKV-K(cyclo[Ff-Nal-R-r-Cit-rQ])-PEG_{12}-K(N_3)-NH_2
Ac-PKKKRKV-K(cyclo[Ff-Nal-Cit-r-R-rQ])-PEG_{12}-K(N_3)-NH_2
Ac-PKKKRKV-K(cyclo[Ff-Nal-R-cit-R-cit-Q])-PEG_{12}-K(N_3)-NH_2
Ac-PKKKRKV-PEG_2-K(cyclo[Ff-Nal-Cit-r-Cit-rQ])-PEG_2-k(N_3)-NH_2
Ac-PKKKRKV-PEG_2-K(cyclo[Ff-Nal-Cit-r-Cit-rQ])-PEG_4-k(N_3)-NH_2
Ac-PKKKRKV-K(cyclo[Ff-Nal-Cit-r-Cit-rQ])-PEG_{12}-k(N_3)-NH_2
Ac-PKKKRKV-PEG_2-K(cyclo[Ff-Nal-Cit-r-Cit-rQ])-PEG_{12}-k(N_3)-NH_2
Ac-PKKKRKV-PEG_2-K(cyclo[Ff-Nal-Cit-r-Cit-rQ])-PEG_{12}-K(N_3)-NH_2

[0309] In the EEVs provided herein, the cCPP can be attached to the linker through AAsc. In embodiments, the cCPP is attached to the linker through a -$(CH_2)_{y'}$- group through AAsc. In embodiments, the cCPP is attached to the linker through a -$(CH_2)_{y'}$- group through AAsc such

that the connection is the structure  wherein n is an integer from 1 to 5. In embodiments, the cCPP can be attached to a lysine component of the linker through AAsc. In embodiments, when AAsc is glutamic acid, n is 1. In the EEVs shown in **Tables 2a-2c** and **Tables 3a-3c**, the cCPP is attached to a lysine component of the linker through AAsc, wherein AAsc is glutamic acid (E) and n is 1 prior to conjugation. After congjuation to the linker, AAsc is shown as glutamine (Q).

[0310] In the EEV sequences provided, lysine can include a protecting group (not shown). In embodiments, the protecting group is a trifluoroacetyl (Tfa) group. In embodiments, the protecting group is an acetyl (Ac) group. It is understood that other protecting group can also be used and that the protecting group can be removed after the EEV is conjugated to a cargo. In embodiments, the EEVs are deprotected after conjugation to the PMOs.

Exocyclic Peptides

[0311] The exocyclic peptide (EP) can comprise from 2 to 10 amino acid residues e.g., 2, 3, 4, 5, 6, 7, 8, 9, or 10 amino acid residues. The EP can comprise 2 to 8 amino acid residues or 2 to 6 amino acid residues. In embodiments, the EP comprises 2 amino acid residues. In embodiments, the EP comprises 3 amino acid residues. In embodiments, the EP comprises 4 amino acid residues. In embodiments, the EP comprises 5 amino acid residues. In embodiments, the EP comprises 6 amino acid residues. In embodiments, the EP comprises 7 amino acid residues. In embodiments, the EP comprises 8 amino acid residues.

[0312] The amino acids in the EP can have D or L stereochemistry. The amino acid residues of the EP may all be D-amino acids. The amino acid residues of the EP may all be L-amino acids. The amino acid residues of the EP may be a combination of D-amino acids and L-amino acids.

[0313] The exocyclic peptide can be acylated at the N-terminus (Ac-EP). It is understood that EP and Ac-EP can be used interchangeably throughout the application. For example, the EP can comprise Ac-PKKKRKV.

[0314] Each amino acid in the exocyclic peptide may be a natural or non-natural amino acid. The term “non-natural amino acid” refers to an organic compound that is a congener of a natural

amino acid in that it has a structure similar to a natural amino acid so that it mimics the structure and reactivity of a natural amino acid. The non-natural amino acid can be a modified amino acid, and/or amino acid analog, that is not one of the 20 common naturally occurring amino acids or the rare natural amino acids selenocysteine or pyrrolysine. Non-natural amino acids can also be the D-isomer of the natural amino acids. Non-natural amino acids can also be the D-isomer of the natural amino acids. Amino acids and/or amino acid residues may be referred to using their full name, their conventional three letter abbreviation, or their conventional one letter abbreviation. When using the one letter abbreviation, capital letters indicate L amino acids or residues, and lower case letters indicate D amino acids or residues. For example, arginine may be referred to as Arg, R, or r.

[0315] The EP can comprise at least one amino acid residue that is positively charged at physiologically relevant pH values. In embodiments, the EP comprises at least one amino acid residue comprising a guanidine group, a terminal amine, an imidazole, or a protonated form thereof. In embodiments, the EP comprises at least one amino acid residue comprising a guanidine group, or a protonated form thereof. In embodiments, the EP comprises at least one amino acid residue comprising a terminal amine or a protonated form thereof. In embodiments, the EP comprises at least one amino acid residue comprising an imidazole, or a protonated form thereof. Protonated forms can mean salt thereof throughout the disclosure.

[0316] The EP can comprise from 1 to 5 amino acids residues comprising a side chain comprising a guanidine group, a terminal amine, an imidazole, or a protonated form thereof. The EP can comprise from 1 to 4 amino acids residues comprising a side chain comprising a guanidine group, a terminal amine, an imidazole, or a protonated form thereof. The EP can comprise from 1 to 3 amino acids residue comprising a side chain comprising a guanidine group, a terminal amine, an imidazole, or a protonated form thereof. The EP can comprise 1 or 2 amino acids residue comprising a side chain comprising a guanidine group, a terminal amine, an imidazole, or a protonated form thereof. The EP can comprise 2 or 3 amino acids residue comprising a side chain comprising a guanidine group, a terminal amine, an imidazole, or a protonated form thereof. The EP can comprise from 2 to 4 amino acids residue comprising a side chain comprising a guanidine group, a terminal amine, an imidazole, or a protonated form thereof.

[0317] In embodiments, the amino acid comprising a guanidine group is arginine. In embodiments, the amino acid comprising a terminal amine is lysine. In embodiments, the amino acid comprising an imidazole is histidine.

[0318] The EP can comprise 1, 2, 3, 4, or 5 amino acids residues comprising a side chain comprising a guanidine group, or a protonated form thereof. The EP can comprise 1 amino acid residue comprising a side chain comprising a guanidine group, or a protonated form thereof. The EP can comprise amino acid residues comprising a side chain comprising a guanidine group, or a protonated form thereof. The EP can comprise 3 amino acid residues comprising a side chain comprising a guanidine group, or a protonated form thereof. The EP can comprise 4 amino acid residues comprising a side chain comprising a guanidine group, or a protonated form thereof. The EP can comprise 5 amino acid residues comprising a side chain comprising a guanidine group, or a protonated form thereof. The amino acid residue comprising a side chain comprising a guanidine group can be an arginine residue. The EP can comprise 1, 2, 3, 4, or 5 arginine residues. The EP can comprise 1 arginine residue. The EP can comprise 2 arginine residues. The EP can comprise 3 arginine residues. The EP can comprise 4 arginine residues. The EP can comprise 5 arginine residues.

[0319] The EP can comprise 1, 2, 3, 4, or 5 amino acid residues comprising a terminal amine, or a protonated form thereof. The EP can comprise 1 amino acid residue comprising a terminal amine, or a protonated form thereof. The EP can comprise 2 amino acid residues comprising a terminal amine, or a protonated form thereof. The EP can comprise 3 amino acid residues comprising a terminal amine, or a protonated form thereof. The EP can comprise 4 amino acid residues comprising a terminal amine, or a protonated form thereof. The EP can comprise 5 amino acid residues comprising a terminal amine, or a protonated form thereof. The amino acid with a terminal amine can be lysine. The EP can comprise 1, 2, 3, 4, or 5 lysine residues. The EP can comprise 1 lysine residue. The EP can comprise 2 lysine residues. The EP can comprise 3 lysine residues. The EP can comprise 4 lysine residues. The EP can comprise 5 lysine residues. The amino group on the side chain of each lysine residue can be substituted with a protecting group, including, for example, trifluoroacetyl (-$COCF_3$), allyloxycarbonyl (Alloc), 1-(4,4-dimethyl-2,6-dioxocyclohexylidene)ethyl (Dde), or (4,4-dimethyl-2,6-dioxocyclohex-1-ylidene-3)-methylbutyl (ivDde) group. The amino group on the side chain of each lysine residue can be substituted with a trifluoroacetyl (-$COCF_3$) group. The protecting group can be included to

enable amide conjugation. The protecting group can be removed after the EP is conjugated to a cCPP.

[0320] The EP can comprise 1, 2, 3, 4, or 5 amino acid residues comprising an imidazole, or a protonated form thereof. The EP can comprise 1 amino acid residue comprising an imidazole, or a protonated form thereof. The EP can comprise 2 amino acid residues comprising an imidazole, or a protonated form thereof. The EP can comprise 3 amino acid residues comprising an imidazole, or a protonated form thereof. The EP can comprise 4 amino acid residues comprising an imidazole, or a protonated form thereof. The EP can comprise 5 amino acid residues comprising an imidazole, or a protonated form thereof. The amino acid comprising an imidazole can be histidine. The EP can comprise 1, 2, 3, 4, or 5 histidine residues. The EP can comprise 1 histidine residue. The EP can comprise 2 histidine residues. The EP can comprise 3 histidine residues. The EP can comprise 4 histidine residues. The EP can comprise 5 histidine residues.

[0321] The EP can comprise 1, 2, 3, or 4 amino acid residues with an uncharged side chain. The uncharged side chain can comprise a hydrophobic side chain. The EP can comprise 1, 2, 3, or 4 amino acid residues with a hydrophobic side chain. The EP can comprise 1, 2, 3, or 4 amino acid residues with an uncharged hydrophobic side chain. The EP can comprise 1 amino acid residue with an uncharged hydrophobic side chain. The EP can comprise 2 amino acid residues with an uncharged hydrophobic side chain. The EP can comprise 3 amino acid residues with an uncharged hydrophobic side chain. The EP can comprise 4 amino acid residues with an uncharged hydrophobic side chain. The amino acid residue with an uncharged hydrophobic side chain can be selected from valine, proline, β-alanine, and glycine. The amino acid residue with a hydrophobic side chain can be valine or proline. The amino acid residue with a hydrophobic side chain can be valine. The amino acid residue with a hydrophobic side chain can be proline. The amino acid residue with a hydrophobic side chain can be β-alanine. The amino acid residue with a hydrophobic side chain can be glycine.

[0322] The EP can comprise at least one positively charged amino acid residue and at least one uncharged hydrophobic residue. The EP can comprise two positively charged amino acid residues and one uncharged hydrophobic residue. The EP can comprise three positively charged amino acid residues and one uncharged hydrophobic residue. The EP can comprise four positively charged amino acid residues and one uncharged hydrophobic residue. The EP can comprise two positively charged amino acid residues and two uncharged hydrophobic residues.

The EP can comprise three positively charged amino acid residues and two uncharged hydrophobic residues. The EP can comprise four positively charged amino acid residues and two uncharged hydrophobic residues.

[0323] The EP can comprise at least one lysine residue and at least one arginine residue. The EP can comprise 2, 3, 4, or 5 lysine residues and/or arginine residues. The EP can comprise 2 lysine and/or arginine residues. The EP can comprise 3 lysine and/or arginine residues. The EP can comprise 4 lysine and/or arginine residues. The EP can comprise 5 lysine and/or arginine residues.

[0324] The EP can comprise one or more amino acid residues selected from lysine (K), arginine (R), histidine (H), glycine (G), β-alanine (B), phenylalanine (F), proline (P), valine (V), or combinations thereof. The EP can comprise one or more amino acid residues selected from lysine (K), arginine (R), histidine (H), glycine (G), β-alanine (B), or combinations thereof. The EP can comprise one proline (P). The EP can comprise one valine (V). The EP can comprise one glycine (G). The EP can comprise one or more lysine (K). The EP can comprise one lysine (K). The EP can comprise two lysine (K). The EP can comprise three lysine (K). The EP can comprise four lysine (K). The EP can comprise one or more arginine (R). The EP can comprise one arginine (R). The EP can comprise two arginine (R). The EP can comprise three arginine (R). The EP can comprise four arginine (R). The EP can comprise five arginine (R). The EP can comprise six arginine (R). The EP can comprise one or more histidine (H). The EP can comprise one histidine (H). The EP can comprise two histidine (H). The EP can comprise three histidine (H). The EP can comprise four histidine (H). The EP can comprise five histidine (H). The EP can comprise six histidine (H). The EP can comprise one or more β-alanine (B). The EP can comprise two β-alanine (B). The EP can comprise two β-alanine (B). The amino acid residues in the EP can have D or L stereochemistry.

[0325] The EP can comprise KK, KR, RR, RK, RF, HH, HK, HR, RH, KKK, KFK, KGK, KBK, KBR, KRK, KRR, RKK, RRR, RHR, RRV, FRR, RFR, RBR, KKH, KHK, HKK, HRR, HRH, HHR, HBH, HHH, HHHH, PKKK, KHKK, KKHK, KKKH, KHKH, HKHK, KKKK, KKRK, KRKK, KRRK, KRKF, RKKR, RRRR, RBRB, BRBR, HRHR, RHRH, HBHB, BHBH, KGKK, KKGK, HBHBH, RBHBH, BRBRB, HBRBH, BHRHR, BRHRH, HBKBH, RRRRR, KKKKK, KKKRK, RKKKK, KRKKK, KKRKK, KKKKR, KKFRK, KBKBK, KRKIL, RBFBR, RBRBR, RBHBR, RFRRF, RIRRI, RKKKKG, KRKKKG, KKRKKG, KKKKRG, KKKRKG,

RKKKKB, KRKKKB, KKRKKB, KKKKRB, KKKRKV, RRRRRR, HHHHHH, RHRHRH, HRHRHR, KRKKKP, PKKKRK, KRKRKR, RKRKRK, RBRBRB, RBRRBR, KBKBKB, PKKKRKV, PGKKRKV, PKGKRKV, PKKGRKV, PKKKGKV, PKKKRGV, or PKKKRKG, wherein B is beta-alanine. The EP can comprise PKKKRKV. The EP can comprise RR, RRR, RHR, RBR, RBRBR, RBRRBR, RBGRBR, RBRGBR, RBRRBG, RBHBR, or HBRBH, wherein B is beta-alanine. The amino acids in the EP can have D or L stereochemistry.

[0326] The EP can comprise kk, kr, rr, rk, rf, hh, hk, hr, rh, kkk, kfk, kgk, kbk, kbr, krk, krr, rkk, rrr, rhr, rrv, frr, rfr, rbr, kkh, khk, hkk, hrr, hrh, hhr, hbh, hhh, hhhh, pkkk, khkk, kkhk, kkkh, khkh, hkhk, kkkk, kkrk, krkk, krrk, krkf, rkkr, rrrr, rbrb, brbr, hrhr, rhrh, hbhb, bhbh, kgkk, kkgk, hbhbh, rbhbh, brbrb, hbrbh, bhrhr, brhrh, hbkbh, rrrrr, kkkkk, kkkrk, rkkkk, krkkk, kkrkk, kkkkr, kkfrk, kbkbk, krkil, rbfbr, rbrbr, rbhbr, rfrrf, rirri, rkkkkg, krkkkg, kkrkkg, kkkkrg, rkkkkb, krkkkb, kkrkkb, kkkkrb, kkkrkv, rrrrrr, hhhhhh, rhrhrh, hrhrhr, krkkkp, pkkkrk, krkrkr, rkrkrk, rbrbrb, rbrrbr, kbkbkb, pkkkrkv, pgkkrkv, pkgkrkv, pkkgrkv, pkkkgkv, pkkkrgv, or pkkkrkg.

[0327] The EP can consist of KK, KR, RR, RK, RF, HH, HK, HR, RH, KKK, KFK, KGK, KBK, KBR, KRK, KRR, RKK, RRR, RHR, RRV, FRR, RFR, RBR, KKH, KHK, HKK, HRR, HRH, HHR, HBH, HHH, HHHH, PKKK, KHKK, KKHK, KKKH, KHKH, HKHK, KKKK, KKRK, KRKK, KRRK, KRKF, RKKR, RRRR, RBRB, BRBR, HRHR, RHRH, HBHB, BHBH, KGKK, KKGK, HBHBH, RBHBH, BRBRB, HBRBH, BHRHR, BRHRH, HBKBH, RRRRR, KKKKK, KKKRK, RKKKK, KRKKK, KKRKK, KKKKR, KKFRK, KBKBK, KRKIL, RBFBR, RBRBR, RBHBR, RFRRF, RIRRI, RKKKKG, KRKKKG, KKRKKG, KKKKRG, KKKRKG, RKKKKB, KRKKKB, KKRKKB, KKKKRB, KKKRKV, RRRRRR, HHHHHH, RHRHRH, HRHRHR, KRKKKP, PKKKRK, KRKRKR, RKRKRK, RBRBRB, RBRRBR, KBKBKB, PKKKRKV, PGKKRKV, PKGKRKV, PKKGRKV, PKKKGKV, PKKKRGV, or PKKKRKG, wherein B is beta-alanine. The EP can consist of PKKKRKV. The EP can consist of RR, RRR, RHR, RBR, RBRBR, RBRRBR, RBGRBR, RBRGBR, RBRRBG, RBHBR, or HBRBH, wherein B is beta-alanine. The amino acids in the EP can have D or L stereochemistry.

[0328] The EP can consist of kk, kr, rr, rk, rf, hh, hk, hr, rh, kkk, kfk, kgk, kbk, kbr, krk, krr, rkk, rrr, rhr, rrv, frr, rfr, rbr, kkh, khk, hkk, hrr, hrh, hhr, hbh, hhh, hhhh, pkkk, khkk, kkhk, kkkh, khkh, hkhk, kkkk, kkrk, krkk, krrk, krkf, rkkr, rrrr, rbrb, brbr, hrhr, rhrh, hbhb, bhbh, kgkk, kkgk, hbhbh, rbhbh, brbrb, hbrbh, bhrhr, brhrh, hbkbh, rrrrr, kkkkk, kkkrk, rkkkk, krkkk, kkrkk, kkkkr, kkfrk, kbkbk, krkil, rbfbr, rbrbr, rbhbr, rfrrf, rirri, rkkkkg, krkkkg, kkrkkg, kkkkrg, rkkkkb,

krkkkb, kkrkkb, kkkkrb, kkkrkv, rrrrrr, hhhhhh, rhrhrh, hrhrhr, rkkkp, pkkkrk, krkrkr, rkrkrk, rbrbrb, rbrrbr, kbkbkb, pkkkrkv, pgkkrkv, pkgkrkv, pkkgrkv, pkkkgkv, pkkkrgv, or pkkkrkg.

[0329] The EP can comprise KK, KR, RR, RK, RF, KKK, KGK, KFK, KBK, KBR, KRK, KRR, RKK, RRR, KKKK, KKRK, KRKK, KRRK, KRKF, RKKR, RRRR, KGKK, KKGK, KKKKK, KKKRK, KBKBK, KKFRK, KRKIL, RFRRF, RIRRI, KKKRKV, PKKKRKV, PGKKRKV, PKGKRKV, PKKGRKV, PKKKGKV, PKKKRGV, or PKKKRKG. The EP can comprise PKKKRKV, RR, RRR, RHR, RBR, RBRBR, RBHBR, or HBRBH, wherein B is beta-alanine.

[0330] The EP can comprise kk, kr, rr, rk, rf, kkk, kgk, kfk, kbk, kbr, krk, krr, rkk, rrr, kkkk, kkrk, krkk, krrk, krkf, rkkr, rrrr, kgkk, kkgk, kkkkk, kkkrk, kbkbk, kkfrk, krkil, rfrrf, rirri, kkkrkv, pkkkrkv, pgkkrkv, pkgkrkv, pkkgrkv, pkkkgkv, pkkkrgv, or pkkkrkg. The EP can comprise pkkkrkv, rr, rrr, rhr, rbr, rbrbr, rbhbr, or hbrbh.

[0331] The EP can consist of KK, KR, RR, RK, RF, KKK, KGK, KFK, KBK, KBR, KRK, KRR, RKK, RRR, KKKK, KKRK, KRKK, KRRK, KRKF, RKKR, RRRR, KGKK, KKGK, KKKKK, KKKRK, KBKBK, KKFRK, KRKIL, RFRRF, RIRRI, KKKRKV, PKKKRKV, PGKKRKV, PKGKRKV, PKKGRKV, PKKKGKV, PKKKRGV, or PKKKRKG. The EP can consist of PKKKRKV, RR, RRR, RHR, RBR, RBRBR, RBHBR, or HBRBH, wherein B is beta-alanine.

[0332] The EP can consist of kk, kr, rr, rk, rf, kkk, kgk, kbk, kfk, kbr, krk, krr, rkk, rrr, kkkk, kkrk, krkk, krrk, krkf, rkkr, rrrr, kgkk, kkgk, kkkkk, kkkrk, kbkbk, kkfrk, krkil, rfrrf, rirri, kkkrkv, pkkkrkv, pgkkrkv, pkgkrkv, pkkgrkv, pkkkgkv, pkkkrgv, or pkkkrkg.

[0333] The EP can comprise one of the following sequences: HBH, HBHBH, or HBRBH. The EP can comprise HHRBFBR. The EP can comprise one of the following sequences: KBK, KBKBK, or KBR. The EP can comprise one of the following sequences: KGK or KGKK. The EP can comprise one of the following sequences: KKGK, KKKK, KKKKR, KKKRK, KKKRKG, or KKRK. The EP can comprise one of the following sequences: KR, KRK, or KRKKK. The EP can comprise PGKKRKV. The EP can comprise one of the following sequences: PKGKRKV, PKK, PKKGRKV, PKKK, PKKKGKV, PKKKRGV, PKKKRKG, PKKRGV, PKKRKG, or PKKRKV. The EP can comprise one of the following sequences: RBHBH, RBHBR, RBR, RBRBR, or RBRRBR. The EP can comprise one of the following sequences: RFR, or RHR. The EP can comprise RKKK. The EP can comprise one of the following sequences: RR, RRR, or RRRRFFF. The EP can comprise one of the following

sequences: RK or RF. The EP can comprise one of the following sequences: KFK or KRKF. The EP can comprise one of the following sequences: KKFRK or KRKIL. The EP can comprise one of the following sequences: RFRRF or RIRRI. The EP can comprise PKKKRKV. B is beta-alanine.

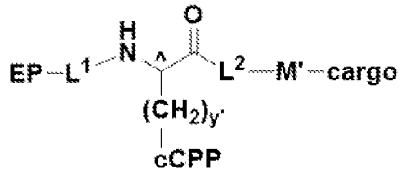
[0334] The EP can comprise an amino acid sequence identified in the art as a nuclear localization sequence (NLS). The EP can consist of an amino acid sequence identified in the art as a nuclear localization sequence (NLS). The EP can comprise an NLS comprising the amino acid sequence PKKKRKV. The EP can consist of an NLS comprising the amino acid sequence PKKKRKV.

[0335] The EP comprise from 2 to 10, 6 to 9, or 4 to 8 consecutive amino acid residues. The EP can comprise between 2 to 10, 6 to 9, or 4 to 8 amino acids, wherein not all amino acids are consecutive, for example, the amino acid residues of the EP may be separated by one or more components of the EEV, for example, the amino acid residues of an EP may be separated by a linker, the cCPP or a combination thereof, thereby forming a "split" EP.

EEV-cargo conjugate

[0336] In embodiments, the EEV can be conjugated to a cargo to form an EEV-cargo conjugate comprising the structure of Formula (A-1):

Formula (A-1):



(A-1)

wherein:

EP is a linear exocyclic peptide defined herein;

cCPP is a cyclic cell penetrating peptide defined herein;

L^1 and L^2, are, independently, a linker arm;

^ indicates D- or L-stereochemistry;

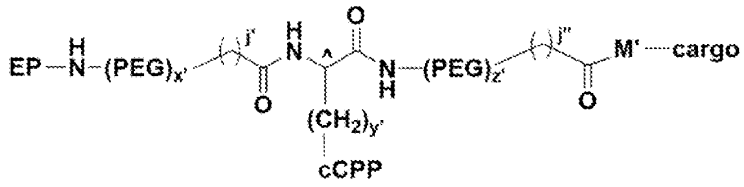
y′ is an integer from 1 to 5;

M′ is a bonding group defined herein; and

cargo is a peptide, an oligonucleotide, a small molecule, or any combinations thereof.

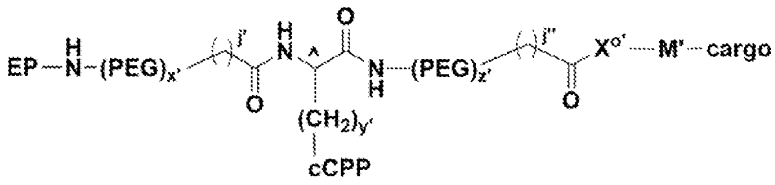
[0337] In embodiments, the EEV-cargo conjugate Formula (A-1) can have a Formula selected from (B-1), (C-1), and (D-1):

Formula (B-1):



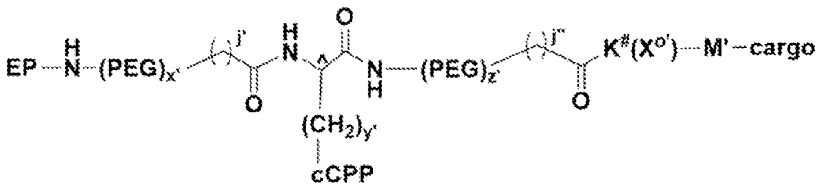
(B-1);

Formula (C-1):



(C-1); and

Formula (D-1):



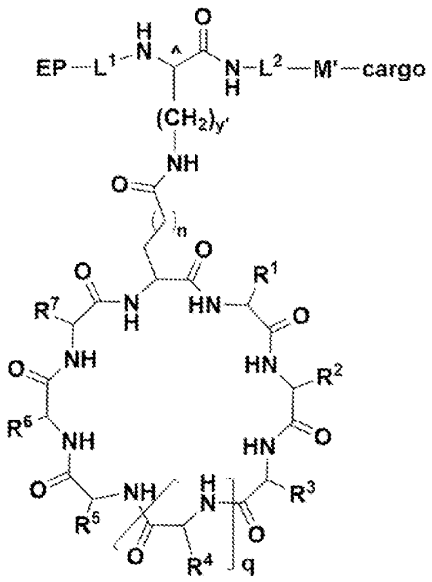
(D-1);

wherein EP, cCPP, x′, j′, ^, y′, z′, j″, X^{o}′, $K^{\#}$, M′, and cargo are as defined herein.

[0338] In embodiments, the EEV-cargo conjugate has the structure of Formula (B-1), Formula (C-1), or Formula (D-1), wherein EP comprises all D-amino acids; cCPP comprises D-amino acids, achiral amino acids and AAsc, wherein AAsc is an amino acid side chain and AAsc conjugates cCPP to the linker.

[0339] In embodiments, the EEV-cargo conjugate has the structure of formula (A-3):

Formula (A-3):



(A-3)

wherein:

EP is a linear exocyclic peptide;

L^1 and L^2 are linker arms,

M′ is a bonding group; and

y′ is an integer from 1-5,

^ indicates D- or L- stereochemistry;

R^1, R^2, and R^3 can each independently be H or an amino acid residue having a side chain comprising an aryl or heteroaryl group;

R^4, R^5, R^6, and R^7 are independently H or a side chain of an amino acid;

q is an integer from 1-4;

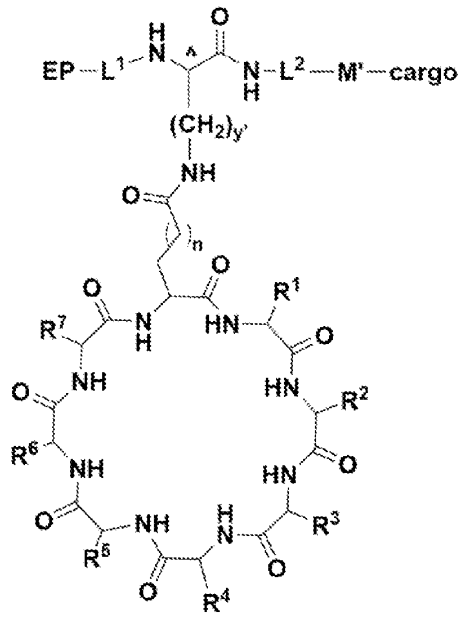
n is an integer from 1-4; and

cargo is a peptide, oligonucleotide, a small molecule, or a combination thereof.

[0340] In embodiments, the EEV-cargo conjugate of Formula (A-3) has a linker structure of Formula (B′). In embodiments, the EEV-cargo conjugate of Formula (A-3) has a linker structure of Formula (C′). In embodiments, the EEV-cargo conjugate of Formula (A-3) has a linker structure of Formula (D′).

[0341] In embodiments, the EEV-cargo conjugate has the structure of formula (A-3a):

Formula (A-3a):



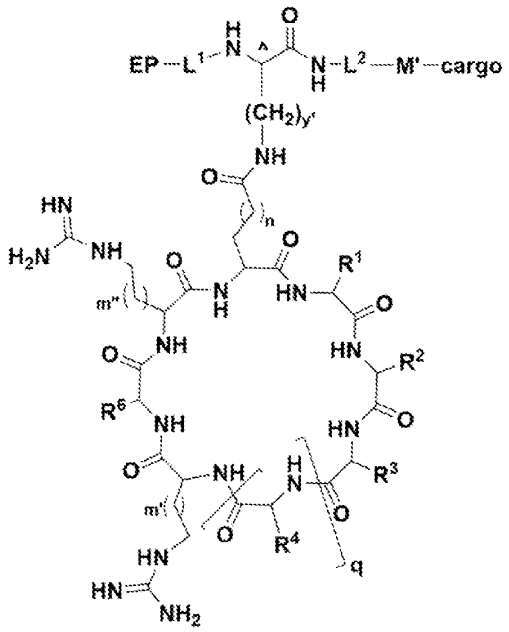
(A-3a)

wherein EP, M′, L^1, L^2, ^, y′, n, R^1, R^2, R^3, R^4, R^5, R^6, and R^7 are as defined herein and cargo is a peptide, oligonucleotide, a small molecule, or a combination thereof.

[0342] In embodiments, the EEV-cargo conjugate of Formula (A-3a) has a linker structure of Formula (B′). In embodiments, the EEV-cargo conjugate of Formula (A-3a) has a linker structure of Formula (C′). In embodiments, the EEV-cargo conjugate of Formula (A-3a) has a linker structure of Formula (D′).

[0343] In embodiments, the EEV-cargo conjugate has the structure of formula (A-3b):

Formula (A-3b):



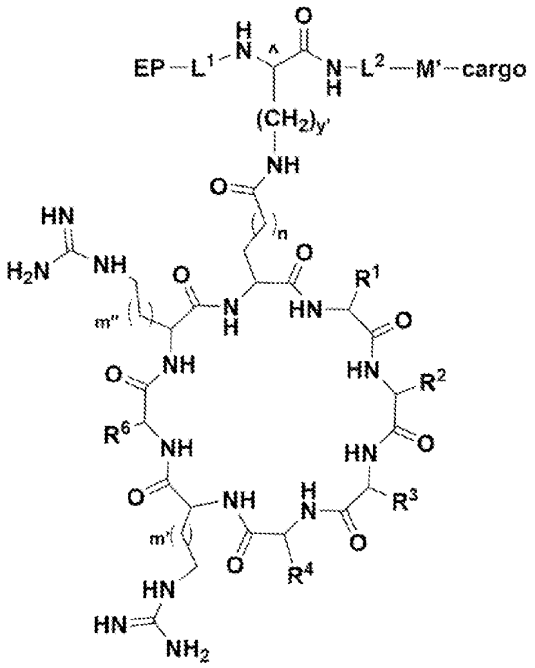
(A-3b)

wherein EP, L^1, L^2, M′, ^, y′, n, m′, m″, q, R^1, R^2, R^3, R^4, and R^6 are as defined herein and cargo is a peptide, oligonucleotide, a small molecule, or a combination thereof.

[0344] In embodiments, the EEV-cargo conjugate of Formula (A-3b) has a linker structure of Formula (B′). In embodiments, the EEV-cargo conjugate of Formula (A-3b) has a linker structure of Formula (C′). In embodiments, the EEV-cargo conjugate of Formula (A-3b) has a linker structure of Formula (D′).

[0345] In embodiments, the EEV-cargo conjugate has the structure of formula (A-3c):

Formula (A-3c):

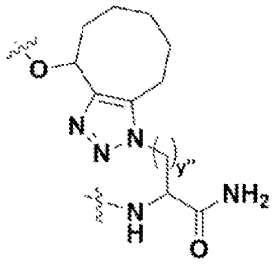
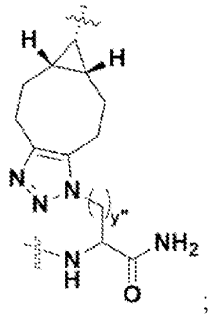
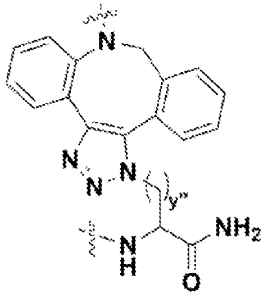
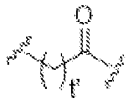
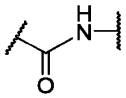


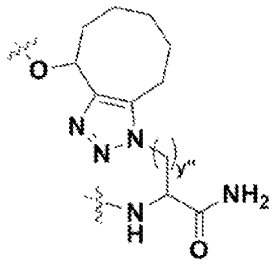
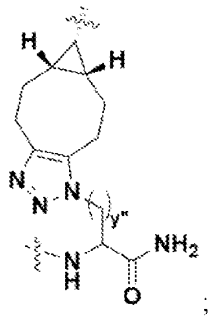
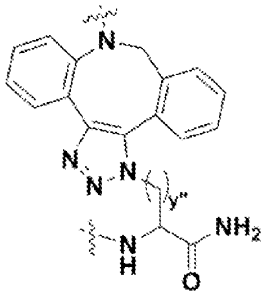
(A-3c);

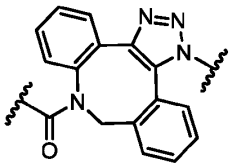
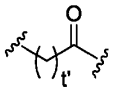
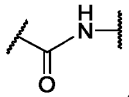
wherein EP, L^1, L^2, M′, ^, y′, n, m′, m″, R^1, R^2, R^3, R^4, and R^6 are as defined herein and cargo is a peptide, oligonucleotide, a small molecule, or a combination thereof.

[0346] In embodiments, the EEV-cargo conjugate of Formula (A-3c) has a linker structure of Formula (B′). In embodiments, the EEV-cargo conjugate of Formula (A-3c) has a linker structure of Formula (C′). In embodiments, the EEV-cargo conjugate of Formula (A-3c) has a linker structure of Formula (D′).

[0347] In embodiments, the cargo is an oligonucleotide. In embodiments, the oligonucleotide is a therapeutic oligonucleotide. In embodiments, the oligonucleotide is an antisense oligonucleotide. In embodiments, the oligonucleotide is a phosphorodiamidate morpholino oligonucleotide (PMO).

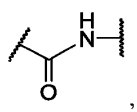
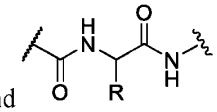
[0348] M′ can comprise: -NH-, -C(O)-, -O-,  ;  ;  ;  , or  , wherein y″ is an integer from 1 to 4 and t′ is 0 to 10.

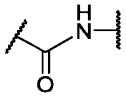
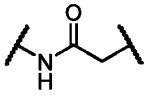
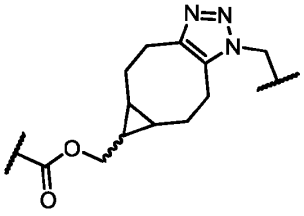
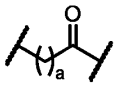
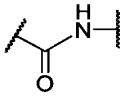
[0349] M′ can comprise: -NH-, -C(O)-, -O-,  ;  ;  ; wherein y″ is an integer from 1 to 4.

[0350] In embodiments, M′ comprises  ,  , or  , wherein t′ is 0 to 10.

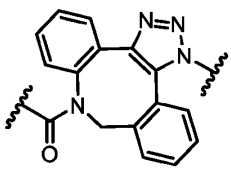
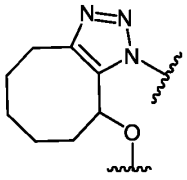
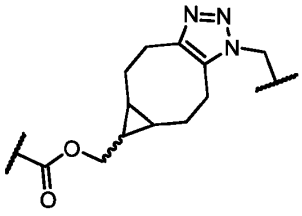
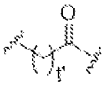
[0351] M′ can comprise -NH-. M′ can comprise -C(O)-. M′ can comprise -O-.

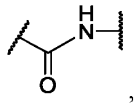
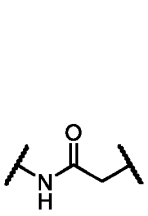
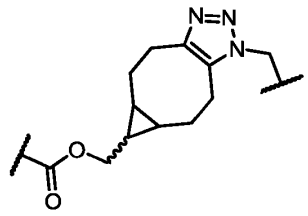
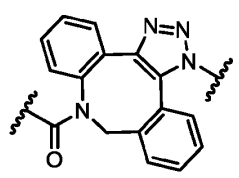
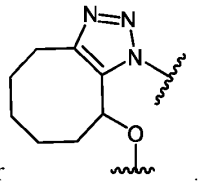
[0352] M′ can comprise a structure selected from:

 , and  , wherein R is alkyl, alkenyl, alkynyl, carbocyclyl, or heterocyclyl.

[0353] M′ comprise a structure selected from:  ,  , or  . M′ can comprise  . M′ can comprise  . M′ can comprise  .

[0354] M′ can comprise -C(O)-. M′ can comprise -NH-. M′ can comprise -O-.

[0355] M′ can comprise  ,  or  . M′ can comprise  , wherein t′ is 0 to 10. M′ can comprise  . M′ can be -O-. M′ can be -NH-. M′ can be -C(O)-.

[0356] Provided herein is an EEV-cargo conjugate comprising an EEV conjugated to a cargo through a bonding group (M′), wherein M′ can comprise -NH-, -C(O)-, -O-,  ,  ,  ,  , or  .

[0357] The linker can be covalently bound to a cargo at any suitable location on the cargo. The linker can be covalently bound to the 3' end or 5′ end of an oligonucleotide cargo. The linker can be covalently bound to the backbone of a cargo.

[0358] M′ forms a bond to the free secondary amine of the morpholino ring of a terminal nucleotide of a PMO cargo. In embodiments, M′ forms a bond to the free secondary amine of the morpholino ring of the 3′ terminal nucleotide of a PMO. In embodiments, M′ forms a bond to the free hydroxyl of the of the 5′ terminal nucleotide of an PMO.

[0359] In embodiments, the EEV-cargo conjugate has a structure shown in Formula (B-1), wherein x′ is 0 and z′ is 12. In embodiments, the EEV-cargo conjugate can have a sequence shown in **Table 9a**.

***Table 9a**: EEV-cargo conjugate sequences*

Ac-PKKKRKV-K(cyclo[Ff-Nal-RrRrQ])-PEG_{12}-M′-cargo
Ac-PKKKRKV-K(cyclo[Ff-Nal-GrGrQ])-PEG_{12}-M′-cargo

[0360] In embodiments, the EEV-cargo conjugate has a structure shown in Formula (B-1), wherein x′ is 2 and z′ is 12. In embodiments, the EEV-cargo conjugate can have a sequence shown in **Table 9b**.

***Table 9b**: EEV-cargo conjugate sequences*

Ac-PKKKRKV-PEG_2-K(cyclo[Ff-Nal-Cit-r-Cit-rQ])-PEG_{12}-M′-cargo
Ac-PKKKRKV-PEG_2-K(cyclo[Ff-Nal-GrGrQ])-PEG_{12}-M′-cargo

Ac-PKKKRKV-PEG_2-K(cyclo[FGFGRGRQ])-PEG_{12}-M′-cargo
Ac-PKKKRKV-PEG_2-K(cyclo[GfFGrGrQ])-PEG_{12}-M′-cargo

[0361] In embodiments, the EEV-cargo conjugate has a structure shown in Formula (B-1), wherein x′ is 2 and z′ is 2. In embodiments, the EEV-cargo conjugate can have a sequence shown in **Table 9c**.

***Table 9c**: EEV-cargo conjugate sequences*

Ac-PKKKRKV-PEG_2-K(cyclo[FGFGRGRQ])-PEG_2-M′-cargo
Ac-PKKKRKV-PEG_2-K(cyclo[GfFGrGrQ])-PEG_2-M′-cargo
Ac-PKKKRKV-PEG_2-K(cyclo[FfFGRGRQ])-PEG_2-M′-cargo

[0362] In embodiments, the EEV-cargo conjugate has a structure shown in Formula (B-1), wherein x′ is 2 and z′ is 12. In embodiments, the EEV-cargo conjugate can have a sequence shown in **Table 9d**.

***Table 9d**: EEV-cargo conjugate structures*

Ac-PKKKRKV-PEG_2-K(cyclo[βhF-F-Nal-SRSRQ])-PEG_{12}-M′-cargo

[0363] In embodiments, the EEV-cargo conjugate has a structure shown in Formula (D-1), wherein x′ is 2 and z′ is 12. In embodiments, the EEV-cargo conjugate can have a sequence shown in **Table 10a**.

***Table 10a**: EEV-cargo conjugate structures*

Ac-PKKKRKV-PEG_2-K(cyclo[FGFGRGRQ])-PEG_{12}-K(Bip)-M′-cargo
Ac-PKKKRKV-PEG_2-K(cyclo[FGFGRGRQ])-PEG_{12}-K(Nal) -M′-cargo

[0364] In embodiments, the EEV-cargo conjugate has a structure shown in Formula (C-1), wherein x′ is 2 and z′ is 12. In embodiments, the EEV-cargo conjugate can have a sequence shown in **Table 10b**.

***Table 10b**: EEV-cargo conjugate structures*

Ac-PKKKRKV-PEG_2-K(cyclo[FGFGRGRQ])-PEG_{12}-Nal-M′-cargo
Ac-PKKKRKV-PEG_2-K(cyclo[FGFGRGRQ])-PEG_{12}-Bip-M′-cargo

[0365] The linker can be covalently bound to a cargo at any suitable location on the cargo. The linker can be covalently bound to the 3′ end or 5′ end of an oligonucleotide cargo. The linker can be covalently bound to the backbone of a cargo.

[0366] In embodiment where the cargo is a PMO, M′ forms a bond to the free secondary amine of the morpholino ring of a terminal nucleotide of a PMO cargo. In embodiments, M′ forms a bond to the free secondary amine of the morpholino ring of the 3′ terminal nucleotide of a PMO. In embodiments, M′ forms a bond to the free hydroxyl of the of the 5′ terminal nucleotide of an PMO.

[0367] Structures of EEV-cargo conjugates are shown in **FIGS. 27-29**.

Cargo

[0368] In embodiments, an EEV is provided that comprises a cyclic peptide, for example, a cyclic cell penetrating peptide (cCPP) conjugated to a cargo. In embodiments, the cargo is an oligonucleotide. In embodiments, the oligonucleotide is a therapeutic oligonucleotide. In embodiments, the oligonucleotide is an antisense oligonucleotide. In embodiments, the oligonucleotide is directed to a target polynucleotide. In embodiments, the antisense oligonucleotide is a phosphorodiamidate morpholino oligonucleotide (PMO). In embodiments, the oligonucleotide comprises siRNA, RNAi, microRNA, antagomir, an aptamer, a ribozyme, an immunostimulatory oligonucleotide, a decoy oligonucleotide, a supermir, a miRNA mimic, a miRNA inhibitor, or a combination thereof. See, for example, Chery, J., "RNA therapeutics: RNAi and antisense mechanisms and clinical applications," Postdoc J, July 2016, 4(7):35-50, and Zhu, et al., "RNA-based therapeutics: an overview and prospectus," Cell Death & Disease, 23 July 2022, 12(644) (doi.org/10.1038/s41419-022-05075-2).

[0369] The term "antisense oligonucleotide" or simply "antisense" refers to oligonucleotides that are complementary to a targeted polynucleotide sequence. Antisense oligonucleotides are single strands of DNA or RNA that are complementary to a chosen sequence, e.g., a target gene mRNA.

[0370] The antisense oligonucleotide may modulate one or more aspects of protein transcription, translation, and expression. In embodiments, an antisense oligonucleotide can be directed to a target sequence within a target pre-mRNA and modulates one or more aspects of pre-mRNA splicing. As used herein, modulation of splicing refers to altering the processing of a pre-mRNA transcript such that the spliced mRNA molecule contains either a different combination of exons as a result of exon skipping or exon inclusion, a deletion in one or more exons, or the deletion or addition of a sequence not normally found in the spliced mRNA (e.g., an intron sequence). In embodiments, hybridization of antisense oligonucleotides to a target sequence in a pre-mRNA molecule restores native splicing to a mutated pre-mRNA sequence. In embodiments, hybridization of antisense oligonucleotides results in alternative splicing of the target pre-mRNA. In embodiments, hybridization of antisense oligonucleotides results in exon inclusion or exon skipping of one or more exons. In embodiments, a skipped exon sequence comprises a frameshift mutation, a nonsense mutation, or a missense mutation. In embodiments, a skipped exon sequence comprises a nucleic acid deletion, substitution, or insertion. In embodiments, a skipped exon itself does not comprise a sequence mutation, but a neighboring exon comprises a mutation leading to a frameshift mutation or a nonsense mutation. In embodiments, hybridization of antisense oligonucleotides to a target sequence within a target pre-mRNA prevents inclusion of an exon sequence in the mature mRNA molecule. In embodiments, hybridization of antisense oligonucleotides to a target sequence within a target pre-mRNA results in preferential expression of a wild-type target protein isomer. In embodiments, hybridization of antisense oligonucleotides to a target sequence within a target pre-mRNA results in expression of a re-spliced target protein comprising an active fragment of a wild-type target protein.

[0371] The mechanism of an antisense oligonucleotide is a result of hybridization of the antisense oligonucleotide with a target nucleic acid. In embodiments, an antisense oligonucleotide that hybridizes to its target sequence suppresses expression of the target protein. In embodiments, hybridization of an antisense oligonucleotide to its target sequence suppresses expression of one or more wild-type target protein isomers. In embodiments, hybridization of an antisense oligonucleotide to its target sequence upregulates expression of the target protein. In embodiments, hybridization of an antisense oligonucleotide to its target sequence increases expression of one or more wild-type target protein isomers.

[0372] Methods of producing antisense oligonucleotides are known in the art and can be readily adapted to produce an antisense oligonucleotide that targets any polynucleotide sequence. Selection of antisense oligonucleotide sequences specific for a given target sequence is based upon analysis of the chosen target sequence and determination of secondary structure, Tm, binding energy, and relative stability. Antisense oligonucleotides may be selected based upon their relative inability to form dimers, hairpins, or other secondary structures that would reduce or prohibit specific binding to a target mRNA in a host cell. Target regions of mRNA can include those regions at or near the AUG translation initiation codon and those sequences that are substantially complementary to 5' regions of the mRNA. These secondary structure analyses and target site selection considerations can be performed, for example, using v.4 of the OLIGO primer analysis software (Molecular Biology Insights) and/or the BLASTN 2.0.5 algorithm software (Altschul et ai, Nucleic Acids Res. 1997, 25(17):3389-402).

[0373] In embodiments, an oligonucleotide, for example, an antisense oligonucleotide, can alter one or more aspects of the splicing, translation, or expression of a target gene, e.g., by altering the splicing of a eukaryotic target pre-mRNA. The oligonucleotide comprises a nucleic acid sequence that is complementary to a sequence found within a target pre-mRNA sequence, for example, at sequence that includes at least a portion of an exon, at least a portion of an intron, or both. The use of these oligonucleotides provides a direct genetic approach that has the ability to modulate splicing of specific disease-causing genes. The principle behind antisense technology is that an antisense oligonucleotide, which hybridizes to a target nucleic acid, modulates gene expression activities such as splicing or translation through one of a number of antisense mechanisms. The sequence-specificity of the oligonucleotide makes this technique extremely attractive as a therapeutic to selectively modulate the splicing of pre-mRNA involved in the pathogenesis of any one of a variety of diseases. Antisense technology is an effective means for changing the expression of one or more specific gene products and can therefore prove to be useful in a number of therapeutic, diagnostic, and research applications.

[0374] The oligonucleotide may contain one or more asymmetric centers and thus give rise to enantiomers, diastereomers, and other stereoisomeric configurations that may be defined, in terms of absolute stereochemistry, as (R) or (S), α or β, or as (D) or (L). All such possible isomers, as well as their racemic and optically pure forms can be included in the oligonucleotide.

Oligonucleotide hybridization site

[0375] Antisense mechanisms rely on hybridization of the antisense oligonucleotide to the target nucleic acid. In embodiments, antisense oligonucleotides are provided that are complementary to a target nucleic acid. In embodiments, the target nucleic acid sequence is present in a pre-mRNA molecule. In embodiments, the target nucleic acid sequence is present in an exon of a pre-mRNA molecule. In embodiments, the target nucleic acid sequence is present in an intron of a pre-mRNA molecule.

[0376] Pre-mRNA molecules are made in the nucleus and are processed before or during transport to the cytoplasm for translation. Processing of the pre-mRNAs includes addition of a 5′ methylated cap and an approximately 200-250 base poly(A) tail to the 3′ end of the transcript. The next step in mRNA processing is splicing of the pre-mRNA, which occurs in the maturation of 90-95% of mammalian mRNAs. Introns (or intervening sequences) are regions of a primary transcript (or the DNA encoding it) that are not included in the coding sequence of the mature mRNA. Exons are regions of a primary transcript that remain in the mature mRNA when it reaches the cytoplasm. The exons are spliced together to form the mature mRNA sequence. Splice junctions are also referred to as splice sites with the 5′ side of the junction often called the "5′ splice site," or "splice donor site", and the 3′ side called the "3′ splice site" or "splice acceptor site." In splicing, the 3′ end of an upstream exon is joined to the 5′ end of the downstream exon. Thus, the unspliced RNA (or pre-mRNA) has an exon/intron junction at the 5′ end of an intron and an intron/exon junction at the 3′ end of an intron. After the intron is removed, the exons are contiguous at what is sometimes referred to as the exon/exon junction or boundary in the mature mRNA. Cryptic splice sites are those which are less often used but may be used when the usual splice site is blocked or unavailable. Alternative splicing, defined as the splicing together of different combinations of exons, often results in multiple mRNA transcripts from a single gene.

[0377] In embodiments, an oligonucleotide can hybridize with a sequence in a splice site. In embodiments, an oligonucleotide can hybridize with a sequence comprising part of a splice site. In embodiments, an oligonucleotide can hybridize with a sequence comprising part or all of a splice site. In embodiments, an oligonucleotide can hybridize with a sequence comprising part or all of a splice donor site. In embodiments, an oligonucleotide can hybridize with a sequence comprising part or all of a splice acceptor site. In embodiments, an oligonucleotide can hybridize

with a sequence comprising part or all of a cryptic splice site. In embodiments, an oligonucleotide can hybridize with a sequence comprising an exon/intron junction.

[0378] Pre-mRNA splicing involves two sequential biochemical reactions. Both reactions involve the spliceosomal transesterification between RNA nucleotides. In a first reaction, the 2′-OH of a specific branch-point nucleotide within an intron, which is defined during spliceosome assembly, performs a nucleophilic attack on the first nucleotide of the intron at the 5′ splice site forming a lariat intermediate. In a second reaction, the 3′-OH of the released 5′ exon performs a nucleophilic attack at the last nucleotide of the intron at the 3′ splice site thus joining the exons and releasing the intron lariat. Pre-mRNA splicing is regulated by intronic silencer sequence (ISS) and terminal stem loop (TSL) sequences. As used herein, the terms "intronic silencer sequences (ISS)" and "terminal stem loop (TSL)" refer to sequence elements within introns and exons, respectively, that control alternative splicing by the binding of trans-acting protein factors within a pre-mRNA thereby resulting in differential use of splice sites. Typically, intronic silencer sequences are between 8 and 16 nucleotides and are less conserved than the splice sites at exon-intron junctions. Terminal stem loop sequences are typically between 12 and 24 nucleotides and form a secondary loop structure due to the complementarity, and hence binding, within the 12-24 nucleotide sequence.

[0379] In embodiments, an oligonucleotide hybridizes with a sequence comprising part or all of an intronic silencer sequence. In embodiments, an oligonucleotide hybridizes with a sequence comprising part or all of a terminal stem loop.

[0380] Up to 50% of human genetic diseases resulting from a point mutation are caused by aberrant splicing. Such point mutations can either disrupt a current splice site or create a new splice site, resulting in mRNA transcripts comprised of a different combination of exons or with deletions in exons. Point mutations also can result in activation of a cryptic splice site or disrupt regulatory cis elements (i.e., splicing enhancers or silencers).

[0381] In embodiments, an oligonucleotide hybridizes with a sequence comprising part or all of an aberrant splice site resulting from a mutation in the target gene. In embodiments, the oligonucleotide hybridizes with a sequence comprising part or all of a regulatory element. Also provided are antisense oligonucleotides targeted to cis regulatory elements. In embodiments, the regulatory element is in an exon. In embodiments, the regulatory element is in an intron.

[0382] In embodiments, the oligonucleotide may be specifically hybridizable with a translation initiation codon region, a 5′ cap region, an intron/exon junction, a coding sequence, a translation termination codon region or sequences in the 5′- or 3′-untranslated region. In embodiments, the oligonucleotide may hybridize with part or all of a pre-mRNA splice site, an exon-exon junction, or an intron-exon junction. In embodiments, the oligonucleotide may hybridize with an aberrant fusion junction due to a rearrangement or a deletion. In embodiments, the oligonucleotide may hybridize with particular exons in alternatively spliced mRNAs.

[0383] In embodiments, the oligonucleotide can hybridize with a sequence between 5 and 50 nucleotides in length, which can also be referred to as the length of the oligonucleotide. In embodiments, the oligonucleotide is from 5 to 50, 10 to 40, 15 to 30, 20 to 30, or 20 to 25 nucleotides in length. In embodiments, the oligonucleotide is from 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 nucleotides in length. In embodiments, the oligonucleotide is at least 15, 16, 17, 18, 19, or 20 and up to 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 nucleotides in length. In embodiments, the oligonucleotide is 10 nucleotides in length. In embodiments, the oligonucleotide is 15 nucleotides in length. In embodiments, the oligonucleotide is 16 nucleotides in length. In embodiments, the oligonucleotide is 17 nucleotides in length. In embodiments, the oligonucleotide is 18 nucleotides in length. In embodiments, the oligonucleotide is 19 nucleotides in length. In embodiments, the oligonucleotide is 20 nucleotides in length. In embodiments, the oligonucleotide is 21 nucleotides in length. In embodiments, the oligonucleotide is 22 nucleotides in length. In embodiments, the oligonucleotide is 23 nucleotides in length. In embodiments, the oligonucleotide is 24 nucleotides in length. In embodiments, the oligonucleotide is 25 nucleotides in length. In embodiments, the oligonucleotide is 26 nucleotides in length. In embodiments, the oligonucleotide is 27 nucleotides in length. In embodiments, the oligonucleotide is 28 nucleotides in length. In embodiments, the oligonucleotide is 29 nucleotides in length. In embodiments, the oligonucleotide is 30 nucleotides in length.

[0384] In embodiments, the oligonucleotide may be less than 100 percent complementary to a target nucleic acid sequence. As used herein, the term "percent complementary" refers to the number of nucleobases of an oligonucleotide that have nucleobase complementarity with a corresponding nucleobase of an oligonucleotide or nucleic acid divided by the total length (number of nucleobases) of the oligonucleotide. One skilled in the art recognizes that the

inclusion of mismatches is possible without eliminating the activity of the antisense oligonucleotide. In embodiments, an oligonucleotide may contain up to 20% nucleotides that disrupt base pairing of the oligonucleotide to the target nucleic acid. In embodiments, the oligonucleotides contain no more than 15%, no more than 10%, no more than 5%, or no mismatches. In embodiments, the oligonucleotides contain no more than 1, 2, 3, 4, or 5 mismatches. In embodiments, the oligonucleotides are at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% complementary to a target nucleic acid. Percent complementarity of an oligonucleotide is calculated by dividing the number of complementary nucleobases by the total number of nucleobases of the oligonucleotide. Percent complementarity of a region of an oligonucleotide is calculated by dividing the number of complementary nucleobases in the region by the total number of nucleobases region.

[0385] In embodiments, incorporation of nucleotide affinity modifications allows for a greater number of mismatches compared to an unmodified oligonucleotide. Similarly, certain oligonucleotide sequences may be more tolerant to mismatches than other oligonucleotide sequences. One of ordinary skill in the art is capable of determining an appropriate number of mismatches between oligonucleotides, or between an oligonucleotide and a target nucleic acid, such as by determining melting temperature (Tm). Tm or ΔTm can be calculated by techniques that are familiar to one of ordinary skill in the art. For example, techniques described in Freier et al. (Nucleic Acids Research, 1997, 25, 22: 4429-4443) allow one of ordinary skill in the art to evaluate nucleotide modifications for their ability to increase the melting temperature of an RNA:DNA duplex.

Antisense mechanisms

[0386] The oligonucleotide can be an antisense oligonucleotide that modulates one or more aspects of protein transcription, translation, and expression. In embodiments, an oligonucleotide that hybridizes to a target sequence within a target pre-mRNA can modulate one or more aspects of pre-mRNA splicing. As used herein, modulation of splicing refers to altering the processing of a pre-mRNA transcript such that the spliced mRNA molecule contains either a different combination of exons as a result of exon skipping or exon inclusion, a deletion in one or more exons, or the deletion or addition of a sequence not normally found in the spliced mRNA (e.g., an intron sequence). In embodiments, oligonucleotide hybridization to a target sequence within a

pre-mRNA molecule restores native splicing to a mutated pre-mRNA sequence. In embodiments, oligonucleotide hybridization results in alternative splicing of the target pre-mRNA. In embodiments, oligonucleotide hybridization results in exon inclusion or exon skipping of one or more exons. In embodiments, the skipped exon sequence comprises a frameshift mutation, a nonsense mutation, or a missense mutation. In embodiments, the skipped exon sequence comprises a nucleic acid deletion, substitution, or insertion. In embodiments, the skipped exon itself does not comprise a sequence mutation, but a neighboring exon comprises a mutation leading to a frameshift mutation or a nonsense mutation. In embodiments, deletion of an exon that does not comprise a sequence mutation restores the reading frame of the mature mRNA. In embodiments, oligonucleotide hybridization to a target sequence within a target pre-mRNA results in preferential expression of a wild-type target protein isomer. In embodiments, oligonucleotide hybridization to a target sequence within a target pre-mRNA results in expression of a re-spliced target protein comprising an active fragment of a wild-type target protein.

[0387] The mechanism of an antisense oligonucleotide includes hybridization of the antisense oligonucleotide with a target nucleic acid. In embodiments, an oligonucleotide that hybridizes to its target sequence suppresses expression of the target protein. In embodiments, an oligonucleotide that hybridizes to its target sequence suppresses expression of one or more wild-type target protein isomers. In embodiments, the oligonucleotide that hybridizes to its target sequence upregulates expression of the target protein. In embodiments, an oligonucleotide that hybridizes to its target sequence increases expression of one or more wild-type target protein isomers.

[0388] The efficacy of oligonucleotides may be assessed by evaluating the antisense activity effected by their administration. As used herein, the term "antisense activity" refers to any detectable and/or measurable activity attributable to the hybridization of an antisense oligonucleotide to its target nucleic acid. Such detection and or measuring may be direct or indirect. In embodiments, antisense activity is assessed by detecting and or measuring the amount of target protein. In embodiments, antisense activity is assessed by detecting and or measuring the amount of re-spliced target protein. In embodiments, antisense activity is assessed by detecting and/or measuring the amount of target nucleic acids and/or cleaved target nucleic acids and/or alternatively spliced target nucleic acids.

Antisense oligonucleotide design

[0389] Design of oligonucleotides will depend upon the sequence being targeted. Targeting an oligonucleotide to a particular target nucleic acid molecule can be a multistep process. The process usually begins with the identification of a target nucleic acid whose expression is to be modulated. As used herein, the terms "target nucleic acid" and "nucleic acid encoding a target gene" encompass DNA encoding a selected target gene, RNA (including pre-mRNA and mRNA) transcribed from such DNA, and also cDNA derived from such RNA. For example, the target nucleic acid can be a cellular gene (or mRNA transcribed from the gene) whose expression is associated with a particular disorder or disease state, or a nucleic acid molecule from an infectious agent.

[0390] One of skill in the art will be able to design, synthesize, and screen antisense oligonucleotides of different nucleobase sequences to identify a sequence that results in antisense activity. For example, antisense oligonucleotides can be designed such that it alters splicing of a target pre-mRNA or inhibits expression of a target protein. Methods for designing, synthesizing, and screening antisense oligonucleotides for antisense activity against a preselected target nucleic acid can be found, for example in "Antisense Drug Technology, Principles, Strategies, and Applications" Edited by Stanley T. Crooke, CRC Press, Boca Raton, Florida, which is incorporated by reference in its entirety for any purpose.

[0391] In embodiments, the antisense oligonucleotides comprise modified nucleosides, modified internucleoside linkages and/or conjugate groups.

[0392] In embodiments, the antisense oligonucleotide is a “tricyclo-DNA (tc-DNA)”, which refers to 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.

Nucleosides

[0393] In embodiments, antisense oligonucleotides are provided comprising linked nucleosides. In embodiments, some or all of the nucleosides are modified nucleosides. In embodiments, one or more nucleosides comprise a modified nucleobase. In embodiments, one or more nucleosides comprises a modified sugar. Chemically modified nucleosides are routinely used for

incorporation into antisense oligonucleotides to enhance one or more properties, such as nuclease resistance, pharmacokinetics or affinity for a target RNA.

[0394] In general, a nucleobase is any group that contains one or more atom or groups of atoms capable of hydrogen bonding to a base of another nucleic acid. In addition to "unmodified" or "natural" nucleobases, such as the purine nucleobases adenine (A) and guanine (G), and the pyrimidine nucleobases thymine (T), cytosine (C) and uracil (U), many modified nucleobases or nucleobase mimetics known to those skilled in the art are amenable with the oligonucleotides described herein. The terms modified nucleobase and nucleobase mimetic can overlap, but generally a modified nucleobase refers to a nucleobase that is similar in structure to the parent nucleobase, such as for example a 7-deaza purine, a 5-methyl cytosine, or a G-clamp, whereas a nucleobase mimetic would include more complicated structures, such as for example a tricyclic phenoxazine nucleobase mimetic. Methods for preparation of the above noted modified nucleobases are well known to those skilled in the art.

[0395] In embodiments, oligonucleotides provided herein comprise one or more nucleosides having a modified sugar moiety. In embodiments, the furanosyl sugar ring of a natural nucleoside can be modified in a number of ways including, but not limited to, addition of a substituent group, bridging of two non-geminal ring atoms to form a bicyclic nucleic acid (BNA) and substitution of an atom or group such as -S-, -N(U)-, or -C(U^1)(U^2) for the ring oxygen at the 4'-position, where U, U^1, and U^2 are each independently selected from H or any suitable substituent. Modified sugar moieties are well known and can be used to alter, typically increase, the affinity of the antisense oligonucleotide for its target and/or increase nuclease resistance. A representative list of modified sugars includes but is not limited to non-bicyclic substituted sugars, especially non-bicyclic 2'-substituted sugars having a 2'-F, 2'-OCH_3, or a 2'-$O(CH_2)_2$-OCH_3 substituent group; and 4'-thio modified sugars. Sugars can also be replaced with sugar mimetic groups among others. In embodiments, the sugar is replaced with a six membered morpholine ring. Methods for the preparations of modified sugars are well known to those skilled in the art.

[0396] In embodiments, nucleosides comprise bicyclic modified sugars (BNA's), including LNA (4'-(CH_2)-O-2' bridge), 2'-thio-LNA (4'-(CH_2)-S-2' bridge), 2'-amino-LNA (4'-(CH_2)-NR-2' bridge), ENA (4'-(CH_2)2-O-2' bridge), 4'-$(CH_2)_3$-2' bridged BNA, 4'-$(CH_2CH(CH_3))$-2' bridged BNA" cEt (4'-$(CH(CH_3)$-O-2' bridge), and cMOE BNAs (4'-$(CH(CH_2OCH_3)$-O-2' bridge).

[0397] Also provided herein are "Locked Nucleic Acids" (LNAs) in which the 2'-hydroxyl group of the ribosyl sugar ring is linked to the 4' carbon atom of the sugar ring thereby forming a 2'-C,4'-C-oxymethylene linkage to form the bicyclic sugar moiety. The linkage can be a methylene (-CH_2-) group bridging the 2' oxygen atom and the 4' carbon atom, for which the term LNA is used for the bicyclic moiety; in the case of an ethylene group in this position, the term ENA™ is used (Singh et al., Chem. Commun., 1998, 4, 455-456; ENA™: Morita et al., Bioorganic Medicinal Chemistry, 2003, 11, 2211-2226). LNA and other bicyclic sugar analogs display very high duplex thermal stabilities with complementary DNA and RNA (Tm = +3 to +10° C), stability towards 3'-exonucleolytic degradation and good solubility properties. Potent and nontoxic antisense oligonucleotides containing LNAs have been described (Wahlestedt et al., Proc. Natl. Acad. Sci. U.S.A., 2000, 97, 5633-5638).

[0398] An isomer of LNA that has also been studied is alpha-L-LNA which has been shown to have improved stability against a 3'-exonuclease. The alpha-L-LNA's were incorporated into antisense gapmers and chimeras that showed potent antisense activity (Frieden et al., Nucleic Acids Research, 2003, 21, 6365-6372).

[0399] The synthesis and preparation of the LNA monomers adenine, cytosine, guanine, 5-methyl-cytosine, thymine and uracil, along with their oligomerization, and nucleic acid recognition properties have been described (Koshkin et al., Tetrahedron, 1998, 54, 3607-3630). LNAs and preparation thereof are also described in WO 98/39352 and WO 99/14226.

[0400] Analogs of LNA, phosphorothioate-LNA and 2'-thio-LNAs, have also been prepared (Kumar et al., Bioorg. Med. Chem. Lett., 1998, 8, 2219-2222). Preparation of locked nucleoside analogs containing oligodeoxyribonucleotide duplexes as substrates for nucleic acid polymerases has also been described (Wengel et al., WO 99/14226). Synthesis of 2'-amino-LNA, a novel conformationally restricted high-affinity oligonucleotide analog has been described in the art (Singh et al., J. Org. Chem., 1998, 63, 10035-10039). In addition, 2'-Amino- and 2'-methylamino-LNA's have been prepared and the thermal stability of their duplexes with complementary RNA and DNA strands has been previously reported.

Internucleoside Linkages

[0401] Described herein are internucleoside linking groups that link the nucleosides or otherwise modified monomer units together thereby forming an antisense oligonucleotide. The two main classes of internucleoside linking groups are defined by the presence or absence of a phosphorus

atom. Representative phosphorus containing internucleoside linkages include, but are not limited to, phosphodiesters, phosphotriesters, methylphosphonates, phosphoramidate (including phosphorodiamidate), and phosphorothioates. Representative non-phosphorus containing internucleoside linking groups include, but are not limited to, methylenemethylimino (-CH_2-$N(CH_3)$-O-CH_2-), thiodiester (-O-C(O)-S-), thionocarbamate (-O-C(O)(NH)-S-), siloxane (-O-$Si(H)_2$-O-), and N,N'-dimethylhydrazine (-CH_2-$N(CH_3)$-$N(CH_3)$-). Antisense oligonucleotides having non-phosphorus internucleoside linking groups are referred to as oligonucleosides. Modified internucleoside linkages, compared to natural phosphodiester linkages, can be used to alter, typically increase, nuclease resistance of the antisense oligonucleotide. Internucleoside linkages having a chiral atom can be prepared racemic, chiral, or as a mixture. Representative chiral internucleoside linkages include, but are not limited to, alkylphosphonates and phosphorothioates. Methods of preparation of phosphorous-containing and non-phosphorous-containing linkages are well known to those skilled in the art.

[0402] In embodiments, a phosphate group can be linked to the 2', 3' or 5' hydroxyl moiety of the sugar. In forming oligonucleotides, the phosphate groups covalently link adjacent nucleosides to one another to form a linear polymeric oligonucleotide. Within oligonucleotides, the phosphate groups are commonly referred to as forming the internucleoside backbone of the oligonucleotide. The normal linkage or backbone of RNA and DNA is a 3' to 5' phosphodiester linkage.

Conjugate Groups

[0403] In embodiments, an oligonucleotide is modified by covalent attachment of one or more conjugate groups. In general, conjugate groups can modify one or more properties of an oligonucleotide, including, but not limited to, pharmacodynamic, pharmacokinetic, binding, absorption, cellular distribution, cellular uptake, charge and clearance. Conjugate groups are routinely used in the chemical arts and are linked directly or via an optional linking moiety or linking group to anoligonucleotide. Conjugate groups include without limitation, intercalators, reporter molecules, polyamines, polyamides, polyethylene glycols, thioethers, polyethers, cholesterols, thiocholesterols, cholic acid moieties, folate, lipids, phospholipids, biotin, phenazine, phenanthridine, anthraquinone, adamantane, acridine, fluoresceins, rhodamines, coumarins and dyes. In embodiments, the conjugate group is a polyethylene glycol (PEG), and the PEG is conjugated to either the oligonucleotide or the cyclic peptide.

[0404] Conjugate groups include lipid moieties such as a cholesterol moiety (Letsinger et al., Proc. Natl. Acad. Sci. USA, 1989, 86, 6553); cholic acid (Manoharan et al., Bioorg. Med. Chem. Lett., 1994, 4, 1053); a thioether, e.g., hexyl-S-tritylthiol (Manoharan et al., Ann. N.Y. Acad. Sci., 1992, 660, 306; Manoharan et al., Bioorg. Med. Chem. Let., 1993, 3, 2765); a thiocholesterol (Oberhauser et al., Nucl. Acids Res., 1992, 20, 533); an aliphatic chain, e.g., dodecandiol or undecyl residues (Saison-Behmoaras et al., EMBO J., 1991, 10, 111; Kabanov et al., FEBS Lett., 1990, 259, 327; Svinarchuk et al., Biochimie, 1993, 75, 49); a phospholipid, e.g., di-hexadecyl-rac-glycerol or triethylammonium-1,2-di-O-hexadecyl-rac-glycero-3-H-phosphonate (Manoharan et al., Tetrahedron Lett., 1995, 36, 3651; Shea et al., Nucl. Acids Res., 1990, 18, 3777); a polyamine or a polyethylene glycol chain (Manoharan et al., Nucleosides & Nucleotides, 1995, 14, 969); adamantane acetic acid (Manoharan et al., Tetrahedron Lett., 1995, 36, 3651); a palmityl moiety (Mishra et al., Biochim. Biophys. Acta, 1995, 1264, 229); or an octadecylamine or hexylamino-carbonyl-oxycholesterol moiety (Crooke et al., J. Pharmacol. Exp. Ther., 1996,277,923).

[0405] Linking groups or bifunctional linking moieties such as those known in the art can be included with the oligonucleotides provided herein. Linking groups are useful for attachment of chemical functional groups, conjugate groups, reporter groups, and other groups to selective sites in an oligonucleotide, such as an antisense oligonucleotide. In embodiments, a bifunctional linking moiety comprises a hydrocarbyl moiety having two functional groups. Any of the linkers described here may be used. In embodiments, the linker comprises a chain structure or an oligomer of repeating units such as ethylene glycol or amino acid units. Examples of functional groups that are used in a bifunctional linking moiety include, but are not limited to, electrophiles for reacting with nucleophilic groups and nucleophiles for reacting with electrophilic groups. In embodiments, bifunctional linking moieties include amino, hydroxyl, carboxylic acid, thiol, unsaturations (e.g., double or triple bonds), and the like. Some nonlimiting examples of bifunctional linking moieties include 8-amino-3,6-dioxaoctanoic acid (ADO), succinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate (SMCC) and 6-aminohexanoic acid (AHEX or AHA). Other linking groups include, but are not limited to, substituted C_1-C_{10} alkyl, substituted or unsubstituted C_2-C_{10} alkenyl or substituted or unsubstituted C_2-C_{10} alkynyl, wherein a nonlimiting list of substituent groups includes hydroxyl, amino, alkoxy, carboxy, benzyl, phenyl, nitro, thiol, thioalkoxy, halogen, alkyl, aryl, alkenyl and alkynyl.

[0406] In embodiments, an oligonucleotide may be linked to a 10 arginine-serine dipeptide repeat. Oligonucleotides linked to 10 arginine-serine dipeptide repeats for the artificial recruitment of splicing enhancer factors have been applied in vitro to induce inclusion of mutated BRCA1 and SMN2 exons that otherwise would be skipped. See Cartegni and Krainer 2003, incorporated by reference herein.

[0407] In embodiments, the cargo is an oligonucleotide. In embodiments, the oligonucleotide is a therapeutic oligonucleotide. In embodiments, the oligonucleotide is an antisense oligonucleotide. In embodiments, the the cargo is an oligonucleotide that can hybridize to a nucleic acid sequence of the human *DMD* gene, which encodes dystrophin. In embodiments, the oligonucleotide can hybridize to at least a portion of exon 50 of the *DMD* gene. In embodiments, the oligonucleotide that hybridizes to at least a portion of exon 50 of the *DMD* gene may also hybridize to at least a portion of 5' flanking intron of exon or at least a portion the 3' flanking intron of exon 50. In embodiments, the oligonucleotide that hybridizes to at least a portion of exon 50 of the *DMD* gene is from 5 to 50, 10 to 40, 15 to 35, or 20 to 30 nucleotides in length, for example, at least 15, 16, 17, 18, 19, or 20, and up to 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, or 35 nucleotides in length. In embodiments, the oligonucleotide that hybridizes to at least a portion of exon 50 of the *DMD* gene is from 15 to 35 nucleic acids in length, for example, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, or 35 nucleotides in length.

[0408] In embodiments, an oligonucleotide can hybridize to a nucleic acid sequence comprising at least a portion of the 5' flanking intron of exon 50 of the *DMD* gene. In embodiments, an oligonucleotide can hybridize to a nucleic acid sequence comprising at least a portion of the 3' flanking intron of exon 50 of the *DMD* gene. In embodiments, an oligonucleotide can hybridize to a nucleic acid sequence within exon 50 of the *DMD* gene. In embodiments, the antisense oligonucleotide can hybridize to a nucleic acid sequence that spans an intron-exon or exon-intron junction of exon 50 of the *DMD* gene.

[0409] In the sequences shown herein, the last nucleotide of the Acceptor intron sequence preceding exon 50 is represented as nucleotide "-1," the first nucleotide of exon 50 is represented as nucleotide "0," and the first nucleotide of the Donor intron sequence following exon 50 is represented as "-1."

[0410] In embodiments, the nucleic acid sequence of exon 50 of the *DMD* gene is shown as SEQ ID NO:1 below (from 5' to 3'):

```
AGGAAGTTAG AAGATCTGAG CTCTGAGTGG AAGGCGGTAA ACCGTTTACT
0    9     19     29     39     49

TCAAGAGCTG AGGGCAAAGC AGCCTGACCT AGCTCCTGGA CTGACCACTA
    59     69     79     89     99

TTGGAGCCT (SEQ ID NO: 1)
  108
```

[0411] In embodiments, the oligonucleotide comprises 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29 or 30 consecutive nucleotides (e.g. the oligonucleotide is a 15-mer, 16-mer, 17-mer, 18-mer, 19-mer, 20-mer, 21-mer, 22-mer, 23-mer, 24-mer, 25-mer, 26-mer, 27-mer, 28-mer, 29-mer, or 30-mer) that are complementary to consecutive nucleotides of SEQ ID NO: 1, wherein the first nucleotide of the oligonucleotide hybridizes to a nucleotide of exon 50 of the *DMD* gene at position -10, -9, -8, -7, -6, -5, -4, -3, -2, -1, 0, +1, +2, +3, +4, +5, +6, +7, +8, +9, +10, +11, +12, +13, +14, +15, +16, +17, +18, +19, +20, +21, +22, +23, +24, +25, +26, +27, +28, +29, +30, +31, +32, +33, +34, +35, +36, +37, +38, +39, +40, +41, +42, +43, +44, +45, +46, +47, +48, +49, +50, +51, +52, +53, +54, +55, +56, +57, +58, +59, +60, +61, +62, +63, +64, +65, +66, +67, +68, +69, +70, +71, +72, +73, +74, +75, +76, +77, +78, +79, +80, +81, +82, +83, +84, +85, +86, +87, +88, +89, +90, +91, +92, +93, +94, +95, +96, +97, +98, +99, +100, +101, +102, +103, +104, +105, +106, +107, +108, +109 of SEQ ID NO: 1. In embodiments, the oligonucleotide comprises 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 consecutive nucleotides (e.g. the oligonucleotide is a 15-mer, 16-mer, 17-mer, 18-mer, 19-mer, 20-mer, 21-mer, 22-mer, 23-mer, 24-mer, 25-mer, 26-mer, 27-mer, 28-mer, 29-mer or 30-mer) that are complementary to consecutive nucleotides of SEQ ID NO: 1, wherein the first nucleotide of the oligonucleotide hybridizes to a nucleotide of exon 50 of the *DMD* gene at position -5, -4, -3, -2, -1, 0, +1, +2, +3, +4, +5, +6, +7, +8, +9, +10, +11, +12, +13, +14, +15, +16, +17, +18, +19, +20, +21, +22, +23, +24, +25, +26, +27, +28, +29, +30, +31, +32, +33, +34, +35, +36, +37, +38, +39, +40, +41, +42, +43, +44, +45, +46, +47, +48, +49, +50, +51, +52, +53, +54, +55, +56, +57, +58, +59, +60, +61, +62, +63, +64, +65, +66, +67, +68, +69, +70, +71, +72, +73, +74, +75, +76, +77, +78, +79, +80, +81, +82, +83, +84, +85, +86, +87, +88, +89, +90, +91, +92, +93, +94, +95, +96, +97, +98, +99, +100, +101, +102, +103, +104, +105, +106, +107, +108, +109 of SEQ ID NO: 1. In

embodiments, the oligonucleotide comprises 20, 21, 22, 23, 24, or 25 consecutive nucleotides (e.g. the oligonucleotide is a 20-mer, 21-mer, 22-mer, 23-mer, 24-mer, or 25-mer that are complementary to consecutive nucleotides of SEQ ID NO: 1, wherein the first nucleotide of the oligonucleotide hybridizes to a nucleotide of exon 50 of the *DMD* gene at position -5, -4, -3, -2, -1. In embodiments, the oligonucleotide comprises 20, 21, 22, 23, 24, or 25 consecutive nucleotides (e.g. the oligonucleotide is a 20-mer, 21-mer, 22-mer, 23-mer, 24-mer, or 25-mer that are complementary to consecutive nucleotides of SEQ ID NO: 1, wherein the first nucleotide of the oligonucleotide hybridizes to a nucleotide of exon 50 of the *DMD* gene at position 0, +1, +2, +3, +4, +5, +6, +7, +8, +9, +10, +11, +12, +13, +14, +15, +16, +17, +18, +19, +20, +21, +22, +23, +24, or +25. In embodiments, the oligonucleotide comprises 20, 21, 22, 23, 24, or 25 consecutive nucleotides (e.g. the oligonucleotide is a 20-mer, 21-mer, 22-mer, 23-mer, 24-mer, or 25-mer that are complementary to consecutive nucleotides of SEQ ID NO: 1, wherein the first nucleotide of the oligonucleotide hybridizes to a nucleotide of exon 50 of the *DMD* gene at position +50, +51, +52, +53, +54, +55, +56, +57, +58, +59, +60, +61, +62, +63, +64, +65, +66, +67, +68, +69, +70, +71, +72, +73, +74, +75, +76, +77, +78, +79, +80, +81, +82, +83, +84, +85, +86, +87, +88, +89, or +90 of SEQ ID NO: 1. As used herein, the "first nucleotide" refers to the first 5' nucleotide of the oligonucleotide cargo.

[0412] The nucleic acid sequence of exon 50 of the *DMD* gene (upper case in bold and underlined) with 50 nt of the 5' flanking upstream intron (not underlined) and 50 nt of the 3' flanking downstream (not underlined) intron is shown as SEQ ID NO:2 below.

[0413] Exon 50 (*lowercase italics* = intron; **UPPERCASE/BOLD and UNDERLINED** = exon). Note: the first nucleotide of the exonic sequence is given the number "0".

5'

atcttcaaag tgttaatcga ataagtaatg tgtatgcttt tctgttaaag
-50 -40 -30 -20 -10

AGGAAGTTAG AAGATCTGAG CTCTGAGTGG AAGGCGGTAA ACCGTTTACT
0 9 19 29 39 49

TCAAGAGCTG AGGGCAAAGC AGCCTGACCT AGCTCCTGGA CTGACCACTA
59 69 79 89 99

TTGGAGCCT*g taagtatact ggatcccatt ctctttggct ctagctattt*
+108/-1 -11 -21

*gttcaaaag 3′ (**SEQ ID NO.2**)*

[0414] The upstream (5') intronic sequence (residues -50 to -1) and downstream (3') intronic sequence (residues -1 to -50) are shown in lowercase and italics; the exonic sequence (residues +0 to +108) is shown in uppercase in bold and underlining. In embodiments, the nucleic acid sequence for human duchenne muscle dystrophy (*DMD*) gene for dystrophin exon 50 comprises the 109 nucleotides shown in uppercase in bold and underlining.

[0415] In embodiments, the oligonucleotide comprises nucleotides that are complementary to one or more nucleotides of the 5' intronic sequence flanking exon 50 of the *DMD* gene. In embodiments, the oligonucleotide comprises nucleotides that are complementary to one or more nucleotides of the 5' intronic sequence flanking exon 50, and one or more nucleotides of the exonic sequence of exon 50. In embodiments, the oligonucleotide comprises nucleotides that are complementary to one or more nucleotides of the 3' intronic sequence following exon 50. In embodiments, the oligonucleotide comprises nucleotides that are complementary to one or more nucleotides of the 3' intronic sequence flanking exon 50, and one or more nucleotides of the exonic sequence of exon 50.

[0416] In embodiments, the oligonucleotide hybridizes to a nucleic acid sequence that spans an intron-exon or exon-intron junction of exon 50 of the *DMD* gene. In embodiments, the oligonucleotide is complementary to a target nucleic acid sequence that includes at least 1, at least 2, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, at least 10, at least 11, at least 12, at least 13, at least 14, at least 15, at least 16, at least 17, at least 18, at least 19, at least 20, or more consecutive nucleotides of the upstream (5') intron preceding exon 50 (i.e., starting at position -20, -19, -18, -17, -16, -15, -14, -13, -12, -11, -10, -9, -8, -7, -6, -5, -4, -3, -2, or -1).

[0417] In embodiments, the oligonucleotide is complementary to a target nucleic acid sequence that includes at least the first nucleotide at the 5' end of exon 50 (i.e, position 0). In embodiments, the oligonucleotide is complementary to a target nucleic acid sequence that includes at least 1, at least 2, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, at least 10, at least 11, at least 12, at least 13, at least 14, at least 15, at least 16, at least 17, at least 18, at least 19, at least 20, at least 21, at least 22, at least 23, at least 24, at least 25, at least 26, at least 27, at least 28, at least 29, at least 30, at least 31, at least 32, at least 33, at least 34, at

least 35, or more consecutive nucleotides of exon 50, including first nucleotide at the 5' end of exon 50 (i.e., starting from position 0).

[0418] In embodiments, the oligonucleotides that hybridizes to exon 50 of the *DMD* gene are shown in nucleic acid sequences within **Tables 11A-11D**, **12A-12D**, or **13,** the reverse complement thereof, or a sequence with at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% nucleic acid sequence identity thereto.

[0419] In embodiments, the oligonucleotide hybridizes to a nucleic acid sequence that spans a portion of the exon 50 of the *DMD* gene. In embodiments, the exonic sequence of SEQ ID NO:2 is shown in bold and underlining (residues 0 to +108). In embodiments, the oligonucleotide has a nucleic acid sequence as shown in **Tables 11A-11D**, **12A-12D**, or **13**, the reverse complement thereof, or a sequence with at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% nucleic acid sequence identity thereto.

[0420] In embodiments, the oligonucleotide comprises 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 consecutive nucleotides (e.g. the oligonucleotide is a 20-mer, 21-mer, 22-mer, 23-mer, 24-mer, 25-mer, 26-mer, 27-mer, 28-mer, 29-mer, or 30-mer) that are complementary to consecutive nucleotides of SEQ ID NO:1, wherein the first nucleotide of the oligonucleotide hybridizes to a nucleotide of exon 50 at position -10, -9, -8, -7, -6, -5, -4, -3, -2, -1, 0, +1, +2, +3, +4, +5, +6, +7, +8, +9, +10, +11, +12, +13, +14, +15, +16, +17, +18, +19, +20, +21, +22, +23, +24, +25, +26, +27, +28, +29, +30, +31, +32, +33, +34, +35 of SEQ ID NO:1, as shown in **Tables 11A-11D**, **12A-12D**, or **13,** the reverse complement thereof, or a sequence with at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% nucleic acid sequence identity thereto. In embodiments, the first nucleotide of the oligonucleotide hybridizes to a nucleotide of exon 50 of the *DMD* gene at position +60, +61, +62, +63, +64, +65, +66, +67, +68, +69, +70, +71, +72, +73, +74, +75, +76, +77, +78, +79, +80, +81, +82, +83, +84, +85, +86, +87, +88, +89, +90, +91, +92, +93, +94, +95, +96, +96, +98, +99, +100, +101, +102, +103, +104, +105, +106, +107, +108, +109 of SEQ ID NO: 1, as shown in **Tables 11A-11D**, **12A-12D**, or **13**, the reverse complement thereof, or a sequence with at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%,

96%, 97%, 98%, or 99% nucleic acid sequence identity thereto. Exon 50 has the following sequence:

```
AGGAAGTTAG AAGATCTGAG CTCTGAGTGG AAGGCGGTAA ACCGTTTACT
0    9     19     29     39     49

TCAAGAGCTG AGGGCAAAGC AGCCTGACCT AGCTCCTGGA CTGACCACTA
    59     69     79     89     99

TTGGAGCCT (SEQ ID NO: 1).
   108
```

[0421] In embodiments, the oligonucleotide hybridizes to a sequence of exon 50 of the *DMD* gene selected from any one of the nucleic acid sequences shown in **Tables 11A-11D, 12A-12D**, or **13.** In embodiments, the oligonucleotide that hybridizes to exon 50 of the *DMD* gene is selected from any one of the nucleic acid sequences shown in **Tables 11A-11D, 12A-12D**, or **13,** the reverse complement thereof, or a sequence with at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% nucleic acid sequence identity thereto. In embodiments, the oligonucleotide that hybridizes to exon 50 of the *DMD* gene comprises one or more modified nucleic acids, one or more modified internucleotide linkages, or a combination thereof. In embodiments, the oligonucleotide that hybridizes to exon 50 comprises one or more morpholine rings, one or more phosphorodiamidate linkages, or a combination thereof. In embodiments, the oligonucleotide that hybridizes to exon 50 of the *DMD* gene is an antisense phosphorodiamidate morpholino oligonucleotide (PMO) with a sequence selected from any one of the nucleic acid sequences within **Tables 11A-11D, 12A-12D**, or **13,** the reverse complement thereof, or a sequence with at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% nucleic acid sequence identity thereto**.**

Table 11A: *21-mer oligonucleotides that hybridize to Exon 50.*

AC Name	Nucleic Acid Sequence (Reverse complement 5' – 3')
21mer-n5	GATCTTCTAACTTCCTcttta
21mer-n4	AGATCTTCTAACTTCCTcttt
21mer-n3	CAGATCTTCTAACTTCCTctt
21mer-n2	TCAGATCTTCTAACTTCCTct
21mer-n1	CTCAGATCTTCTAACTTCCTc
21mer-00	GCTCAGATCTTCTAACTTCCT

21mer-01	AGCTCAGATCTTCTAACTTCC
21mer-02	GAGCTCAGATCTTCTAACTTC
21mer-03	AGAGCTCAGATCTTCTAACTT
21mer-04	CAGAGCTCAGATCTTCTAACT
21mer-05	TCAGAGCTCAGATCTTCTAAC
21mer-06	CTCAGAGCTCAGATCTTCTAA
21mer-07	ACTCAGAGCTCAGATCTTCTA
21mer-08	CACTCAGAGCTCAGATCTTCT
21mer-09	CCACTCAGAGCTCAGATCTTC
21mer-10	TCCACTCAGAGCTCAGATCTT
21mer-11	TTCCACTCAGAGCTCAGATCT
21mer-12	CTTCCACTCAGAGCTCAGATC
21mer-13	CCTTCCACTCAGAGCTCAGAT
21mer-14	GCCTTCCACTCAGAGCTCAGA
21mer-15	CGCCTTCCACTCAGAGCTCAG
21mer-16	CCGCCTTCCACTCAGAGCTCA
21mer-17	ACCGCCTTCCACTCAGAGCTC
21mer-18	TACCGCCTTCCACTCAGAGCT
21mer-19	TTACCGCCTTCCACTCAGAGC
21mer-20	TTTACCGCCTTCCACTCAGAG
21mer-21	GTTTACCGCCTTCCACTCAGA
21mer-22	GGTTTACCGCCTTCCACTCAG
21mer-23	CGGTTTACCGCCTTCCACTCA
21mer-24	ACGGTTTACCGCCTTCCACTC
21mer-25	AACGGTTTACCGCCTTCCACT
21mer-26	AAACGGTTTACCGCCTTCCAC
21mer-59	AGGTCAGGCTGCTTTGCCCTC
21mer-60	TAGGTCAGGCTGCTTTGCCCT
21mer-61	CTAGGTCAGGCTGCTTTGCCC
21mer-62	GCTAGGTCAGGCTGCTTTGCC
21mer-63	AGCTAGGTCAGGCTGCTTTGC
21mer-64	GAGCTAGGTCAGGCTGCTTTG
21mer-65	GGAGCTAGGTCAGGCTGCTTT
21mer-66	AGGAGCTAGGTCAGGCTGCTT
21mer-67	CAGGAGCTAGGTCAGGCTGCT
21mer-68	CCAGGAGCTAGGTCAGGCTGC
21mer-69	TCCAGGAGCTAGGTCAGGCTG
21mer-70	GTCCAGGAGCTAGGTCAGGCT
21mer-71	AGTCCAGGAGCTAGGTCAGGC
21mer-72	CAGTCCAGGAGCTAGGTCAGG
21mer-73	TCAGTCCAGGAGCTAGGTCAG
21mer-74	GTCAGTCCAGGAGCTAGGTCA
21mer-75	GGTCAGTCCAGGAGCTAGGTC
21mer-76	TGGTCAGTCCAGGAGCTAGGT
21mer-77	GTGGTCAGTCCAGGAGCTAGG

21mer-78	AGTGGTCAGTCCAGGAGCTAG
21mer-79	TAGTGGTCAGTCCAGGAGCTA
21mer-93	cttacAGGCTCCAATAGTGGT
21mer-94	acttacAGGCTCCAATAGTGG
21mer-95	tacttacAGGCTCCAATAGTG
21mer-96	atacttacAGGCTCCAATAGT
21mer-97	tatacttacAGGCTCCAATAG
21mer-98	gtatacttacAGGCTCCAATA
21mer-99	agtatacttacAGGCTCCAAT
21mer-100	cagtatacttacAGGCTCCAA
21mer-101	ccagtatacttacAGGCTCCA

***Table 11B**: 22-mer oligonucleotide that hybridize to exon 50.*

AC Name	Nucleic Acid Sequence (Reverse complement 5' – 3')
22mer-n1	GCTCAGATCTTCTAACTTCCTc
22mer-n2	CTCAGATCTTCTAACTTCCTct
22mer-n3	TCAGATCTTCTAACTTCCTctt
22mer-n4	CAGATCTTCTAACTTCCTcttt
22mer-n5	AGATCTTCTAACTTCCTcttta
22mer-00	AGCTCAGATCTTCTAACTTCCT
22mer-01	GAGCTCAGATCTTCTAACTTCC
22mer-02	AGAGCTCAGATCTTCTAACTTC
22mer-03	CAGAGCTCAGATCTTCTAACTT
22mer-04	TCAGAGCTCAGATCTTCTAACT
22mer-05	CTCAGAGCTCAGATCTTCTAAC
22mer-06	ACTCAGAGCTCAGATCTTCTAA
22mer-07	CACTCAGAGCTCAGATCTTCTA
22mer-08	CCACTCAGAGCTCAGATCTTCT
22mer-09	TCCACTCAGAGCTCAGATCTTC
22mer-10	TTCCACTCAGAGCTCAGATCTT
22mer-11	CTTCCACTCAGAGCTCAGATCT
22mer-12	CCTTCCACTCAGAGCTCAGATC
22mer-13	GCCTTCCACTCAGAGCTCAGAT
22mer-14	CGCCTTCCACTCAGAGCTCAGA
22mer-15	CCGCCTTCCACTCAGAGCTCAG
22mer-16	ACCGCCTTCCACTCAGAGCTCA
22mer-17	TACCGCCTTCCACTCAGAGCTC
22mer-18	TTACCGCCTTCCACTCAGAGCT
22mer-19	TTTACCGCCTTCCACTCAGAGC
22mer-20	GTTTACCGCCTTCCACTCAGAG
22mer-21	GGTTTACCGCCTTCCACTCAGA
22mer-22	CGGTTTACCGCCTTCCACTCAG
22mer-23	ACGGTTTACCGCCTTCCACTCA

22mer-24	AACGGTTTACCGCCTTCCACTC
22mer-25	AAACGGTTTACCGCCTTCCACT
22mer-26	TAAACGGTTTACCGCCTTCCAC
22mer-59	TAGGTCAGGCTGCTTTGCCCTC
22mer-60	CTAGGTCAGGCTGCTTTGCCCT
22mer-61	GCTAGGTCAGGCTGCTTTGCCC
22mer-62	AGCTAGGTCAGGCTGCTTTGCC
22mer-63	GAGCTAGGTCAGGCTGCTTTGC
22mer-64	GGAGCTAGGTCAGGCTGCTTTG
22mer-65	AGGAGCTAGGTCAGGCTGCTTT
22mer-66	CAGGAGCTAGGTCAGGCTGCTT
22mer-67	CCAGGAGCTAGGTCAGGCTGCT
22mer-68	TCCAGGAGCTAGGTCAGGCTGC
22mer-69	GTCCAGGAGCTAGGTCAGGCTG
22mer-70	AGTCCAGGAGCTAGGTCAGGCT
22mer-71	CAGTCCAGGAGCTAGGTCAGGC
22mer-72	TCAGTCCAGGAGCTAGGTCAGG
22mer-73	GTCAGTCCAGGAGCTAGGTCAG
22mer-74	GGTCAGTCCAGGAGCTAGGTCA
22mer-75	TGGTCAGTCCAGGAGCTAGGTC
22mer-76	GTGGTCAGTCCAGGAGCTAGGT
22mer-77	AGTGGTCAGTCCAGGAGCTAGG
22mer-78	TAGTGGTCAGTCCAGGAGCTAG
22mer-79	ATAGTGGTCAGTCCAGGAGCTA
22mer-80	AATAGTGGTCAGTCCAGGAGCT
22mer-81	CAATAGTGGTCAGTCCAGGAGC
22mer-82	CCAATAGTGGTCAGTCCAGGAG
22mer-83	TCCAATAGTGGTCAGTCCAGGA
22mer-84	CTCCAATAGTGGTCAGTCCAGG
22mer-85	GCTCCAATAGTGGTCAGTCCAG
22mer-87	GGCTCCAATAGTGGTCAGTCCA
22mer-88	cAGGCTCCAATAGTGGTCAGTC
22mer-93	acttacAGGCTCCAATAGTGGT
22mer-94	tacttacAGGCTCCAATAGTGG
22mer-95	atacttacAGGCTCCAATAGTG
22mer-96	tatacttacAGGCTCCAATAGT
22mer-97	gtatacttacAGGCTCCAATAG
22mer-98	agtatacttacAGGCTCCAATA
22mer-99	cagtatacttacAGGCTCCAAT
22mer-100	ccagtatacttacAGGCTCCAA
22mer-101	tccagtatacttacAGGCTCCA
22mer-102	atccagtatacttacAGGCTCC
22mer-103	gatccagtatacttacAGGCTC
22mer-104	ggatccagtatacttacAGGCT

***Table 11C**: 23-mer oligonucleotide that hybridize to exon 50.*

AC Name	Nucleic Acid Sequence (Reverse complement 5' – 3')
23mer-n1	AGCTCAGATCTTCTAACTTCCTc
23mer-n2	GCTCAGATCTTCTAACTTCCTct
23mer-n3	CTCAGATCTTCTAACTTCCTctt
23mer-n4	TCAGATCTTCTAACTTCCTcttt
23mer-n5	CAGATCTTCTAACTTCCTcttta
23mer-0	GAGCTCAGATCTTCTAACTTCCT
23mer-1	AGAGCTCAGATCTTCTAACTTCC
23mer-2	CAGAGCTCAGATCTTCTAACTTC
23mer-3	TCAGAGCTCAGATCTTCTAACTT
23mer-4	CTCAGAGCTCAGATCTTCTAACT
23mer-5	ACTCAGAGCTCAGATCTTCTAAC
23mer-6	CACTCAGAGCTCAGATCTTCTAA
23mer-7	CCACTCAGAGCTCAGATCTTCTA
23mer-8	TCCACTCAGAGCTCAGATCTTCT
23mer-9	TTCCACTCAGAGCTCAGATCTTC
23mer-10	CTTCCACTCAGAGCTCAGATCTT
23mer-11	CCTTCCACTCAGAGCTCAGATCT
23mer-12	GCCTTCCACTCAGAGCTCAGATC
23mer-13	CGCCTTCCACTCAGAGCTCAGAT
23mer-14	CCGCCTTCCACTCAGAGCTCAGA
23mer-15	ACCGCCTTCCACTCAGAGCTCAG
23mer-16	TACCGCCTTCCACTCAGAGCTCA
23mer-17	TTACCGCCTTCCACTCAGAGCTC
23mer-18	TTTACCGCCTTCCACTCAGAGCT
23mer-19	GTTTACCGCCTTCCACTCAGAGC
23mer-20	GGTTTACCGCCTTCCACTCAGAG
23mer-21	CGGTTTACCGCCTTCCACTCAGA
23mer-22	ACGGTTTACCGCCTTCCACTCAG
23mer-23	AACGGTTTACCGCCTTCCACTCA
23mer-24	AAACGGTTTACCGCCTTCCACTC
23mer-25	TAAACGGTTTACCGCCTTCCACT
23mer-26	GTAAACGGTTTACCGCCTTCCAC
23mer-59	CTAGGTCAGGCTGCTTTGCCCTC
23mer-60	GCTAGGTCAGGCTGCTTTGCCCT
23mer-61	AGCTAGGTCAGGCTGCTTTGCCC
23mer-62	GAGCTAGGTCAGGCTGCTTTGCC
23mer-63	GGAGCTAGGTCAGGCTGCTTTGC
23mer-64	AGGAGCTAGGTCAGGCTGCTTTG
23mer-65	CAGGAGCTAGGTCAGGCTGCTTT
23mer-66	CCAGGAGCTAGGTCAGGCTGCTT
23mer-67	TCCAGGAGCTAGGTCAGGCTGCT

23mer-68	GTCCAGGAGCTAGGTCAGGCTGC
23mer-69	AGTCCAGGAGCTAGGTCAGGCTG
23mer-70	CAGTCCAGGAGCTAGGTCAGGCT
23mer-71	TCAGTCCAGGAGCTAGGTCAGGC
23mer-72	GTCAGTCCAGGAGCTAGGTCAGG
23mer-73	GGTCAGTCCAGGAGCTAGGTCAG
23mer-74	TGGTCAGTCCAGGAGCTAGGTCA
23mer-75	GTGGTCAGTCCAGGAGCTAGGTC
23mer-76	AGTGGTCAGTCCAGGAGCTAGGT
23mer-77	TAGTGGTCAGTCCAGGAGCTAGG
23mer-78	ATAGTGGTCAGTCCAGGAGCTAG
23mer-79	AATAGTGGTCAGTCCAGGAGCTA
23mer-80	CAATAGTGGTCAGTCCAGGAGCT
23mer-81	CCAATAGTGGTCAGTCCAGGAGC
23mer-82	TCCAATAGTGGTCAGTCCAGGAG
23mer-83	CTCCAATAGTGGTCAGTCCAGGA
23mer-84	GCTCCAATAGTGGTCAGTCCAGG
23mer-87	cAGGCTCCAATAGTGGTCAGTCC
23mer-88	acAGGCTCCAATAGTGGTCAGTC
23mer-92	acttacAGGCTCCAATAGTGGTC
23mer-93	tacttacAGGCTCCAATAGTGGT
23mer-94	atacttacAGGCTCCAATAGTGG
23mer-95	tatacttacAGGCTCCAATAGTG
23mer-96	gtatacttacAGGCTCCAATAGT
23mer-97	agtatacttacAGGCTCCAATAG
23mer-98	cagtatacttacAGGCTCCAATA
23mer-99	ccagtatacttacAGGCTCCAAT
23mer-100	gccagtatacttacAGGCTCCAA
23mer-101	atccagtatacttacAGGCTCCA
23mer-102	gatccagtatacttacAGGCTCC
23mer-103	ggatccagtatacttacAGGCTC
23mer-104	gggatccagtatacttacAGGCT

***Table 11D**: 24-mer oligonucleotide that hybridize to exon 50.*

AC Name	Nucleic Acid Sequence (Reverse complement 5' – 3')
24mer-n6	CAGATCTTCTAACTTCCTctttaa
24mer-n7	AGATCTTCTAACTTCCTctttaac
24mer-n8	GATCTTCTAACTTCCTctttaaca
24mer-n9	ATCTTCTAACTTCCTctttaacag
24mer-n4	CTCAGATCTTCTAACTTCCTcttt
24mer-n3	GCTCAGATCTTCTAACTTCCTctt
24mer-n2	AGCTCAGATCTTCTAACTTCCTct
24mer-n1	GAGCTCAGATCTTCTAACTTCCTc

24mer-n0	AGAGCTCAGATCTTCTAACTTCCT
24mer-01	CAGAGCTCAGATCTTCTAACTTCC
24mer-02	TCAGAGCTCAGATCTTCTAACTTC
24mer-03	CTCAGAGCTCAGATCTTCTAACTT
24mer-04	ACTCAGAGCTCAGATCTTCTAACT
24mer-05	CACTCAGAGCTCAGATCTTCTAAC
24mer-06	CCACTCAGAGCTCAGATCTTCTAA
24mer-07	TCCACTCAGAGCTCAGATCTTCTA
24mer-08	TTCCACTCAGAGCTCAGATCTTCT
24mer-09	CTTCCACTCAGAGCTCAGATCTTC
24mer-11	GCCTTCCACTCAGAGCTCAGATCT
24mer-12	CGCCTTCCACTCAGAGCTCAGATC
24mer-13	CCGCCTTCCACTCAGAGCTCAGAT
24mer-14	ACCGCCTTCCACTCAGAGCTCAGA
24mer-15	TACCGCCTTCCACTCAGAGCTCAG
24mer-16	TTACCGCCTTCCACTCAGAGCTCA
24mer-17	TTTACCGCCTTCCACTCAGAGCTC
24mer-18	GTTTACCGCCTTCCACTCAGAGCT
24mer-19	GGTTTACCGCCTTCCACTCAGAGC
24mer-20	CGGTTTACCGCCTTCCACTCAGAG
24mer-21	ACGGTTTACCGCCTTCCACTCAGA
24mer-22	AACGGTTTACCGCCTTCCACTCAG
24mer-56	AGGTCAGGCTGCTTTGCCCTCAGC
24mer-57	TAGGTCAGGCTGCTTTGCCCTCAG
24mer-58	CTAGGTCAGGCTGCTTTGCCCTCA
24mer-59	GCTAGGTCAGGCTGCTTTGCCCTC
24mer-60	AGCTAGGTCAGGCTGCTTTGCCCT
24mer-61	GAGCTAGGTCAGGCTGCTTTGCCC
24mer-62	GGAGCTAGGTCAGGCTGCTTTGCC
24mer-63	AGGAGCTAGGTCAGGCTGCTTTGC
24mer-64	CAGGAGCTAGGTCAGGCTGCTTTG
24mer-65	CCAGGAGCTAGGTCAGGCTGCTTT
24mer-66	TCCAGGAGCTAGGTCAGGCTGCTT
24mer-67	GTCCAGGAGCTAGGTCAGGCTGCT
24mer-68	AGTCCAGGAGCTAGGTCAGGCTGC
24mer-69	CAGTCCAGGAGCTAGGTCAGGCTG
24mer-71	GTCAGTCCAGGAGCTAGGTCAGGC
24mer-72	GGTCAGTCCAGGAGCTAGGTCAGG
24mer-73	TGGTCAGTCCAGGAGCTAGGTCAG
24mer-74	GTGGTCAGTCCAGGAGCTAGGTCA
24mer-75	AGTGGTCAGTCCAGGAGCTAGGTC
24mer-76	TAGTGGTCAGTCCAGGAGCTAGGT
24mer-77	ATAGTGGTCAGTCCAGGAGCTAGG
24mer-78	AATAGTGGTCAGTCCAGGAGCTAG
24mer-79	CAATAGTGGTCAGTCCAGGAGCTA

24mer-80	CCAATAGTGGTCAGTCCAGGAGCT
24mer-81	TCCAATAGTGGTCAGTCCAGGAGC
24mer-82	CTCCAATAGTGGTCAGTCCAGGAG
24mer-86	cAGGCTCCAATAGTGGTCAGTCCA
24mer-87	acAGGCTCCAATAGTGGTCAGTCC
24mer-88	tacAGGCTCCAATAGTGGTCAGTC
24mer-89	ttacAGGCTCCAATAGTGGTCAGT
24mer-91	acttacAGGCTCCAATAGTGGTCA
24mer-92	tacttacAGGCTCCAATAGTGGTC
24mer-93	atacttacAGGCTCCAATAGTGGT
24mer-94	tatacttacAGGCTCCAATAGTGG
24mer-96	agtatacttacAGGCTCCAATAGT
24mer-97	cagtatacttacAGGCTCCAATAG
24mer-98	ccagtatacttacAGGCTCCAATA
24mer-99	tccagtatacttacAGGCTCCAAT
24mer-101	gatccagtatacttacAGGCTCCA

Table 12A. *21-mer oligonucleotides that hybridize to exon 50.*

AC Name	Nucleic Acid Sequence (Reverse complement 5' – 3')
21mer-09	CCACTCAGAGCTCAGATCTTC
21mer-10	TCCACTCAGAGCTCAGATCTT
21mer-16	CCGCCTTCCACTCAGAGCTCA
21mer-17	ACCGCCTTCCACTCAGAGCTC
21mer-59	AGGTCAGGCTGCTTTGCCCTC
21mer-60	TAGGTCAGGCTGCTTTGCCCT
21mer-66	AGGAGCTAGGTCAGGCTGCTT
21mer-67	CAGGAGCTAGGTCAGGCTGCT
21mer-77	GTGGTCAGTCCAGGAGCTAGG

Table 12B. *22-mer oligonucleotides that hybridize to exon 50.*

AC Name	Nucleic Acid Sequence (Reverse complement 5' – 3')
22mer-10	TTCCACTCAGAGCTCAGATCTT
22mer-15	CCGCCTTCCACTCAGAGCTCAG
22mer-16	ACCGCCTTCCACTCAGAGCTCA
22mer-17	TACCGCCTTCCACTCAGAGCTC
22mer-59	TAGGTCAGGCTGCTTTGCCCTC
22mer-60	CTAGGTCAGGCTGCTTTGCCCT
22mer-61	GCTAGGTCAGGCTGCTTTGCCC
22mer-64	GGAGCTAGGTCAGGCTGCTTTG

22mer-65	AGGAGCTAGGTCAGGCTGCTTT
22mer-74	GGTCAGTCCAGGAGCTAGGTCA
22mer-75	TGGTCAGTCCAGGAGCTAGGTC
22mer-84	CTCCAATAGTGGTCAGTCCAGG
22mer-85	GCTCCAATAGTGGTCAGTCCAG

Table 12C. *23-mer oligonucleotides that hybridize to exon 50.*

AC Name	Nucleic Acid Sequence (Reverse complement 5' – 3')
23mer-n5	CAGATCTTCTAACTTCCTCTTTA
23mer-9	TTCCACTCAGAGCTCAGATCTTC
23mer-10	CTTCCACTCAGAGCTCAGATCTT
23mer-15	ACCGCCTTCCACTCAGAGCTCAG
23mer-16	TACCGCCTTCCACTCAGAGCTCA
23mer-22	ACGGTTTACCGCCTTCCACTCAG
23mer-75	GTGGTCAGTCCAGGAGCTAGGTC
23mer-76	AGTGGTCAGTCCAGGAGCTAGGT
23mer-88	acAGGCTCCAATAGTGGTCAGTC
23mer-104	gggatccagtatacttacAGGCT

Table 12D. *24-mer oligonucleotides that hybridize to exon 50.*

AC Name	Nucleic Acid Sequence (Reverse complement 5' – 3')
24mer-14	ACCGCCTTCCACTCAGAGCTCAGA
24mer-15	TACCGCCTTCCACTCAGAGCTCAG
24mer-16	TTACCGCCTTCCACTCAGAGCTCA
24mer-56	AGGTCAGGCTGCTTTGCCCTCAGC
24mer-57	TAGGTCAGGCTGCTTTGCCCTCAG
24mer-74	GTGGTCAGTCCAGGAGCTAGGTCA
24mer-75	AGTGGTCAGTCCAGGAGCTAGGTC
24mer-76	TAGTGGTCAGTCCAGGAGCTAGGT
24mer-88	tacAGGCTCCAATAGTGGTCAGTC
24mer-101	gatccagtatacttacAGGCTCCA

Table 13*. 20- to 30- mer oligonucleotides that hybridize to exon 50.*

AC Description	Reverse Complement Nucleic Acid Sequence (5' – 3')
24-mer-76	AGTGGTCAGTCCAGGAGCTAGGTC
21-mer-78	GTGGTCAGTCCAGGAGCTAGG
22-mer-75	GGTCAGTCCAGGAGCTAGGTCA

24-mer-77	TAGTGGTCAGTCCAGGAGCTAGGT
22-mer-86	GCTCCAATAGTGGTCAGTCCAG
24-mer-14	ACCGCCTTCCACTCAGAGCTCAGA
24-mer-16	TTACCGCCTTCCACTCAGAGCTCA
22-mer-65	GGAGCTAGGTCAGGCTGCTTTG
23-mer-76	GTGGTCAGTCCAGGAGCTAGGTC
22-mer-66	AGGAGCTAGGTCAGGCTGCTTT

[0422] In embodiments, the oligonucleotide that hybridizes to exon 50 of the *DMD* gene is an antisense phosphorodiamidate morpholino oligonucleotide (PMO) with a sequence of 5'-AGTGGTCAGTCCAGGAGCTAGGTC -3' (24-mer-76), the reverse complement thereof, or a sequence with at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% nucleic acid sequence identity thereto. In embodiments, the oligonucleotide that hybridizes to exon 50 of the *DMD* gene is an antisense phosphorodiamidate morpholino oligonucleotide (PMO) with a sequence of 5'-AGTGGTCAGTCCAGGAGCTAGGTC -3' (24-mer-76). In embodiments, the oligonucleotide that hybridizes to exon 50 of the *DMD* gene is an antisense phosphorodiamidate morpholino oligonucleotide (PMO) with a sequence of 5'- GTGGTCAGTCCAGGAGCTAGG -3' (21-mer-78), the reverse complement thereof, or a sequence with at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% nucleic acid sequence identity thereto. In embodiments, the oligonucleotide that hybridizes to exon 50 of the *DMD* gene is an antisense phosphorodiamidate morpholino oligonucleotide (PMO) with a sequence of 5'- GTGGTCAGTCCAGGAGCTAGG -3' (21-mer-78). In embodiments, the oligonucleotide that hybridizes to exon 50 of the *DMD* gene is an antisense phosphorodiamidate morpholino oligonucleotide (PMO) with a sequence of 5'- GGTCAGTCCAGGAGCTAGGTCA -3' (22-mer-75), the reverse complement thereof, or a sequence with at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% nucleic acid sequence identity thereto. In embodiments, the oligonucleotide that hybridizes to exon 50 of the *DMD* gene is an antisense phosphorodiamidate morpholino oligonucleotide (PMO) with a sequence of 5'- GGTCAGTCCAGGAGCTAGGTCA -3' (22-mer-75). In embodiments, the oligonucleotide that hybridizes to exon 50 of the *DMD* gene is an antisense phosphorodiamidate morpholino oligonucleotide (PMO) with a sequence of 5'-TAGTGGTCAGTCCAGGAGCTAGGT -3' (24-mer-77), the reverse complement thereof, or a

sequence with at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% nucleic acid sequence identity thereto. In embodiments, the oligonucleotide that hybridizes to exon 50 of the *DMD* gene is an antisense phosphorodiamidate morpholino oligonucleotide (PMO) with a sequence of 5'-TAGTGGTCAGTCCAGGAGCTAGGT -3' (24-mer-77). In embodiments, the oligonucleotide that hybridizes to exon 50 of the *DMD* gene is an antisense phosphorodiamidate morpholino oligonucleotide (PMO) with a sequence of 5'- GCTCCAATAGTGGTCAGTCCAG -3' (22-mer-86), the reverse complement thereof, or a sequence with at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% nucleic acid sequence identity thereto. In embodiments, the oligonucleotide that hybridizes to exon 50 of the *DMD* gene is an antisense phosphorodiamidate morpholino oligonucleotide (PMO) with a sequence of 5'- GCTCCAATAGTGGTCAGTCCAG -3' (22-mer-86). In embodiments, the oligonucleotide that hybridizes to exon 50 of the *DMD* gene is an antisense phosphorodiamidate morpholino oligonucleotide (PMO) with a sequence of 5'-ACCGCCTTCCACTCAGAGCTCAGA -3' (24-mer-14), the reverse complement thereof, or a sequence with at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% nucleic acid sequence identity thereto. In embodiments, the oligonucleotide that hybridizes to exon 50 of the *DMD* gene is an antisense phosphorodiamidate morpholino oligonucleotide (PMO) with a sequence of 5'-ACCGCCTTCCACTCAGAGCTCAGA -3' (24-mer-14). In embodiments, the oligonucleotide that hybridizes to exon 50 of the *DMD* gene is an antisense phosphorodiamidate morpholino oligonucleotide (PMO) with a sequence of 5'- TTACCGCCTTCCACTCAGAGCTCA -3' (24-mer-16), the reverse complement thereof, or a sequence with at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% nucleic acid sequence identity thereto. In embodiments, the oligonucleotide that hybridizes to exon 50 of the *DMD* gene is an antisense phosphorodiamidate morpholino oligonucleotide (PMO) with a sequence of 5'- TTACCGCCTTCCACTCAGAGCTCA -3' (24-mer-16). In embodiments, the oligonucleotide that hybridizes to exon 50 of the *DMD* gene is an antisense phosphorodiamidate morpholino oligonucleotide (PMO) with a sequence of 5'-GGAGCTAGGTCAGGCTGCTTTG -3' (22-mer-65), the reverse complement thereof, or a sequence with at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%,

92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% nucleic acid sequence identity thereto. In embodiments, the oligonucleotide that hybridizes to exon 50 of the *DMD* gene is an antisense phosphorodiamidate morpholino oligonucleotide (PMO) with a sequence of 5'-GGAGCTAGGTCAGGCTGCTTTG -3' (22-mer-65). In embodiments, the oligonucleotide that hybridizes to exon 50 of the *DMD* gene is an antisense phosphorodiamidate morpholino oligonucleotide (PMO) with a sequence of 5'- GTGGTCAGTCCAGGAGCTAGGTC -3' (23-mer-76), the reverse complement thereof, or a sequence with at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% nucleic acid sequence identity thereto. In embodiments, the oligonucleotide that hybridizes to exon 50 of the *DMD* gene is an antisense phosphorodiamidate morpholino oligonucleotide (PMO) with a sequence of 5'- GTGGTCAGTCCAGGAGCTAGGTC -3' (23-mer-76). In embodiments, the oligonucleotide that hybridizes to exon 50 of the *DMD* gene is an antisense phosphorodiamidate morpholino oligonucleotide (PMO) with a sequence of 5'-AGGAGCTAGGTCAGGCTGCTTT -3' (22-mer-66), the reverse complement thereof, or a sequence with at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% nucleic acid sequence identity thereto. In embodiments, the oligonucleotide that hybridizes to exon 50 of the *DMD* gene is an antisense phosphorodiamidate morpholino oligonucleotide (PMO) with a sequence of 5'-AGGAGCTAGGTCAGGCTGCTTT -3' (22-mer-66).

[0423] In embodiments, the oligonucleotide that hybridizes to exon 50 of the *DMD* gene comprises a sequence comprising: 5'-XX ACC GCC XXC CAC XCA GAG CXC AGA-3', wherein X = U or T, the reverse complement thereof, or a sequence with at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% sequence identity thereto. In embodiments, the oligonucleotide that hybridizes to exon 50 of the *DMD* gene comprises a sequence comprising at least 15, 16, 17, 18, 19, 20, 21, 22, 23, 24 ,25 or 26 contiguous nucleic acids of a base sequence comprising: 5'-XX ACC GCC XXC CAC XCA GAG CXC AGA-3', wherein X = U or T. In embodiments, the oligonucleotide that hybridizes to exon 50 of the *DMD* gene comprises a sequence comprising 15 contiguous nucleic acids of a base sequence comprising: 5'-XX ACC GCC XXC CAC XCA GAG CXC AGA-3', wherein X = U or T. In embodiments, the oligonucleotide that hybridizes to exon 50 of the *DMD* gene comprises a sequence comprising 16 contiguous nucleic acids of a base sequence comprising: 5'-

XX ACC GCC XXC CAC XCA GAG CXC AGA-3', wherein X = U or T. In embodiments, the oligonucleotide that hybridizes to exon 50 of the *DMD* gene comprises a sequence comprising 17 contiguous nucleic acids of a base sequence comprising: 5'-XX ACC GCC XXC CAC XCA GAG CXC AGA-3', wherein X = U or T. In embodiments, the oligonucleotide that hybridizes to exon 50 of the *DMD* gene comprises a sequence comprising 18 contiguous nucleic acids of a base sequence comprising: 5'-XX ACC GCC XXC CAC XCA GAG CXC AGA-3', wherein X = U or T. In embodiments, the oligonucleotide that hybridizes to exon 50 of the *DMD* gene comprises a sequence comprising 19 contiguous nucleic acids of a base sequence comprising: 5'-XX ACC GCC XXC CAC XCA GAG CXC AGA-3', wherein X = U or T. In embodiments, the oligonucleotide that hybridizes to exon 50 of the *DMD* gene comprises a sequence comprising 20 contiguous nucleic acids of a base sequence comprising: 5'-XX ACC GCC XXC CAC XCA GAG CXC AGA-3', wherein X = U or T. In embodiments, the oligonucleotide that hybridizes to exon 50 of the *DMD* gene comprises a sequence comprising 21 contiguous nucleic acids of a base sequence comprising: 5'-XX ACC GCC XXC CAC XCA GAG CXC AGA-3', wherein X = U or T. In embodiments, the oligonucleotide that hybridizes to exon 50 of the *DMD* gene comprises a sequence comprising 22 contiguous nucleic acids of a base sequence comprising: 5'-XX ACC GCC XXC CAC XCA GAG CXC AGA-3', wherein X = U or T. In embodiments, the oligonucleotide that hybridizes to exon 50 of the *DMD* gene comprises a sequence comprising 23 contiguous nucleic acids of a base sequence comprising: 5'-XX ACC GCC XXC CAC XCA GAG CXC AGA-3', wherein X = U or T. In embodiments, the oligonucleotide that hybridizes to exon 50 of the *DMD* gene comprises a sequence comprising 24 contiguous nucleic acids of a base sequence comprising: 5'-XX ACC GCC XXC CAC XCA GAG CXC AGA-3', wherein X = U or T. In embodiments, the oligonucleotide that hybridizes to exon 50 of the *DMD* gene comprises a sequence comprising 25 contiguous nucleic acids of a base sequence comprising: 5'-XX ACC GCC XXC CAC XCA GAG CXC AGA-3', wherein X = U or T. In embodiments, the oligonucleotide that hybridizes to exon 50 of the *DMD* gene comprises a sequence comprising 26 contiguous nucleic acids of a base sequence comprising: 5'-XX ACC GCC XXC CAC XCA GAG CXC AGA-3', wherein X = U or T. In embodiments, the base sequence can vary at up to 1, 2, 3, or 4 or 5 positions. In embodiments, the oligonucleotide comprises one or more modified oligonucleotides. In embodiments, oligonucleotide comprises at least one phosphorodiamidate

morpholino oligonucleotide (PMO). In embodiments, each nucleotide in the oligonucleotide is a phosphorodiamidate morpholino oligonucleotide (PMO).

[0424] In embodiments, any oligonucleotide described herein, including the oligonucleotide in **Tables 11A-11D, 12A-12D,** the reverse complement thereof, or a sequence with at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% nucleic acid sequence identity thereto, comprise at least one modified nucleotide or nucleic acid selected from a phosphorothioate (PS) nucleotide, a phosphorodiamidate morpholino oligonucleotide (PMO), a locked nucleic acid (LNA), a peptide nucleic acid (PNA), a nucleotide comprising a 2'-O-methyl (2'-OMe) modified backbone, a 2'O-methoxy-ethyl (2'-MOE) nucleotide, a 2',4' constrained ethyl (cEt) nucleotide, and a 2'-deoxy-2'-fluoro-beta-D-arabinonucleic acid (2'F-ANA). In embodiments, hybridization of the oligonucleotide with the target sequence promotes or induces splicing of exon 50. In embodiments, the oligonucleotide comprises at least one phosphorodiamidate morpholino oligonucleotide (PMO). In embodiments, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 1,4 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 2,7 28, 29, 30 or more of the nucleoties are modified. In embodiments, each nucleotide in the oligonucleotide is a phosphorodiamidate morpholino oligonucleotide (PMO).

Cytosolic Delivery Efficiency

[0425] Modifications to an endosomal escape vehicle (EEV) or a component thereof, such as a cyclic cell penetrating peptide (cCPP), exocyclic peptide (EP) or linker, may improve cytosolic delivery efficiency. Improved cytosolic uptake efficiency can be measured by comparing the cytosolic delivery efficiency of the modified EEV to a control sequence, wherein the control does not include a particularmodification, for example to the cCPP, EP, or linker, but is otherwise identical.

[0426] In embodiments, the EEV-cargo conjugate has an improved cytosolic uptake efficiency compared to the cargo alone. Cytosolic uptake efficiency can be measured by comparing the cytosolic delivery efficiency of the EEV-cargo conjugate to the cytosolic delivery efficiency of the cargo alone. In embodiments, the cargo is an oligonucleotide. In embodiments, the oligonucleotide is a therapeutic oligonucleotide. In embodiments, the oligonucleotide is an antisense oligonucleotide. In embodiments, the oligonucleotide is a PMO.

[0427] As used herein cytosolic delivery efficiency refers to the ability of an EEV, EEV-cargo conjugate, or cargo to traverse a cell membrane and enter the cytosol of a cell. Cytosolic delivery efficiency of the EEV, EEV-cargo conjugate, or cargo is not necessarily dependent on a receptor or a cell type. Cytosolic delivery efficiency can refer to absolute cytosolic delivery efficiency or relative cytosolic delivery efficiency.

[0428] Absolute cytosolic delivery efficiency is the ratio of cytosolic concentration of an EEV, EEV-cargo conjugate, or cargo over the concentration of the EEV, EEV-cargo conjugate, or cargo in the growth medium. Relative cytosolic delivery efficiency refers to the concentration of an EEV, EEV-cargo conjugate, or cargo in the cytosol compared to the concentration of a control EEV, EEV-cargo conjugate, or cargo in the cytosol. Quantification can be achieved by fluorescently labeling the EEV, EEV-cargo conjugate, or cargo (e.g., with a FITC dye) and measuring the fluorescence intensity using techniques well-known in the art.

[0429] Relative cytosolic delivery efficiency is determined by comparing (i) the amount of an EEV, EEV-cargo conjugate, or cargo internalized by a cell type (e.g., HeLa cells) to (ii) the amount of a control EEV, EEV-cargo conjugate, or cargo internalized by the same cell type. To measure relative cytosolic delivery efficiency, the cell type may be incubated in the presence of an EEV, EEV-cargo conjugate, or cargo for a specified period of time (e.g., 30 minutes, 1 hour, 2 hours, etc.) after which the amount of the cCPP internalized by the cell is quantified using methods known in the art, e.g., fluorescence microscopy. Separately, the same concentration of the control EEV, EEV-cargo conjugate, or cargo is incubated in the presence of the cell type over the same period of time, and the amount of the control EEV, EEV-cargo conjugate, or cargo internalized by the cell is quantified.

[0430] Relative cytosolic delivery efficiency can be determined by measuring the IC_{50} of a modified EEV, EEV-cargo conjugate, or cargo for an intracellular target and comparing the IC_{50} of the modified EEV, EEV-cargo conjugate, or cargo to a control sequence.

Re-spliced target proteins

[0431] As used herein, a "target protein" is the amino acid sequence resulting from transcription and translation of the target gene. A "re-spliced target protein," as used herein, refers to a protein encoded as a result of binding of an oligonucleotide to a target pre-mRNA transcribed from the target gene. A "wild-type target protein" refers to a naturally occurring, correctly translated protein isomer resulting from proper splicing of a target pre-mRNA encoded by a wild-type

target gene. The re-spliced target protein may contain one or more amino acid substitutions, deletions, and/or insertions as compared to a wild-type target protein. In embodiments, the re-spliced target protein retains some wild-type target protein activity. In embodiments, the re-spliced target protein is homologous to a wild-type target protein. In embodiments, the re-spliced target protein has an amino acid sequence that is at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91 %, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% and up to 100% identical to a wild-type target protein. In embodiments, the re-spliced target protein is substantially identical to a wild-type target protein. In embodiments, the amino acid sequence of the re-spliced target protein is at least 50% identical to the amino acid sequence of a wild-type target protein. In embodiments, the amino acid sequence of the re-spliced target protein is at least 75% identical to the amino acid sequence of a wild-type target protein. In embodiments, the amino acid sequence of the re-spliced target protein is at least 90% identical to the amino acid sequence of a wild-type target protein. In embodiments, the re-spliced target protein is a shortened version of a wild-type target protein.

[0432] In embodiments, the re-spliced target protein can rescue one or more phenotypes that are lost due to a disease associated with the transcription and translation of the target gene or ameliorate one or more symptoms of a disease associated with the transcription and translation of the target gene. In embodiments, the re-spliced target protein can rescue one or more phenotypes or ameliorate one or more symptoms of a disease associated with the expression of the target protein. In embodiments, the re-spliced target protein is an active fragment of a wild-type target protein. In embodiments, the re-spliced target protein functions in a substantially similar manner to the wild-type target protein. In embodiments, the re-spliced target protein allows the cell to function substantially similar to a similar cell which expresses a wild-type target protein. In embodiments, the re-spliced target protein does not cure the disease associated with the target gene or with the target protein but ameliorates one or more symptoms of the disease. In embodiments, the re-spliced target protein results in an improvement of target protein function of at least 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 15%, 205, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, or 95%, and up to 100%.

[0433] In embodiments, the re-spliced target protein may have an amino acid sequence that is reduced from the size of a wild-type target protein by 1 or more amino acids, e.g., from 5, 10, 15,

20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 90, 95, 100, 105, 110, 115, 120, 125, 130, 135, 140, 145, 150, 155, 160, 165, 170, 175, or 180 or more amino acids.

[0434] In embodiments, the re-spliced target protein may have one or more properties that are improved relative to the target protein. In embodiments, the re-spliced target protein may have one or more properties that are improved relative to a wild-type target protein. In embodiments, the enzymatic activity or stability may be enhanced by promoting different splicing of the target pre-mRNA. In embodiments, the re-spliced target protein may have a sequence identical or substantially similar to a wild-type target protein isomer having improved properties compared to another wild-type target protein isomer.

[0435] In embodiments, one or more properties of the target protein are either not present (eliminated) or are reduced in the re-spliced target protein. In embodiments, one or more properties of the wild-type target protein are either not present (eliminated) or are reduced in the re-spliced target protein. Non-limiting examples of properties that may be reduced or eliminated include immunogenic, angiogenic, thrombogenic, aggregation, and ligand-binding activity.

[0436] In embodiments, the re-spliced target protein contains one or more amino acid substitutions compared to a wild-type target protein. In embodiments, the substitutions may be conservative substitutions or non-conservative substitutions. Examples of conservative amino acid substitutions include substitution of one amino acid for another amino acid within one from one of the following groups: basic amino acids (arginine, lysine and histidine), acidic amino acids (glutamic acid and aspartic acid), polar amino acids (glutamine and asparagine), hydrophobic amino acids (leucine, isoleucine and valine), aromatic amino acids (phenylalanine, tryptophan and tyrosine), and small amino acids (glycine, alanine, serine, threonine and methionine). In embodiments, structurally similar amino acids are substituted to reverse the charge of a residue (*e.g.*, glutamine for glutamic acid or vice-versa, aspartic acid for asparagine or vice-versa). In embodiments, tyrosine is substituted for phenylalanine or vice-versa. Other non-limiting examples of amino acid substitutions are described, for example, by H. Neurath and R. L. Hill, 1979, *In, The Proteins*, Academic Press, New York. Common substitutions are Ala/Ser, Val/Ile, Asp/Glu, Thr/Ser, Ala/Gly, Ala/Thr, Ser/Asn, Ala/Val, Ser/Gly, Tyr/Phe, Ala/Pro, Lys/Arg, Asp/Asn, Leu/Ile, Leu/Val, Ala/Glu, and Asp/Gly.

[0437] In embodiments, the re-spliced target protein may comprise a substitution, deletion, and/or insertion at one or more (*e.g.*, several) positions compared to a wild-type target protein. In

embodiments, the number of amino acid substitutions, deletions and/or insertions in the re-spliced target protein amino acid sequence is not more than 200, not more than 150, not more than 100, not more than 50, not more than 40, not more than 30, not more than 20, or not more than 10, e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10.

Methods of Treatment

[0438] In embodiments, an EEV-cargo conjugate is administered to a patient diagnosed with Duchenne muscular dystrophy (DMD). In embodiments, the cargo is an oligonucleotide. In embodiments, the oligonucleotide is a therapeutic oligonucleotide. In embodiments, the oligonucleotide is an antisense oligonucleotide. In embodiments, the EEV-cargo conjugate is administered to the patient at a dose from 0.1 mg/kg to 1000 mg/kg, including all values and ranges therein and in between.

[0439] A method of treating Duchenne Muscular Dystrophy (DMD) in a subject in need thereof is provided, comprising administering an EEV-cargo conjugate disclosed herein. In embodiments, the target gene is *DMD*. In embodiments, the target sequence includes at least a portion of exon 50 of the *DMD* gene, at least a portion of a 3' intron flanking exon 50 of the *DMD* gene, at least a portion of a 5' intron flanking exon 50 of the *DMD* gene, or a combination thereof.

[0440] In embodiments, treatment refers to partial or complete alleviation, amelioration, relief, inhibition, delaying onset, reducing severity and/or incidence of one or more symptoms in a subject.

[0441] In embodiments, a method is provided for altering the expression of a target gene in a subject in need thereof, comprising administering an EEV-cargo conjugate disclosed herein. In embodiments, the treatment results in the lowered expression of a target protein. In embodiments, the treatment results in the expression of a re-spliced target protein. In embodiments, the treatment results in the preferential expression of a wild-type target protein isomer.

[0442] In embodiments, treatment results in decreased expression of a target protein in a subject by more than 5%, e.g., 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, and 100%, as compared to the average level of the target protein in the subject before the treatment or of one or more control individuals with similar disease without treatment. In embodiments, treatment results in increased expression of a re-

spliced target protein in a subject by more than 5%, e.g., 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, and 100%, as compared to the average level of the target protein in the subject before the treatment or of one or more control individuals with similar disease without treatment. In embodiments, treatment results in increased or decreased expression of a wild-type target protein isomer in a subject by more than 5%, e.g., 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, and 100%, as compared to the average level of the target protein in the subject before the treatment or of one or more control individuals with similar disease without treatment

[0443] The terms, "improve," "increase," "reduce," "decrease," and the like, as used herein, indicate values that are relative to a control. In embodiments, a suitable control is a baseline measurement, such as a measurement in the same individual prior to initiation of the treatment described herein, or a measurement in a control individual (or multiple control individuals) in the absence of the treatment described herein. A "control individual" is an individual afflicted with the same disease, who is about the same age and/or gender as the individual being treated (to ensure that the stages of the disease in the treated individual and the control individual(s) are comparable).

[0444] The individual (also referred to as "patient" or "subject") being treated is an individual (fetus, infant, child, adolescent, or adult human) having a disease or having the potential to develop a disease. The individual may have a disease mediated by aberrant gene expression or aberrant gene splicing. In embodiments, the individual having the disease may have wild-type target protein expression or activity levels that are from 1% to 99% of normal protein expression or activity levels in an individual not afflicted with the disease. In embodiments, the range includes, but is not limited to, 80-99%, 65-80%, 50-65%, 30-50%, 25-30%, 20-25%, 15-20%, 10-15%, 5-10%, or 1-5% of normal dystrophin expression or activity levels. In embodiments, the individual may have target protein expression or activity levels that are from 1% to 500% higher than normal wild-type target protein expression or activity levels. In embodiments, the range includes, but is not limited to, 1-10%, 10-50%, 50-100%, 100-200%, 200-300%, 300-400%, 400-500%, or 500-1000% higher target protein expression or activity level.

[0445] In embodiments, the individual is an individual who has been recently diagnosed with the disease. Typically, early treatment (treatment commencing as soon as possible after diagnosis) is important to minimize the effects of the disease and to maximize the benefits of treatment.

[0446] In embodiments, the efficacy of the EEV-cargo conjugate is evaluated in an animal model of DMD. Animal models are valuable resources for studying the pathogenesis of disease and provide a means to test dystrophin-related activity. In embodiments, 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), are utilized to evaluate the EEV-cargo conjugate. In embodiments, the C57BL/10ScSn-Dmdmdx/J (Bl10/mdx) or the D2.B10-Dmdmdx/J (D2/mdx) mouse model is utilized to evaluate the EEV-cargo conjugate. In embodiments, a transgenic mouse harboring the human *DMD* gene and lacking the mouse *Dmd* gene (hDMD/Dmd-null mouse) is used to evaluate the EEV-cargo conjugate. This mouse can be generated by cross-breeding male hDMD mice (available from Jackson Laboratory, Bar Harbor, ME) with female DMD-null mice. Each of the following references describe these models and are incorporated by reference in their entirety herein: J Neuromuscul Dis. 2018; 5(4): 407–417.; Proc Natl Acad Sci U S A. 1984;81(4):1189–92.; Am J Pathol. 2010;176(5):2414–24.; J Clin Invest. 2009;119(12):3703–12; International Publication No. WO2019014772. These and other animal models can be used to measure the functional activity of various dystrophin proteins.

[0447] In embodiments, an in vitro model is used to evaluate the efficacy of the compositions of the disclosure. In embodiments, the in vitro model is an immortalized muscle cell model. This model is described in the following articles which is incorporated by reference in its entirety herein: Nguyen et al. J Pers Med. 2017 Dec; 7(4):13.

Methods of Making

[0448] The EEV-cargo conjugates can be prepared in a variety of ways known to one skilled in the art of organic synthesis or variations thereon as appreciated by those skilled in the art. The EEV-cargo conjugates can be prepared from readily available starting materials. Reaction conditions can vary with the particular reactants or solvents used, but such conditions can be determined by one skilled in the art.

[0449] Variations on the EEV-cargo conjugates include the addition, subtraction, or movement of the various components of the EEV-cargo conjugate. Similarly, when one or more chiral centers are present, the chirality of the molecule can be changed. Additionally, synthesis can

involve the protection and deprotection of various chemical groups. The use of protection and deprotection, and the selection of appropriate protecting groups can be determined by one skilled in the art. The chemistry of protecting groups can be found, for example, in Wuts and Greene, Protective Groups in Organic Synthesis, 4th Ed., Wiley & Sons, 2006, which is incorporated herein by reference in its entirety.

[0450] The starting materials and reagents used in preparing the EEV-cargo conjugates and compositions thereof are either available from commercial suppliers such as Aldrich Chemical Co., (Milwaukee, WI), Acros Organics (Morris Plains, NJ), Fisher Scientific (Pittsburgh, PA), Sigma (St. Louis, MO), Pfizer (New York, NY), GlaxoSmithKline (Raleigh, NC), Merck (Whitehouse Station, NJ), Johnson & Johnson (New Brunswick, NJ), Aventis (Bridgewater, NJ), AstraZeneca (Wilmington, DE), Novartis (Basel, Switzerland), Wyeth (Madison, NJ), Bristol-Myers-Squibb (New York, NY), Roche (Basel, Switzerland), Lilly (Indianapolis, IN), Abbott (Abbott Park, IL), Schering Plough (Kenilworth, NJ), or Boehringer Ingelheim (Ingelheim, Germany), or are prepared by methods known to those skilled in the art following procedures set forth in references such as Fieser and Fieser's Reagents for Organic Synthesis, Volumes 1-17 (John Wiley and Sons, 1991); Rodd's Chemistry of Carbon Compounds, Volumes 1-5 and Supplementals (Elsevier Science Publishers, 1989); Organic Reactions, Volumes 1-40 (John Wiley and Sons, 1991); March's Advanced Organic Chemistry, (John Wiley and Sons, 4th Edition); and Larock's Comprehensive Organic Transformations (VCH Publishers Inc., 1989). Other materials, such as the pharmaceutical carriers can be obtained from commercial sources.

[0451] Reactions to produce the EEV-cargo conjugates can be carried out in solvents, which can be selected by one of skill in the art of organic synthesis. Solvents can be substantially nonreactive with the starting materials (reactants), the intermediates, or products under the conditions at which the reactions are carried out, *i.e.*, temperature and pressure. Reactions can be carried out in one solvent or a mixture of more than one solvent. Product or intermediate formation can be monitored according to any suitable method known in the art. For example, product formation can be monitored by spectroscopic means, such as nuclear magnetic resonance spectroscopy (*e.g.*, ^{1}H or ^{13}C) infrared spectroscopy, spectrophotometry (*e.g.*, UV-visible), or mass spectrometry, or by chromatography such as high performance liquid chromatography (HPLC) or thin layer chromatography.

[0452] The EEV and components thereof, including the EP and cCPP, and EEV-cargo conjugates can be prepared by solid phase peptide synthesis wherein the amino acid α-N-terminal is protected by an acid or base protecting group. Such protecting groups should have the properties of being stable to the conditions of peptide linkage formation while being readily removable without destruction of the growing peptide chain or racemization of any of the chiral centers contained therein. Suitable protecting groups are 9-fluorenylmethyloxycarbonyl (Fmoc), t-butyloxycarbonyl (Boc), benzyloxycarbonyl (Cbz), biphenylisopropyloxycarbonyl, t-amyloxycarbonyl, isobornyloxycarbonyl, α,α-dimethyl-3,5-dimethoxybenzyloxycarbonyl, o-nitrophenylsulfenyl, 2-cyano-t-butyloxycarbonyl, and the like. The 9-fluorenylmethyloxycarbonyl (Fmoc) protecting group can be used. Other side chain protecting groups are, for side chain amino groups like lysine and arginine, 2,2,5,7,8-pentamethylchroman-6-sulfonyl (pmc), nitro, p-toluenesulfonyl, 4-methoxybenzene- sulfonyl, Cbz, Boc, and adamantyloxycarbonyl; for tyrosine, benzyl, o-bromobenzyloxy-carbonyl, 2,6-dichlorobenzyl, isopropyl, t-butyl (t-Bu), cyclohexyl, cyclopentyl and acetyl (Ac); for serine, t-butyl, benzyl and tetrahydropyranyl; for histidine, trityl, benzyl, Cbz, p-toluenesulfonyl and 2,4-dinitrophenyl; for tryptophan, formyl; for asparticacid and glutamic acid, benzyl and t-butyl and for cysteine, triphenylmethyl (trityl). In the solid phase peptide synthesis method, the α-C-terminal amino acid is attached to a suitable solid support or resin. Suitable solid supports useful for the above synthesis are those materials which are inert to the reagents and reaction conditions of the stepwise condensation-deprotection reactions, as well as being insoluble in the media used. Solid supports for synthesis of α-C-terminal carboxy peptides is 4-hydroxymethylphenoxymethyl-copoly(styrene-1% divinylbenzene) or 4-(2',4'-dimethoxyphenyl-Fmoc-aminomethyl)phenoxyacetamidoethyl resin available from Applied Biosystems (Foster City, Calif.). The α-C-terminal amino acid is coupled to the resin by means of N,N'-dicyclohexylcarbodiimide (DCC), N,N'-diisopropylcarbodiimide (DIC) or O-benzotriazol-1-yl-N,N,N',N'-tetramethyluroniumhexafluorophosphate (HBTU), with or without 4-dimethylaminopyridine (DMAP), 1-hydroxybenzotriazole (HOBT), benzotriazol-1-yloxy-tris(dimethylamino)phosphoniumhexafluorophosphate (BOP) or bis(2-oxo-3-oxazolidinyl)phosphine chloride (BOPCl), mediated coupling for from 1 to 24 hours at a temperature of between 10 °C and 50 °C in a solvent such as dichloromethane or DMF. When the solid support is 4-(2',4'-dimethoxyphenyl-Fmoc-aminomethyl)phenoxy-acetamidoethyl resin,

the Fmoc group is cleaved with a secondary amine, for example, piperidine, prior to coupling with the α-C-terminal amino acid as described above. One method for coupling to the deprotected 4 (2',4'-dimethoxyphenyl-Fmoc-aminomethyl)phenoxy-acetamidoethyl resin is O-benzotriazol-1-yl-N,N,N',N'-tetramethyluroniumhexafluorophosphate (HBTU, 1 equiv.) and 1-hydroxybenzotriazole (HOBT, 1 equiv.) in DMF. The coupling of successive protected amino acids can be carried out in an automatic polypeptide synthesizer. In one example, the α-N-terminal in the amino acids of the growing peptide chain are protected with Fmoc. The removal of the Fmoc protecting group from the α-N-terminal side of the growing peptide is accomplished by treatment with a secondary amine, for example, piperidine. Each protected amino acid is then introduced in 3-fold molar excess, and the coupling is carried out in DMF. The coupling agent can be O-benzotriazol-1-yl-N,N,N',N'-tetramethyluroniumhexafluorophosphate (HBTU, 1 equiv.) and 1-hydroxybenzotriazole (HOBT, 1 equiv.). At the end of the solid phase synthesis, the polypeptide is removed from the resin and deprotected, either in successively or in a single operation. Removal of the polypeptide and deprotection can be accomplished in a single operation by treating the resin-bound polypeptide with a cleavage reagent comprising thioanisole, water, ethanedithiol and trifluoroacetic acid. In cases wherein the α-C-terminal of the polypeptide is an alkylamide, the resin is cleaved by aminolysis with an alkylamine. Alternatively, the peptide can be removed by transesterification, *e.g.,* with methanol, followed by aminolysis or by direct transamidation. The protected peptide can be purified at this point or taken to the next step directly. The removal of the side chain protecting groups can be accomplished using the cleavage cocktail described above. The fully deprotected peptide can be purified by a sequence of chromatographic steps employing any or all of the following types: ion exchange on a weakly basic resin (acetate form); hydrophobic adsorption chromatography on underivatized polystyrene-divinylbenzene (for example, Amberlite XAD); silica gel adsorption chromatography; ion exchange chromatography on carboxymethylcellulose; partition chromatography, *e.g.,* on Sephadex G-25, LH-20 or countercurrent distribution; high performance liquid chromatography (HPLC), especially reverse-phase HPLC on octyl- or octadecylsilyl-silica bonded phase column packing.

[0453] The above polymers, such as PEG groups, can be attached to the oligonucleotide under any suitable conditions used to react a protein with an activated polymer molecule. Any means known in the art can be used, including via acylation, reductive alkylation, Michael addition,

thiol alkylation or other chemoselective conjugation/ligation methods through a reactive group on the PEG moiety (e.g., an aldehyde, amino, ester, thiol, α-haloacetyl, maleimido or hydrazino group) to a reactive group on the oligonucleotide (e.g., an aldehyde, amino, ester, thiol, α-haloacetyl, maleimido or hydrazino group). Activating groups which can be used to link the water soluble polymer to one or more proteins include without limitation sulfone, maleimide, sulfhydryl, thiol, triflate, tresylate, azidirine, oxirane, 5-pyridyl, and alpha-halogenated acyl group (e.g., α-iodo acetic acid, α-bromoacetic acid, α-chloroacetic acid). If attached to the oligonucleotide by reductive alkylation, the polymer selected should have a single reactive aldehyde so that the degree of polymerization is controlled. See, for example, Kinstler et al., Adv. Drug. Delivery Rev. 54: 477-485 (2002); Roberts et al., Adv. Drug Delivery Rev. 54: 459-476 (2002); and Zalipsky et al., Adv. Drug Delivery Rev. 16: 157-182 (1995).

[0454] In order to direct covalently link the oligonucleotide to the cyclic peptide, appropriate amino acid residues of the CPP may be reacted with an organic derivatizing agent that is capable of reacting with a selected side chain or the N- or C-termini of an amino acids. Reactive groups on the peptide or conjugate moiety include, e.g., an aldehyde, amino, ester, thiol, α-haloacetyl, maleimido or hydrazino group. Derivatizing agents include, for example, maleimidobenzoyl sulfosuccinimide ester (conjugation through cysteine residues), N-hydroxysuccinimide (through lysine residues), glutaraldehyde, succinic anhydride, or other agents known in the art.

[0455] Methods of synthesizing oligonucleotides are known in the art. The present disclosure is not limited by the method of synthesizing the oligonucleotide. In embodiments, reactive phosphorus groups useful for forming internucleoside linkages include for example phosphodiester and phosphorothioate internucleoside linkages. Methods of preparation and/or purification of precursors or oligonucleotides are not a limitation of the compositions or methods provided herein. Methods for synthesis and purification of DNA, RNA, and the oligonucleotides are well known to those skilled in the art.

[0456] Oligomerization of modified and unmodified nucleosides can be routinely performed according to literature procedures for DNA (Protocols for Oligonucleotides and Analogs, Ed. Agrawal (1993), Humana Press) and/or RNA (Scaringe, Methods (2001), 23, 206-217. Gait et al., Applications of Chemically synthesized RNA in RNA: Protein Interactions, Ed. Smith (1998), 1-36. Gallo et al., Tetrahedron (2001), 57, 5707-5713).

[0457] Oligonucleotides provided herein can be conveniently and routinely made through the well-known technique of solid phase synthesis. Equipment for such synthesis is sold by several vendors including, for example, Applied Biosystems (Foster City, CA). Any other means for such synthesis known in the art may additionally or alternatively be employed. It is well known to use similar techniques to prepare oligonucleotides such as the phosphorothioates and alkylated derivatives. The invention is not limited by the method of oligonucleotide synthesis.

[0458] Methods of oligonucleotide purification and analysis are known to those skilled in the art. Analysis methods include capillary electrophoresis (CE) and electrospray-mass spectroscopy. Such synthesis and analysis methods can be performed in multi-well plates. The method of the invention is not limited by the method of oligomer purification.

Methods of Administration

[0459] *In vivo* administration of the EEV-cargo conjugates disclosed herein, and compositions containing them, can be accomplished by any suitable method and technique presently or prospectively known to those skilled in the art. For example, the EEV-cargo conjugate can be formulated in a physiologically- or pharmaceutically-acceptable form and administered by any suitable route known in the art including, for example, oral and parenteral routes of administration. As used herein, the term parenteral includes subcutaneous, intradermal, intravenous, intramuscular, intraperitoneal, intrasternal, and intrathecal administration, such as by injection. Administration of the disclosed EEV-cargo conjugate or compositions can be a single administration, or at continuous or distinct intervals as can be readily determined by a person skilled in the art.

[0460] The EEV-cargo conjugates disclosed herein, and compositions comprising them, can also be administered utilizing liposome technology, slow-release capsules, implantable pumps, and biodegradable containers. These delivery methods can, advantageously, provide a uniform dosage over an extended period of time. The EEV-cargo conjugate can also be administered in their salt derivative forms or crystalline forms.

[0461] The EEV-cargo conjugate can be formulated according to known methods for preparing pharmaceutically acceptable compositions. Formulations are described in detail in a number of sources which are well known and readily available to those skilled in the art. For example, *Remington's Pharmaceutical Science* by E.W. Martin (1995) describes formulations that can be used in connection with the disclosed methods. In general, the EEV-cargo conjugate can be

formulated such that an effective amount of the EEV-cargo conjugate is combined with a suitable carrier in order to facilitate effective administration of the EEV-cargo conjugate. The compositions used can also be in a variety of forms. These include, for example, solid, semi-solid, and liquid dosage forms, such as tablets, pills, powders, liquid solutions or suspension, suppositories, injectable and infusible solutions, and sprays. The form depends on the intended mode of administration and therapeutic application. The compositions also include conventional pharmaceutically-acceptable carriers and diluents which are known to those skilled in the art. Examples of carriers or diluents include ethanol, dimethyl sulfoxide, glycerol, alumina, starch, saline, and equivalent carriers and diluents. To provide for the administration of such dosages for the desired therapeutic treatment, compositions can comprise between 0.1% and 100% by weight of the total of one or more EEV-cargo conjugates based on the weight of the total composition including carrier or diluent.

[0462] Formulations suitable for administration include, for example, aqueous sterile injection solutions, which can contain antioxidants, buffers, bacteriostats, and solutes that render the formulation isotonic with the blood of the intended recipient; and aqueous and nonaqueous sterile suspensions, which can include suspending agents and thickening agents. The formulations can be presented in unit-dose or multi-dose containers, for example sealed ampoules and vials, and can be stored in a freeze dried (lyophilized) condition requiring only the condition of the sterile liquid carrier, for example, water for injections, prior to use. Extemporaneous injection solutions and suspensions can be prepared from sterile powder, granules, tablets, *etc*. It should be understood that in addition to the ingredients particularly mentioned above, the compositions can include other agents conventional in the art having regard to the type of formulation in question.

[0463] EEV-cargo conjugates and compositions comprising them, can be delivered to a cell either through direct contact with the cell or via a carrier means. Carrier means are known in the art and include, for example, encapsulating the composition in a liposome moiety. Another means for delivery comprises attaching the EEV-cargo conjugate to a protein or nucleic acid that is targeted for delivery to the target cell. U.S. Patent No. 6,960,648 and U.S. Application Publication Nos. 20030032594 and 20020120100 disclose amino acid sequences that can be coupled to another composition and that allows the composition to be translocated across biological membranes. U.S. Application Publication No. 20020035243 also describes

compositions for transporting biological moieties across cell membranes for intracellular delivery. EEV-cargo conjugates can also be incorporated into polymers, examples of which include poly (D-L lactide-co-glycolide) polymer for intracranial tumors; poly[bis(p-carboxyphenoxy) propane:sebacic acid] in a 20:80 molar ratio (as used in GLIADEL); chondroitin; chitin; and chitosan.

[0464] EEV-cargo conjugates and compositions thereof, including pharmaceutically acceptable salts or prodrugs thereof, can be administered intravenously, intramuscularly, or intraperitoneally by infusion or injection. Solutions of the active agent or its salts can be prepared in water, optionally mixed with a nontoxic surfactant. Dispersions can also be prepared in glycerol, liquid polyethylene glycols, triacetin, and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations can contain a preservative to prevent the growth of microorganisms.

[0465] The pharmaceutical dosage forms suitable for injection or infusion can include sterile aqueous solutions or dispersions or sterile powders comprising the active ingredient, which are adapted for the extemporaneous preparation of sterile injectable or infusible solutions or dispersions, optionally encapsulated in liposomes. The ultimate dosage form should be sterile, fluid and stable under the conditions of manufacture and storage. The liquid carrier or vehicle can be a solvent or liquid dispersion medium comprising, for example, water, ethanol, a polyol (for example, glycerol, propylene glycol, liquid polyethylene glycols, and the like), vegetable oils, nontoxic glyceryl esters, and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the formation of liposomes, by the maintenance of the required particle size in the case of dispersions or by the use of surfactants. Optionally, the prevention of the action of microorganisms can be brought about by various other antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. In many cases, isotonic agents are included, for example, sugars, buffers or sodium chloride. Prolonged absorption of the injectable compositions can be brought about by the inclusion of agents that delay absorption, for example, aluminum monostearate and gelatin.

[0466] Sterile injectable solutions are prepared by incorporating an EEV-cargo conjugate in the required amount in the appropriate solvent with various other ingredients enumerated above, as required, followed by filter sterilization. In the case of sterile powders for the preparation of sterile injectable solutions, the methods of preparation include vacuum drying and the freeze

drying techniques, which yield a powder of the active ingredient plus any additional desired ingredient present in the previously sterile-filtered solutions.

[0467] Useful dosages of the EEV-cargo conjugate and pharmaceutical compositions thereof can be determined by comparing their *in vitro* activity, and *in vivo* activity in animal models. Methods for the extrapolation of effective dosages in mice, and other animals, to humans are known to the art.

[0468] The dosage ranges for the administration of the compositions are those large enough to produce the desired effect in which the symptoms or disorder are affected. The dosage should not be so large as to cause adverse side effects, such as unwanted cross-reactions, anaphylactic reactions, and the like. Generally, the dosage will vary with the age, condition, sex and extent of the disease in the patient and can be determined by one of skill in the art. The dosage can be adjusted by the individual physician in the event of any counterindications. Dosage can vary, and can be administered in one or more dose administrations daily, for one or several days.

[0469] Also disclosed are pharmaceutical compositions that comprise an EEV-cargo conjugate in combination with a pharmaceutically acceptable carrier. Pharmaceutical compositions adapted for oral, topical or parenteral administration, comprising an amount of an EEV-cargo conjugate are provided. The dose administered to a patient, particularly a human, should be sufficient to achieve a therapeutic response in the patient over a reasonable time frame, without lethal toxicity, and causing no more than an acceptable level of side effects or morbidity. One skilled in the art will recognize that dosage will depend upon a variety of factors including the condition (health) of the subject, the body weight of the subject, kind of concurrent treatment, if any, frequency of treatment, therapeutic ratio, as well as the severity and stage of the pathological condition.

[0470] Also disclosed are kits that comprise an EEV-cargo conjugate in one or more containers. The disclosed kits can optionally include pharmaceutically acceptable carriers and/or diluents. In embodiments, a kit includes one or more other components, adjuncts, or adjuvants as described herein. In embodiments, a kit includes one or more anti-cancer agents, such as those agents described herein. In embodiments, a kit includes instructions or packaging materials that describe how to administer an EEV-cargo conjugate or composition thereof. Containers of the kit can be of any suitable material, *e.g.*, glass, plastic, metal, *etc.*, and of any suitable size, shape, or configuration. In embodiments, an EEV-cargo conjugate is provided in the kit as a solid, such as

a tablet, pill, or powder form. In embodiments, an EEV-cargo conjugate is provided in the kit as a liquid or solution. In embodiments, the kit comprises an ampoule or syringe containing an EEV-cargo conjugate in liquid or solution form.

[0471] A number of embodiments of the invention have been described. Nevertheless, it will be understood that various modifications may be made without departing from the spirit and scope of the invention. Accordingly, other embodiments are within the scope of the following claims.

Certain Definitions

[0472] As used in the description and the appended claims, the singular forms "a," "an," and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to "a composition" includes mixtures of two or more such compositions, reference to "an agent" includes mixtures of two or more such agents, reference to "the component" includes mixtures of two or more such components, and the like.

[0473] As used herein, the term "cyclic cell penetrating peptide" or "cCPP" refers to a peptide that facilitates the delivery of a cargo to the cytosol of a cell.

[0474] As used herein, the term "endosomal escape vehicle" (EEV) refers to a cCPP that is conjugated by a chemical linkage (i.e., a covalent bond or non-covalent interaction) to a linker and/or an exocyclic peptide (EP).

[0475] As used herein, the term "EEV-cargo conjugate" refers to an endosomal escape vehicle (EEV) defined herein conjugated by a chemical linkage (i.e., a covalent bond or non-covalent interaction) to an oligonucleotide. The oligonucleotide can be delivered into a cell by the EEV.

[0476] As used herein, the term "exocyclic peptide" (EP) refers to two or more amino acid residues linked by a peptide bond that can be conjugated to a cyclic cell penetrating peptide (cCPP). The EP, when conjugated to a cCPP, may alter the tissue distribution and/or retention of the compound. Typically, the EP comprises at least one positively charged amino acid residue, e.g., at least one lysine residue and/or at least one arginine residue. Non-limiting examples of EP are described herein. The EP can be a peptide that has been identified in the art as a "nuclear localization sequence" (NLS). Non-limiting examples of nuclear localization sequences include the nuclear localization sequence of the SV40 virus large T-antigen, the minimal functional unit of which is the seven amino acid sequence PKKKRKV, the nucleoplasmin bipartite NLS with the sequence NLSKRPAAIKKAGQAKKKK, the c-myc nuclear localization sequence having the amino acid sequence PAAKRVKLD or RQRRNELKRSF, the sequence

RMRKFKNKGKDTAELRRRRVEVSVELRKAKKDEQILKRRNV of the IBB domain from importin-alpha, the sequences VSRKRPRP and PPKKARED of the myoma T protein, the sequence PQPKKKPL of human p53, the sequence SALIKKKKKMAP of mouse c-abl IV, the sequences DRLRR and PKQKKRK of the influenza virus NS1, the sequence RKLKKKIKKL of the Hepatitis virus delta antigen and the sequence REKKKFLKRR of the mouse Mxl protein, the sequence KRKGDEVDGVDEVAKKKSKK of the human poly(ADP-ribose) polymerase and the sequence RKCLQAGMNLEARKTKK of the steroid hormone receptors (human) glucocorticoid. International Publication No. 2001/038547 describes additional examples of NLSs and is incorporated by reference herein in its entirety.

[0477] As used herein, "linker" or "L" refers to a moiety that covalently bonds one or more moieties (*e.g.*, an exocyclic peptide (EP) and an oligonucleotide to the cyclic cell penetrating peptide (cCPP). The linker can comprise a natural or non-natural amino acid or polypeptide. The linker can be a synthetic compound containing two or more appropriate functional groups. The linker can comprise a polyethylene glycol (PEG) component. The linker can comprise one or more amino acid (AA) components. The linker can comprise one or more hydrophobic groups (X).

[0478] As used herein, the term "oligonucleotide" refers to an oligomeric compound comprising a plurality of linked nucleotides or nucleosides. One or more nucleotides of an oligonucleotide can be modified. An oligonucleotide can comprise ribonucleic acid (RNA) or deoxyribonucleic acid (DNA). Oligonucleotides can be composed of natural and/or modified nucleobases, sugars and covalent internucleoside linkages, and can further include non-nucleic acid conjugates. In embodiments, the oligonucleotide is a therapeutic oligonucleotide.

[0479] The terms "peptide," "protein," and "polypeptide" are used interchangeably to refer to a natural or synthetic molecule comprising two or more amino acids linked by the carboxyl group of one amino acid to the alpha amino group of another. Two or more amino acid residues can be linked by the carboxyl group of one amino acid to the alpha amino group. Two or more amino acids of the polypeptide can be joined by a peptide bond. The polypeptide can include a peptide backbone modification in which two or more amino acids are covalently attached by a bond other than a peptide bond. The polypeptide can include one or more non-natural amino acids, amino acid analogs, or other synthetic molecules that are capable of integrating into a polypeptide. The term polypeptide includes naturally occurring and artificially occurring amino

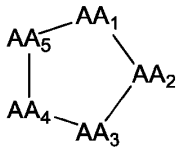
acids. The term polypeptide includes peptides, for example, that include from 2 to 100 amino acid residues as well as proteins, that include more than 100 amino acid residues, or more than 1000 amino acid residues, including, but not limited to therapeutic proteins such as antibodies, enzymes, receptors, soluble proteins and the like.

[0480] As used herein, "polyethylene glycol" and "PEG" are used interchangeably. PEG refers to the group of the chemical formula $-CH_2CH_2O-$.

[0481] The term "PEG2"refers to 2-[2-[2-aminoethoxy]ethoxy]acetic acid.

[0482] The term "therapeutic polypeptide" refers to a polypeptide that has therapeutic, prophylactic or other biological activity. The therapeutic polypeptide can be produced in any suitable manner. For example, the therapeutic polypeptide may be isolated or purified from a naturally occurring environment, may be chemically synthesized, may be recombinantly produced, or a combination thereof.

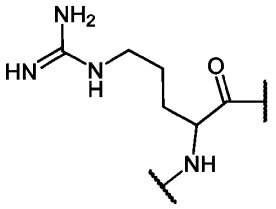
[0483] The term "small molecule" refers to an organic compound with pharmacological activity and a molecular weight of less than 2000 Daltons, or less than 1000 Daltons, or less than 500 Daltons. Small molecule therapeutics are typically manufactured by chemical synthesis.

[0484] As used herein, the term "contiguous" refers to two amino acids, which are connected by a covalent bond. For example, in the context of a representative cyclic cell penetrating peptide (cCPP) such as 

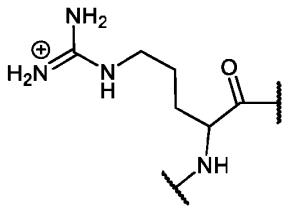
, AA_1/AA_2, AA_2/AA_3, AA_3/AA_4, and AA_5/AA_1 exemplify pairs of contiguous amino acids.

[0485] A residue of a chemical species, as used herein, refers to a derivative of the chemical species that is present in a particular product. To form the product, at least one atom of the species is replaced by a bond to another moiety, such that the product contains a derivative, or residue, of the chemical species. For example, the cyclic cell penetrating peptides (cCPP) described herein have amino acids (e.g., arginine) incorporated therein through formation of one or more peptide bonds. The amino acids incorporated into the cCPP may be referred to as

residues, or simply as an amino acid. Thus, arginine or an arginine residue refers to



[0486] The term "protonated form thereof" refers to a protonated form of an amino acid. For example, the guanidine group on the side chain of arginine may be protonated to form a



guanidinium group. The structure of a protonated form of arginine is

[0487] As used herein, the term "chirality" refers to a molecule that has more than one stereoisomer that differs in the three-dimensional spatial arrangement of atoms, in which one stereoisomer is a non-superimposable mirror image of the other. Amino acids, except for glycine, have a chiral carbon atom adjacent to the carboxyl group. The term "enantiomer" refers to stereoisomers that are chiral. The chiral molecule can be an amino acid residue having a "D" and "L" enantiomer. Molecules without a chiral center, such as glycine, can be referred to as "achiral."

[0488] As used herein, the term "hydrophobic" refers to a moiety that is not soluble in water or has minimal solubility in water. Generally, neutral moieties and/or non-polar moieties, or moieties that are predominately neutral and/or non-polar are hydrophobic.

[0489] As used herein "aromatic" refers to an unsaturated cyclic molecule having $4n + 2\ \pi$ electrons, wherein n is any integer. The term "non-aromatic" refers to any unsaturated cyclic molecule which does not fall within the definition of aromatic.

[0490] "Alkyl", "alkyl chain" or "alkyl group" refer to a fully saturated, straight or branched hydrocarbon chain radical having from one to forty carbon atoms, and which is attached to the rest of the molecule by a single bond. Alkyls comprising any number of carbon atoms from 1 to 40 are included. An alkyl comprising up to 40 carbon atoms is a C_1-C_{40} alkyl, an alkyl comprising up to 10 carbon atoms is a C_1-C_{10} alkyl, an alkyl comprising up to 6 carbon atoms is

a C_1-C_6 alkyl and an alkyl comprising up to 5 carbon atoms is a C_1-C_5 alkyl. A C_1-C_5 alkyl includes C_5 alkyls, C_4 alkyls, C_3 alkyls, C_2 alkyls and C_1 alkyl (*i.e.*, methyl). A C_1-C_6 alkyl includes all moieties described above for C_1-C_5 alkyls but also includes C_6 alkyls. A C_1-C_{10} alkyl includes all moieties described above for C_1-C_5 alkyls and C_1-C_6 alkyls, but also includes C_7, C_8, C_9 and C_{10} alkyls. Similarly, a C_1-C_{12} alkyl includes all the foregoing moieties, but also includes C_{11} and C_{12} alkyls. Non-limiting examples of C_1-C_{12} alkyl include methyl, ethyl, *n*-propyl, *i*-propyl, *sec*-propyl, *n*-butyl, *i*-butyl, *sec*-butyl, *t*-butyl, *n*-pentyl, *t*-amyl, *n*-hexyl, *n*-heptyl, *n*-octyl, *n*-nonyl, *n*-decyl, *n*-undecyl, and *n*-dodecyl. Unless stated otherwise specifically in the specification, an alkyl group can be optionally substituted.

[0491] "Alkylene", "alkylene chain" or "alkylene group" refers to a fully saturated, straight or branched divalent hydrocarbon chain radical, having from one to forty carbon atoms. Non-limiting examples of C_2-C_{40} alkylene include ethylene, propylene, *n*-butylene, ethenylene, propenylene, *n*-butenylene, propynylene, *n*-butynylene, and the like. Unless stated otherwise specifically in the specification, an alkylene chain can be optionally substituted.

[0492] "Alkenyl", "alkenyl chain" or "alkenyl group" refers to a straight or branched hydrocarbon chain radical having from two to forty carbon atoms and having one or more carbon-carbon double bonds. Each alkenyl group is attached to the rest of the molecule by a single bond. Alkenyl groups comprising any number of carbon atoms from 2 to 40 are included. An alkenyl group comprising up to 40 carbon atoms is a C_2-C_{40} alkenyl, an alkenyl comprising up to 10 carbon atoms is a C_2-C_{10} alkenyl, an alkenyl group comprising up to 6 carbon atoms is a C_2-C_6 alkenyl and an alkenyl comprising up to 5 carbon atoms is a C_2-C_5 alkenyl. A C_2-C_5 alkenyl includes C_5 alkenyls, C_4 alkenyls, C_3 alkenyls, and C_2 alkenyls. A C_2-C_6 alkenyl includes all moieties described above for C_2-C_5 alkenyls but also includes C_6 alkenyls. A C_2-C_{10} alkenyl includes all moieties described above for C_2-C_5 alkenyls and C_2-C_6 alkenyls, but also includes C_7, C_8, C_9 and C_{10} alkenyls. Similarly, a C_2-C_{12} alkenyl includes all the foregoing moieties, but also includes C_{11} and C_{12} alkenyls. Non-limiting examples of C_2-C_{12} alkenyl include ethenyl (vinyl), 1-propenyl, 2-propenyl (allyl), iso-propenyl, 2-methyl-1-propenyl, 1-butenyl, 2-butenyl, 3-butenyl, 1-pentenyl, 2-pentenyl, 3-pentenyl, 4-pentenyl, 1-hexenyl, 2-hexenyl, 3-hexenyl, 4-hexenyl, 5-hexenyl, 1-heptenyl, 2-heptenyl, 3-heptenyl, 4-heptenyl, 5-heptenyl, 6-heptenyl, 1-octenyl, 2-octenyl, 3-octenyl, 4-octenyl, 5-octenyl, 6-octenyl, 7-octenyl, 1-nonenyl, 2-nonenyl, 3-nonenyl, 4-nonenyl, 5-nonenyl, 6-nonenyl, 7-nonenyl, 8-nonenyl, 1-decenyl, 2-decenyl, 3-

decenyl, 4-decenyl, 5-decenyl, 6-decenyl, 7-decenyl, 8-decenyl, 9-decenyl, 1-undecenyl, 2-undecenyl, 3-undecenyl, 4-undecenyl, 5-undecenyl, 6-undecenyl, 7-undecenyl, 8-undecenyl, 9-undecenyl, 10-undecenyl, 1-dodecenyl, 2-dodecenyl, 3-dodecenyl, 4-dodecenyl, 5-dodecenyl, 6-dodecenyl, 7-dodecenyl, 8-dodecenyl, 9-dodecenyl, 10-dodecenyl, and 11-dodecenyl. Unless stated otherwise specifically in the specification, an alkyl group can be optionally substituted.

[0493] "Alkenylene", "alkenylene chain" or "alkenylene group" refers to a straight or branched divalent hydrocarbon chain radical, having from two to forty carbon atoms, and having one or more carbon-carbon double bonds. Non-limiting examples of C_2-C_{40} alkenylene include ethene, propene, butene, and the like. Unless stated otherwise specifically in the specification, an alkenylene chain can be optionally.

[0494] "Alkoxy" or "alkoxy group" refers to the group -OR, where R is alkyl, alkenyl, alkynyl, cycloalkyl, or heterocyclyl as defined herein. Unless stated otherwise specifically in the specification, an alkoxy group can be optionally substituted.

[0495] "Acyl" or "acyl group" refers to the group -C(O)R, where R is hydrogen, alkyl, alkenyl, alkynyl, carbocyclyl, or heterocyclyl, as defined herein. Unless stated otherwise specifically in the specification, acyl can be optionally substituted.

[0496] "Alkylcarbamoyl" or "alkylcarbamoyl group" refers to the group -O-C(O)-NR_aR_b, where R_a and R_b are the same or different and are independently an alkyl, alkenyl, alkynyl, aryl, heteroaryl, as defined herein, or R_aR_b can be taken together to form a cycloalkyl group or heterocyclyl group, as defined herein. Unless stated otherwise specifically in the specification, an alkylcarbamoyl group can be optionally substituted.

[0497] "Alkylcarboxamidyl" or "alkylcarboxamidyl group" refers to the group –C(O)-NR_aR_b, where R_a and R_b are the same or different and are independently an alkyl, alkenyl, alkynyl, aryl, heteroaryl, cycloalkyl, cycloalkenyl, cycloalkynyl, or heterocyclyl group, as defined herein, or R_aR_b can be taken together to form a cycloalkyl group, as defined herein. Unless stated otherwise specifically in the specification, an alkylcarboxamidyl group can be optionally substituted.

[0498] "Aryl" refers to a hydrocarbon ring system radical comprising hydrogen, 6 to 18 carbon atoms and at least one aromatic ring. For purposes of this invention, the aryl radical can be a monocyclic, bicyclic, tricyclic, or tetracyclic ring system, which can include fused or bridged ring systems. Aryl radicals include, but are not limited to, aryl radicals derived from aceanthrylene, acenaphthylene, acephenanthrylene, anthracene, azulene, benzene, chrysene,

fluoranthene, fluorene, *as*-indacene, *s*-indacene, indane, indene, naphthalene, phenalene, phenanthrene, pleiadene, pyrene, and triphenylene. Unless stated otherwise specifically in the specification, the term "aryl" refers to aryl radicals that are optionally substituted.

[0499] "Heteroaryl" refers to a 5- to 20-membered ring system radical comprising hydrogen atoms, one to thirteen carbon atoms, one to six heteroatoms selected from nitrogen, oxygen and sulfur, and at least one aromatic ring. For purposes of this invention, the heteroaryl radical can be a monocyclic, bicyclic, tricyclic, or tetracyclic ring system, which can include fused or bridged ring systems; and the nitrogen, carbon, or sulfur atoms in the heteroaryl radical can be optionally oxidized; the nitrogen atom can be optionally quaternized. Examples include, but are not limited to, azepinyl, acridinyl, benzimidazolyl, benzothiazolyl, benzindolyl, benzodioxolyl, benzofuranyl, benzooxazolyl, benzothiazolyl, benzothiadiazolyl, benzo[*b*][1,4]dioxepinyl, 1,4-benzodioxanyl, benzonaphthofuranyl, benzoxazolyl, benzodioxolyl, benzodioxinyl, benzopyranyl, benzopyranonyl, benzofuranyl, benzofuranonyl, benzothienyl (benzothiophenyl), benzotriazolyl, benzo[4,6]imidazo[1,2-a]pyridinyl, carbazolyl, cinnolinyl, dibenzofuranyl, dibenzothiophenyl, furanyl, furanonyl, isothiazolyl, imidazolyl, indazolyl, indolyl, indazolyl, isoindolyl, indolinyl, isoindolinyl, isoquinolyl, indolizinyl, isoxazolyl, naphthyridinyl, oxadiazolyl, 2-oxoazepinyl, oxazolyl, oxiranyl, 1-oxidopyridinyl, 1-oxidopyrimidinyl, 1-oxidopyrazinyl, 1-oxidopyridazinyl, 1-phenyl-1*H*-pyrrolyl, phenazinyl, phenothiazinyl, phenoxazinyl, phthalazinyl, pteridinyl, purinyl, pyrrolyl, pyrazolyl, pyridinyl, pyrazinyl, pyrimidinyl, pyridazinyl, quinazolinyl, quinoxalinyl, quinolinyl, quinuclidinyl, isoquinolinyl, tetrahydroquinolinyl, thiazolyl, thiadiazolyl, triazolyl, tetrazolyl, triazinyl, and thiophenyl (i.e., thienyl). Unless stated otherwise specifically in the specification, a heteroaryl group can be optionally substituted.

[0500] The term "substituted" used herein refers to any of the above groups (*i.e.*, alkyl, alkenyl, alkynyl, cycloalkyl, cycloalkenyl, cycloalkynyl, heterocyclyl, aryl, heteroaryl, alkoxy, aryloxy, acyl, alkylcarbamoyl, alkylcarboxamidyl, alkoxycarbonyl, alkylthio, or arylthio) wherein at least one atom is replaced by a non-hydrogen atoms such as, but not limited to: a halogen atom such as F, Cl, Br, and I; an oxygen atom in groups such as hydroxyl groups, alkoxy groups, and ester groups; a sulfur atom in groups such as thiol groups, thioalkyl groups, sulfone groups, sulfonyl groups, and sulfoxide groups; a nitrogen atom in groups such as amines, amides, alkylamines, dialkylamines, arylamines, alkylarylamines, diarylamines, N-oxides, imides, and enamines; a

silicon atom in groups such as trialkylsilyl groups, dialkylarylsilyl groups, alkyldiarylsilyl groups, and triarylsilyl groups; and other heteroatoms in various other groups. "Substituted" also refers to any of the above groups in which one or more atoms are replaced by a higher-order bond (e.g., a double- or triple-bond) to a heteroatom such as oxygen in oxo, carbonyl, carboxyl, and ester groups; and nitrogen in groups such as imines, oximes, hydrazones, and nitriles. For example, "substituted" includes any of the above groups in which one or more atoms are replaced

with $-NR_gR_h$, $-NR_gC(=O)R_h$, $-NR_gC(=O)NR_gR_h$, $-NR_gC(=O)OR_h$, $-NR_gSO_2R_h$, $-OC(=O)NR_gR_h$, $-OR_g$, $-SR_g$, $-SOR_g$, $-SO_2R_g$, $-OSO_2R_g$, $-SO_2OR_g$, $=NSO_2R_g$, and $-SO_2NR_gR_h$. "Substituted" also refers to any of the above groups in which one or more hydrogen atoms are replaced

with $-C(=O)R_g$, $-C(=O)OR_g$, $-C(=O)NR_gR_h$, $-CH_2SO_2R_g$, $-CH_2SO_2NR_gR_h$. In the foregoing, R_g and R_h are the same or different and independently hydrogen, alkyl, alkenyl, alkynyl, alkoxy, alkylamino, thioalkyl, aryl, aralkyl, cycloalkyl, cycloalkenyl, cycloalkynyl, cycloalkylalkyl, haloalkyl, haloalkenyl, haloalkynyl, heterocyclyl, *N*-heterocyclyl, heterocyclylalkyl, heteroaryl, *N*-heteroaryl and/or heteroarylalkyl. "Substituted" further refers to any of the above groups in which one or more atoms are replaced by an amino, cyano, hydroxyl, imino, nitro, oxo, thioxo, halo, alkyl, alkenyl, alkynyl, alkoxy, alkylamino, thioalkyl, aryl, aralkyl, cycloalkyl, cycloalkenyl, cycloalkynyl, cycloalkylalkyl, haloalkyl, haloalkenyl, haloalkynyl, heterocyclyl, *N*-heterocyclyl, heterocyclylalkyl, heteroaryl, *N*-heteroaryl and/or heteroarylalkyl group. "Substituted" can also mean an amino acid in which one or more atoms on the side chain are replaced by alkyl, alkenyl, alkynyl, acyl, alkylcarboxamidyl, alkoxycarbonyl, carbocyclyl, heterocyclyl, aryl, or heteroaryl. In addition, each of the foregoing substituents can also be optionally substituted with one or more of the above substituents.

[0501] As used herein, "phenyl" refers to a cyclic group of atoms with the formula C_6H_5, which can be covalently attached to a molecule as a functional group. Phenyl can be abbreviated herein as "Ph".

[0502] As used herein, "subject" refers to an individual. Thus, the "subject" can include domesticated animals (*e.g.*, cats, dogs, etc.), livestock (*e.g.*, cattle, horses, pigs, sheep, goats, etc.), laboratory animals (*e.g.*, mouse, rabbit, rat, guinea pig, etc.), and birds. "Subject" can also include a mammal, such as a primate or a human. Thus, the subject can be a human or veterinary patient. The term "patient" refers to a subject under the treatment of a clinician, e.g., physician.

[0503] The term "treatment" refers to the medical management of a patient with the intent to cure, ameliorate, stabilize, or prevent a disease, pathological condition, or disorder. This term includes active treatment, that is, treatment directed specifically toward the improvement of a disease, pathological condition, or disorder, and also includes causal treatment, that is, treatment directed toward removal of the cause of the associated disease, pathological condition, or disorder. In addition, this term includes palliative treatment, that is, treatment designed for the relief of symptoms rather than the curing of the disease, pathological condition, or disorder; preventative treatment, that is, treatment directed to minimizing or partially or completely inhibiting the development of the associated disease, pathological condition, or disorder; and supportive treatment, that is, treatment employed to supplement another specific therapy directed toward the improvement of the associated disease, pathological condition, or disorder.

[0504] The term "therapeutically effective" refers to the amount of the composition used is of sufficient quantity to ameliorate one or more causes or symptoms of a disease or disorder. Such amelioration only requires a reduction or alteration, not necessarily elimination.

[0505] The term "pharmaceutically acceptable" refers to those compounds, materials, compositions, and/or dosage forms which are, within the scope of sound medical judgment, suitable for use in contact with the tissues of human beings and animals without excessive toxicity, irritation, allergic response, or other problems or complications commensurate with a reasonable benefit/risk ratio.

[0506] The term "carrier" refers to a compound, composition, substance, or structure that, when in combination with a compound or composition, aids or facilitates preparation, storage, administration, delivery, effectiveness, selectivity, or any other feature of the compound or composition for its intended use or purpose. For example, a carrier can be selected to minimize any degradation of the active ingredient and to minimize any adverse side effects in the subject.

[0507] The term "pharmaceutically acceptable carrier" refers to sterile aqueous or nonaqueous solutions, dispersions, suspensions or emulsions, as well as sterile powders for reconstitution into sterile injectable solutions or dispersions just prior to use. Examples of suitable aqueous and nonaqueous carriers, diluents, solvents or vehicles include water, ethanol, polyols (such as glycerol, propylene glycol, polyethylene glycol and the like), carboxymethylcellulose 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. These compositions can also contain adjuvants such as preservatives, wetting agents, emulsifying agents and dispersing agents. Prevention of the action of microorganisms can be ensured by the inclusion of various antibacterial and antifungal agents such as paraben, chlorobutanol, phenol, sorbic acid and the like. It can also be desirable to include isotonic agents such as sugars, sodium chloride and the like. The injectable formulations can be sterilized, for example, by filtration through a bacterial-retaining filter or by incorporating sterilizing agents in the form of sterile solid compositions which can be dissolved or dispersed in sterile water or other sterile injectable media just prior to use. Suitable inert carriers can include sugars such as lactose.

[0508] The term "sequence identity" refers to the percentage of amino acids between two polypeptide sequences that are the same and in the same relative position. As such one polypeptide sequence has a certain percentage of sequence identity compared to another polypeptide sequence. For sequence comparison, typically one sequence acts as a reference sequence, to which test sequences are compared. Those of ordinary skill in the art will appreciate that two sequences are generally considered to be "substantially identical" if they contain identical residues in corresponding positions. In embodiments, the sequence identity between two amino acid sequences may be determined using the Needleman-Wunsch algorithm (Needleman and Wunsch, 1970, *J. Mol. Biol.* 48: 443-453) as implemented in the Needle program of the EMBOSS package (EMBOSS: The European Molecular Biology Open Software Suite, Rice et al., 2000, *Trends Genet.* 16: 276-277), in the version that exists as of the date of filing. The parameters used are gap open penalty of 10, gap extension penalty of 0.5, and the EBLOSUM62 (EMBOSS version of BLOSUM62) substitution matrix. The output of Needle labeled "longest identity" (obtained using the –nobrief option) is used as the percent identity and is calculated as follows: (Identical Residues×100)/(Length of Alignment–Total Number of Gaps in Alignment)

[0509] In embodiments, sequence identity may be determined using the Smith-Waterman algorithm, in the version that exists as of the date of filing.

[0510] The term, "sequence homology" refers to the percentage of amino acids between two polypeptide sequences that are homologous and in the same relative position. As such one polypeptide sequence has a certain percentage of sequence homology compared to another

polypeptide sequence. As will be appreciated by those of ordinary skill in the art, two sequences are generally considered to be "substantially homologous" if they contain homologous residues in corresponding positions. Homologous residues may be identical residues. Alternatively, homologous residues may be non-identical residues with appropriately similar structural and/or functional characteristics. For example, as is well known by those of ordinary skill in the art, certain amino acids are typically classified as "hydrophobic" or "hydrophilic" amino acids, and/or as having "polar" or "non-polar" side chains, and substitution of one amino acid for another of the same type may often be considered a "homologous" substitution.

[0511] As is well known in this art, amino acid sequences may be compared using any of a variety of algorithms, including those available in commercial computer programs such as BLASTP, gapped BLAST, and PSI-BLAST, in existence as of the date of filing. Such programs are described in Altschul, et al., Basic local alignment search tool, *J. Mol. Biol.,* 215(3): 403-410, 1990; Altschul, et al., *Methods in Enzymology*; Altschul, et al., "Gapped BLAST and PSI-BLAST: a new generation of protein database search programs", *Nucleic Acids Res.* 25:3389-3402, 1997; Baxevanis, et al., *Bioinformatics A Practical Guide to the Analysis of Genes and Proteins*, Wiley, 1998; and Misener, et al., (eds.), *Bioinformatics Methods and Protocols* (Methods in Molecular Biology, Vol. 132), Humana Press, 1999. In addition to identifying homologous sequences, the programs mentioned above typically provide an indication of the degree of homology.

[0512] The term "antisense oligonucleotide" refers to a polymeric nucleic acid structure (which can also be referred to as an oligonucleotide or polynucleotide) which is at least partially complementary to a target nucleic acid molecule to which it hybridizes. The oligonucleotide may be a short (in embodiments, less than 50 base pair) polynucleotide or polynucleotide homologue comprising a sequence complimentary to a target sequence in a target pre-mRNA strand. The oligonucleotide may be formed of natural nucleic acids, synthetic nucleic acids, nucleic acid homologues, or any combination thereof. In embodiments, the oligonucleotide comprises oligonucleosides. In embodiments, oligonucleotide comprises antisense oligonucleotides. In embodiments, the oligonucleotide comprises conjugate groups. Nonlimiting examples of oligonucleotides include, but are not limited to, primers, probes, antisense oligonucleotides, external guide sequence (EGS) oligonucleotides, alternate splicers, siRNAs, oligonucleotides, oligonucleosides, oligonucleotide analogs, oligonucleotide mimetics, and chimeric combinations

of these. As such, these compounds can be introduced in the form of single-stranded, double-stranded, circular, branched or hairpins and can contain structural elements such as internal or terminal bulges or loops. Oligomeric double-stranded compounds can be two strands hybridized to form double-stranded compounds or a single strand with sufficient self complementarity to allow for hybridization and formation of a fully or partially double-stranded compound. In embodiments, an oligonucleotide modulates (increases, decreases, or changes) expression of a target nucleic acid. Various modifications may be made to the polymeric nucleic acid structure, such as phosphorodiamidate morpholino oligonucleotide (PMO). Therefore, oligonucleotide as used herein encompasses any modification described herein, such as a PMO.

[0513] The terms "pre-mRNA" and "primary transcript" as used herein refer to a newly synthesized eukaryotic mRNA molecule directly after DNA transcription. A pre-mRNA must be capped with a 5' cap, modified with a 3' poly-A tail, and spliced to produce a mature mRNA sequence.

[0514] As used herein, the terms “targeting” or “targeted to” refer to the association of an oligonucleotide, for example, a therapeutic oligonucleotide such as an antisense oligonucleotide with a target nucleic acid molecule or a region of a target nucleic acid molecule. In embodiments, the oligonucleotide is capable of hybridizing to a target nucleic acid under physiological conditions. In embodiments, the oligonucleotide targets a specific portion or site within the target nucleic acid, for example, a portion of the target nucleic acid having at least one identifiable structure, function, or characteristic such as a particular exon or intron, or selected nucleobases or motifs within an exon or intron. In embodiments, the oligonucleotide targets a region comprising the intron-exon junction of a gene that is associated with a disease or disorder. In embodiment, the oligonucleotide targets exon 50 of the dystrophin gene. In embodiments, the oligonucleotide targets a region comprising the intron-exon junction of exon 50 of the dystrophin gene. In embodiments, the oligonucleotide targets a region that comprises an intronic nucleotide sequence upstream (or 5 ') of exon 50 of the dystrophin gene. In embodiments, the oligonucleotide targets a region that comprises an intronic nucleotide sequence downstream (or 3') of exon 50 of the dystrophin gene.

[0515] As used herein, the terms "target nucleic acid" and "target sequence" refer to a nucleic acid molecule having a nucleic acid sequence to which an oligonucleotide binds, or hybridizes. Target nucleic acids include, but are not limited to, RNA (including, but not limited to pre-

mRNA and mRNA or portions thereof), cDNA derived from such RNA, as well as non-translated RNA, such as miRNA. For example, in embodiments, a target nucleic acid can be a cellular gene (or mRNA transcribed from such gene) whose expression is associated with a particular disorder or disease state, or a nucleic acid molecule from an infectious agent. In embodiments, the target nucleic acid is a target RNA. In embodiments, the target nucleic acid is a target mRNA. In embodiments, the target nucleic acid is a target pre-mRNA.

[0516] As used herein, the terms "splicing" and "processing" refer to the modification of a pre-mRNA following transcription, in which introns are removed and exons are joined. Splicing occurs in a series of reactions that are catalyzed by a large RNA-protein complex composed of five small nuclear ribonucleoproteins (snRNPs) referred to as a spliceosome. Within an intron, a 3′ splice site, a 5′ splice site, and a branch site are required for splicing. The RNA components of snRNPs interact with the intron and may be involved in catalysis

[0517] As used herein, the term "exon" refers to a portion of a pre-mRNA which, after splicing, is typically included in the mature mRNA.

[0518] As used herein, the term "intron" refers to a portion of a pre-mRNA which, after splicing, is typically excluded from the mature mRNA.

[0519] As used herein, the term "flanking" refers to an intron located immediately upstream (5') or immediately downstream (3') of an associated exon. For example, the 5' flanking intron of exon 50 refers to the intron that is immediately upstream of (i.e., directly coupled to the 5' end of) exon 50. For example, the 3' flanking intron of exon 50 refers to the intron that is immediately downstream of (i.e., directly coupled to the 5' end of) exon 50.

[0520] The "target pre-mRNA" is the pre-mRNA comprising the target sequence to which the oligonucleotide hybridizes.

[0521] The "target mRNA" is the mRNA sequence resulting from splicing of the target pre-mRNA sequence. In embodiments, the target mRNA does not encode a functional protein. In embodiments, the target mRNA retains one or more intron sequences.

[0522] As used herein, the term "gene" refers to a nucleic acid molecule having a nucleic acid sequence that encompasses a 5' promoter region associated with the expression of the gene product, and any intron and exon regions and 3' untranslated regions ("UTR") associated with the expression of the gene product.

[0523] The "target gene" refers to the gene that encodes the target pre-mRNA.

[0524] The "target protein" refers to the amino acid sequence encoded by the target mRNA. In embodiments, the target protein may not be a functional protein.

[0525] "Wild-type target protein" refers to a native, functional protein isomer produced by a wild type, normal, or unmutated version of the target gene. The wild-type target protein also refers to the protein resulting from a target pre-mRNA that has been properly spliced.

[0526] As used herein, the term “transcript” refers to an RNA molecule transcribed from DNA and includes, but is not limited to mRNA, mature mRNA, pre -mRNA, and partially processed RNA.

[0527] A "re-spliced target protein", as used herein, refers to the protein encoded by the mRNA resulting from the splicing of the target pre-mRNA to which the oligonucleotide hybridizes. Re-spliced target protein may be identical to a wild-type target protein, may be homologous to a wild-type target protein, may be a functional variant of a wild-type target protein, or may be an active fragment of a wild-type target protein.

[0528] As used herein, "functional fragment" or "active fragment" refers to a portion of a eukaryotic wild-type target protein that exhibits an activity, such as one or more activities of a full-length wild-type target protein, or that possesses another activity. In embodiments, a re-spliced target protein that shares at least one biological activity of wild-type target protein is considered to be an active fragment of the wild-type target protein. Activity can be any percentage of activity (*i.e.*, more or less) of the full-length wild-type target protein, including but not limited to, 1% of the activity, 2%, 3%, 4%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, 96%, 97%, 98%, 99%, 100%, 200%, 300%, 400%, 500%, or more (including all values and ranges inbetween these values) activity compared to the wild-type target protein. Thus, in embodiments, the active fragment may retain at least a portion of one or more biological activities of wild-type target protein. In embodiments, the active fragment may enhance one or more biological activities of wild- type target protein.

[0529] As used herein, the term "nucleoside" refers to a glycosylamine comprising a nucleobase and a sugar. Nucleosides includes, but are not limited to, natural nucleosides, abasic nucleosides, modified nucleosides, and nucleosides having mimetic bases and/or sugar groups. A "natural nucleoside" or "unmodified nucleoside" is a nucleoside comprising a natural nucleobase and a natural sugar. Natural nucleosides include RNA and DNA nucleosides.

[0530] As used herein, the term "natural sugar" refers to a sugar of a nucleoside that is unmodified from its naturally occurring form in RNA (2'-OH) or DNA (2'-H).

[0531] As used herein, the term "nucleotide" refers to a nucleoside having a phosphate group covalently linked to the sugar. Nucleotides may be modified with any of a variety of substituents.

[0532] As used herein, the term "nucleobase" refers to the base portion of a nucleoside or nucleotide. A nucleobase may comprise any atom or group of atoms capable of hydrogen bonding to a base of another nucleic acid. A natural nucleobase is a nucleobase that is unmodified from its naturally occurring form in RNA or DNA.

[0533] As used herein, the term "heterocyclic base moiety" refers to a nucleobase comprising a heterocycle.

[0534] As used herein "oligonucleoside" refers to an oligonucleotide in which the internucleoside linkages do not contain a phosphorus atom.

[0535] As used herein, the term "oligonucleotide" refers to an oligomeric compound comprising a plurality of linked nucleotides or nucleosides. In embodiments, one or more nucleotides of an oligonucleotide is modified. In embodiments, an oligonucleotide comprises ribonucleic acid (RNA) or deoxyribonucleic acid (DNA). In embodiments, oligonucleotides are composed of natural and/or modified nucleobases, sugars and covalent internucleoside linkages, and may further include non-nucleic acid conjugates.

[0536] As used herein "internucleoside linkage" refers to a covalent linkage between adjacent nucleosides.

[0537] As used herein "natural internucleotide linkage" refers to a 3' to 5' phosphodiester linkage.

[0538] As used herein, the term "modified internucleoside linkage" refers to any linkage between nucleosides or nucleotides other than a naturally occurring internucleoside linkage.

[0539] As used herein the term "chimeric antisense oligonucletode" refers to an antisense oligonucleotide, having at least one sugar, nucleobase and/or internucleoside linkage that is differentially modified as compared to the other sugars, nucleobases and internucleoside linkages within the same oligonucleotide. The remainder of the sugars, nucleobases and internucleoside linkages can be independently modified or unmodified. A chimeric antisense oligonucletode can have modified nucleosides that can be in isolated positions or grouped together in regions that

will define a particular motif. Any combination of modifications and or mimetic groups can comprise a chimeric antisense oligonucletode as described herein.

[0540] As used herein, the term "mixed-backbone antisense oligonucleotide" refers to an antisense oligonucleotide wherein at least one internucleoside linkage of the antisense oligonucleotide is different from at least one other internucleotide linkage of the antisense oligonucleotide.

[0541] As used herein, the term "nucleobase complementarity" refers to a nucleobase that is capable of base pairing with another nucleobase. For example, in DNA, adenine (A) is complementary to thymine (T). For example, in RNA, adenine (A) is complementary to uracil (U). In embodiments, complementary nucleobase refers to a nucleobase of an antisense oligonucleotide that is capable of base pairing with a nucleobase of its target nucleic acid. For example, if a nucleobase at a certain position of an antisense oligonucleotide is capable of hydrogen bonding with a nucleobase at a certain position of a target nucleic acid, then the position of hydrogen bonding between the oligonucleotide and the target nucleic acid is considered to be complementary at that nucleobase pair.

[0542] As used herein, the term "non-complementary nucleobase" refers to a pair of nucleobases that do not form hydrogen bonds with one another or otherwise support hybridization.

[0543] As used herein, the term "complementary" refers to the capacity of an oligomeric compound to hybridize to another oligomeric compound or nucleic acid through nucleobase complementarity. In embodiments, an antisense oligonucleotide and its target are complementary to each other when a sufficient number of corresponding positions in each molecule are occupied by nucleobases that can bond with each other to allow stable association between the antisense oligonucleotide and the target. One skilled in the art recognizes that the inclusion of mismatches is possible without eliminating the ability of the oligomeric compounds to remain in association. Therefore, described herein are antisense oligonucleotides that may comprise up to 20% nucleotides that are mismatched (i.e., are not nucleobase complementary to the corresponding nucleotides of the target). In embodiments, the antisense oligonucleotides contain no more than 15%, not more than 10%, not more than 5% or no mismatches. The remaining nucleotides are nucleobase complementary or otherwise do not disrupt hybridization (e.g., universal bases). One of ordinary skill in the art would recognize the compounds provided herein are at least 80%, at

least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% or 100% nucleobase complementary to a target nucleic acid.

[0544] As used herein, "hybridization" or "hybridize" refers to the pairing of complementary oligomeric compounds (e.g., an antisense oligonucleotide and its target nucleic acid). While not limited to a particular mechanism, the most common mechanism of pairing involves hydrogen bonding, which may be Watson-Crick, Hoogsteen or reversed Hoogsteen hydrogen bonding, between complementary nucleoside or nucleotide bases (nucleobases). For example, the natural base adenine is nucleobase complementary to the natural nucleobases thymidine and uracil which pair through the formation of hydrogen bonds. The natural base guanine is nucleobase complementary to the natural bases cytosine and 5-methyl cytosine. Hybridization can occur under varying circumstances.

[0545] As used herein, the term "specifically hybridizes" refers to the ability of an oligomeric compound to hybridize to one nucleic acid site with greater affinity than it hybridizes to another nucleic acid site. In embodiments, an antisense oligonucleotide specifically hybridizes to more than one target site. In embodiments, an oligomeric compound specifically hybridizes with its target under stringent hybridization conditions.

[0546] The terms “modulate”, “modulating” and “modulation” refer to a perturbation of expression, function or activity when compared to the level of expression, function or activity prior to modulation. Modulation can include an increase (stimulation or induction) or a decrease (inhibition or reduction) in expression, function or activity. In embodiments, modulation can include perturbation of splice site selection during pre-mRNA processing.

[0547] The terms “inhibit”, “inhibiting” or “inhibition” refer to a decrease in an activity, expression, function or other biological parameter and can include, but does not require complete ablation of the activity, expression, function or other biological parameter. Inhibition can include, for example, at least a 10% reduction in the activity, response, condition, or disease as compared to a control. In embodiments, expression, activity or function of a gene or protein is decreased by a statistically significant amount.

[0548] As used herein, the term "expression" refers to all the functions and steps by which a gene's coded information is converted into structures present and operating in a cell. Such structures include, but are not limited to, the products of transcription and translation.

[0549] As used herein, the term "2'-modified" or "2'-substituted" refers to a sugar comprising substituent at the 2' position other than H or OH. 2'-modified monomers, include, but are not limited to, BNA's and monomers (e.g., nucleosides and nucleotides) with 2'- substituents, such as allyl, amino, azido, thio, O-allyl, O-C_1-C_{10} alkyl, -OCF_3, O-$(CH_2)_2$-O-CH_3, 2'-O$(CH_2)_2SCH_3$, O-$(CH_2)_2$-O-N(R_m)(R_n), or O-CH_2-C(=O)-N(R_m)(R_n), where each Rm and Rn is, independently, H or substituted or unsubstituted C_1-C_{10} alkyl.

[0550] As used herein, the term "MOE" refers to a 2'-O-methoxyethyl substituent.

[0551] As used herein, the term "high-affinity modified nucleotide" refers to a nucleotide having at least one modified nucleobase, internucleoside linkage or sugar moiety, such that the modification increases the affinity of an antisense oligonucleotide comprising the modified nucleotide to a target nucleic acid. High-affinity modifications include, but are not limited to, BNAs, LNAs and 2'-MOE.

[0552] As used herein the term "mimetic" refers to groups that are substituted for a sugar, a nucleobase, and/ or internucleoside linkage in an oligonucleotide. Generally, a mimetic is used in place of the sugar or sugar-internucleoside linkage combination, and the nucleobase is maintained for hybridization to a selected target. Representative examples of a sugar mimetic include, but are not limited to, cyclohexenyl or morpholino. Representative examples of a mimetic for a sugar-internucleoside linkage combination include, but are not limited to, peptide nucleic acids (PNA) and morpholino groups linked by uncharged achiral linkages. In some instances, a mimetic is used in place of the nucleobase. Representative nucleobase mimetics are well known in the art and include, but are not limited to, tricyclic phenoxazine analogs and universal bases (Berger et al., Nuc Acid Res. 2000, 28:2911-14, incorporated herein by reference). Methods of synthesis of sugar, nucleoside and nucleobase mimetics are well known to those skilled in the art.

[0553] As used herein, the term "bicyclic nucleoside" or "BNA" refers to a nucleoside wherein the furanose portion of the nucleoside includes a bridge connecting two atoms on the furanose ring, thereby forming a bicyclic ring system. BNAs include, but are not limited to, α-L-LNA, β-D-LNA, ENA, Oxyamino BNA (2'-O-N(CH_3)-CH_2-4') and Aminooxy BNA (2'-N(CH_3)-O-CH_2-4').

[0554] As used herein, the term "4' to 2' bicyclic nucleoside" refers to a BNA wherin the bridge connecting two atoms of the furanose ring bridges the 4' carbon atom and the 2' carbon atom of the furanose ring, thereby forming a bicyclic ring system.

[0555] As used herein, a "locked nucleic acid" or "LNA" refers to a nucleotide modified such that the 2'-hydroxyl group of the ribosyl sugar ring is linked to the 4' carbon atom of the sugar ring via a methylene groups, thereby forming a 2'-C,4'-C-oxymethylene linkage. LNAs include, but are not limited to, α-L-LNA, and β-D-LNA.

[0556] As used herein, the term "cap structure" or "terminal cap moiety" refers to chemical modifications, which have been incorporated at either end of an oligonucleotide.

[0557] As used herein, the term "dosage unit" refers to a form in which a pharmaceutical agent is provided. In embodiments, a dosage unit is a vial comprising lyophilized antisense oligonucleotide. In embodiments, a dosage unit is a vial comprising reconstituted antisense oligonucleotide.

[0558] As used herein, “Bip” refers to p-phenyl-phenylalanine.

[0559] A number of embodiments have been described. Nevertheless, it will be understood that various modifications may be made without departing from the spirit and scope of the invention. Other embodiments are within the scope of the following claims.

[0560] All publications, patents and patent applications mentioned in the specification are indicative of the level of skill of those skilled in the art to which this invention pertains. All publications, patents and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

EXAMPLES

Example 1. Conjugation of oligonucleotide with cell penetrating peptide.

[0561] **Conjugation of oligonucleotide to a cCPP**. As shown in **FIG. 1A**, an oligonucleotide with a (NH_2-$(CH_2)_5$-CH_2-) linker on the 5’ phosphorothioate end is conjugated to a cCPP via a carboxylate or an N-hydroxysuccinimide ester (NHS ester) functional group on the peptide.

[0562] **Conjugation of PMO oligos with cell penetrating peptides.** As shown in **FIG. 1B**, phosphorodiamidate morpholino oligonucleotide (PMO) is conjugated to cell penetrating peptide

(CPP) via either amide bond formation (left) or click chemistry. The linker/CPP is be installed either on the 5' end, or on the 3' end of the oligonucleotide.

[0563] **Synthesis of oligonucleotide-peptide conjugate with PEG component.** As shown in **FIGS. 2A** and **2B**, an oligonucleotide-peptide conjugate is synthesized without (**FIG. 2A**) and with (**FIG. 2B**) a PEG (polyethylene glycol) component inserted between oligonucleotide moiety and peptide. "R" in the figure represents a palmitoyl group.

[0564] Antisense oligonucleotides are found in **Tables 11A-11D, 12A-12D,** and **13** the reverse complement thereof, or a sequence with at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% nucleic acid sequence identity thereto. CPPs are found throughout this disclosure.

Example 2. *In vitro* screening of oligonucleotide sequences for Exon 50 skipping at 5 µM.

[0565] **Purpose:** This study employed the CRL-2061™ muscle cell line (SJ-RH30) to study the effect of the oligonucleotides of **Tables 14A-14D** on skipping of exon 50 of the *DMD* gene.

[0566] **Study design:** CRL-2061™ muscle cell line was established from the bone marrow of a patient with rhabdomyosarcoma. CRL-2061™ cells do not express dystrophin due to retention of introns 40, 58, and 70. CRL-2061™ cells are plated in 48-well plates (0.03 million cells/well) plate and cultured in 500 µL of RPMI1640 (ATCC) containing 10% FBS (Gibco) and 1x Penicillin-Streptomycin (Gibco) under conditions of 37 °C and 5% CO_2 overnight.

[0567] CRL-2061™ cells were treated with 5 µM of 21-, 22-, 23-, or 24-mer PMO and 3 µL of Endo-Porter for 24 hours. Endo-Porter is a weak-base, amphiphilic peptide used to deliver morpholino oligonucleotides to cells. The Endo-Porter peptide is described in the following reference: Summerton JE. Ann N Y Acad Sci. 2005 Nov; 1058: 62-75.

[0568] The oligonucleotide sequences are shown in **Tables 14A-14D: Table 14A** (21-mer PMO); **Table 14B** (22mer PMO); **Table 14C** (23mer PMO) and **Table 14D** (24mer PMO). In the absence of treatment with an oligonucleotide-PMO, exon skipping was not observed.

***Table 14A:** 21-mer 5µM PMO tested.*

Oligonucleotide Name	Nucleic Acid Sequence (Reverse complement 5' – 3')
21mer-n5	GATCTTCTAACTTCCTcttta
21mer-n4	AGATCTTCTAACTTCCTcttt
21mer-n3	CAGATCTTCTAACTTCCTctt
21mer-n2	TCAGATCTTCTAACTTCCTct

21mer-n1	CTCAGATCTTCTAACTTCCTc
21mer-00	GCTCAGATCTTCTAACTTCCT
21mer-01	AGCTCAGATCTTCTAACTTCC
21mer-02	GAGCTCAGATCTTCTAACTTC
21mer-03	AGAGCTCAGATCTTCTAACTT
21mer-04	CAGAGCTCAGATCTTCTAACT
21mer-05	TCAGAGCTCAGATCTTCTAAC
21mer-06	CTCAGAGCTCAGATCTTCTAA
21mer-07	ACTCAGAGCTCAGATCTTCTA
21mer-08	CACTCAGAGCTCAGATCTTCT
21mer-09	CCACTCAGAGCTCAGATCTTC
21mer-10	TCCACTCAGAGCTCAGATCTT
21mer-11	TTCCACTCAGAGCTCAGATCT
21mer-12	CTTCCACTCAGAGCTCAGATC
21mer-13	CCTTCCACTCAGAGCTCAGAT
21mer-14	GCCTTCCACTCAGAGCTCAGA
21mer-15	CGCCTTCCACTCAGAGCTCAG
21mer-16	CCGCCTTCCACTCAGAGCTCA
21mer-17	ACCGCCTTCCACTCAGAGCTC
21mer-18	TACCGCCTTCCACTCAGAGCT
21mer-19	TTACCGCCTTCCACTCAGAGC
21mer-20	TTTACCGCCTTCCACTCAGAG
21mer-21	GTTTACCGCCTTCCACTCAGA
21mer-22	GGTTTACCGCCTTCCACTCAG
21mer-23	CGGTTTACCGCCTTCCACTCA
21mer-24	ACGGTTTACCGCCTTCCACTC
21mer-25	AACGGTTTACCGCCTTCCACT
21mer-26	AAACGGTTTACCGCCTTCCAC
21mer-59	AGGTCAGGCTGCTTTGCCCTC
21mer-60	TAGGTCAGGCTGCTTTGCCCT
21mer-61	CTAGGTCAGGCTGCTTTGCCC
21mer-62	GCTAGGTCAGGCTGCTTTGCC
21mer-63	AGCTAGGTCAGGCTGCTTTGC
21mer-64	GAGCTAGGTCAGGCTGCTTTG
21mer-65	GGAGCTAGGTCAGGCTGCTTT
21mer-66	AGGAGCTAGGTCAGGCTGCTT
21mer-67	CAGGAGCTAGGTCAGGCTGCT
21mer-68	CCAGGAGCTAGGTCAGGCTGC
21mer-69	TCCAGGAGCTAGGTCAGGCTG
21mer-70	GTCCAGGAGCTAGGTCAGGCT
21mer-71	AGTCCAGGAGCTAGGTCAGGC
21mer-72	CAGTCCAGGAGCTAGGTCAGG
21mer-73	TCAGTCCAGGAGCTAGGTCAG
21mer-74	GTCAGTCCAGGAGCTAGGTCA
21mer-75	GGTCAGTCCAGGAGCTAGGTC

21mer-76	TGGTCAGTCCAGGAGCTAGGT
21mer-77	GTGGTCAGTCCAGGAGCTAGG
21mer-78	AGTGGTCAGTCCAGGAGCTAG
21mer-79	TAGTGGTCAGTCCAGGAGCTA
21mer-93	cttacAGGCTCCAATAGTGGT
21mer-94	acttacAGGCTCCAATAGTGG
21mer-95	tacttacAGGCTCCAATAGTG
21mer-96	atacttacAGGCTCCAATAGT
21mer-97	tatacttacAGGCTCCAATAG
21mer-98	gtatacttacAGGCTCCAATA
21mer-99	agtatacttacAGGCTCCAAT
21mer-100	cagtatacttacAGGCTCCAA
21mer-101	ccagtatacttacAGGCTCCA

***Table 14B**: 22-mer 5μM PMO tested.*

Oligonucleotide Name	Nucleic Acid Sequence (Reverse complement 5' – 3')
22mer-n1	GCTCAGATCTTCTAACTTCCTc
22mer-n2	CTCAGATCTTCTAACTTCCTct
22mer-n3	TCAGATCTTCTAACTTCCTctt
22mer-n4	CAGATCTTCTAACTTCCTcttt
22mer-n5	AGATCTTCTAACTTCCTcttta
22mer-00	AGCTCAGATCTTCTAACTTCCT
22mer-01	GAGCTCAGATCTTCTAACTTCC
22mer-02	AGAGCTCAGATCTTCTAACTTC
22mer-03	CAGAGCTCAGATCTTCTAACTT
22mer-04	TCAGAGCTCAGATCTTCTAACT
22mer-05	CTCAGAGCTCAGATCTTCTAAC
22mer-06	ACTCAGAGCTCAGATCTTCTAA
22mer-07	CACTCAGAGCTCAGATCTTCTA
22mer-08	CCACTCAGAGCTCAGATCTTCT
22mer-09	TCCACTCAGAGCTCAGATCTTC
22mer-10	TTCCACTCAGAGCTCAGATCTT
22mer-11	CTTCCACTCAGAGCTCAGATCT
22mer-12	CCTTCCACTCAGAGCTCAGATC
22mer-13	GCCTTCCACTCAGAGCTCAGAT
22mer-14	CGCCTTCCACTCAGAGCTCAGA
22mer-15	CCGCCTTCCACTCAGAGCTCAG
22mer-16	ACCGCCTTCCACTCAGAGCTCA
22mer-17	TACCGCCTTCCACTCAGAGCTC
22mer-18	TTACCGCCTTCCACTCAGAGCT
22mer-19	TTTACCGCCTTCCACTCAGAGC
22mer-20	GTTTACCGCCTTCCACTCAGAG
22mer-21	GGTTTACCGCCTTCCACTCAGA

22mer-22	CGGTTTACCGCCTTCCACTCAG
22mer-23	ACGGTTTACCGCCTTCCACTCA
22mer-24	AACGGTTTACCGCCTTCCACTC
22mer-25	AAACGGTTTACCGCCTTCCACT
22mer-26	TAAACGGTTTACCGCCTTCCAC
22mer-59	TAGGTCAGGCTGCTTTGCCCTC
22mer-60	CTAGGTCAGGCTGCTTTGCCCT
22mer-61	GCTAGGTCAGGCTGCTTTGCCC
22mer-62	AGCTAGGTCAGGCTGCTTTGCC
22mer-63	GAGCTAGGTCAGGCTGCTTTGC
22mer-64	GGAGCTAGGTCAGGCTGCTTTG
22mer-65	AGGAGCTAGGTCAGGCTGCTTT
22mer-66	CAGGAGCTAGGTCAGGCTGCTT
22mer-67	CCAGGAGCTAGGTCAGGCTGCT
22mer-68	TCCAGGAGCTAGGTCAGGCTGC
22mer-69	GTCCAGGAGCTAGGTCAGGCTG
22mer-70	AGTCCAGGAGCTAGGTCAGGCT
22mer-71	CAGTCCAGGAGCTAGGTCAGGC
22mer-72	TCAGTCCAGGAGCTAGGTCAGG
22mer-73	GTCAGTCCAGGAGCTAGGTCAG
22mer-74	GGTCAGTCCAGGAGCTAGGTCA
22mer-75	TGGTCAGTCCAGGAGCTAGGTC
22mer-76	GTGGTCAGTCCAGGAGCTAGGT
22mer-77	AGTGGTCAGTCCAGGAGCTAGG
22mer-78	TAGTGGTCAGTCCAGGAGCTAG
22mer-79	ATAGTGGTCAGTCCAGGAGCTA
22mer-80	AATAGTGGTCAGTCCAGGAGCT
22mer-81	CAATAGTGGTCAGTCCAGGAGC
22mer-82	CCAATAGTGGTCAGTCCAGGAG
22mer-83	TCCAATAGTGGTCAGTCCAGGA
22mer-84	CTCCAATAGTGGTCAGTCCAGG
22mer-85	GCTCCAATAGTGGTCAGTCCAG
22mer-87	GGCTCCAATAGTGGTCAGTCCA
22mer-88	cAGGCTCCAATAGTGGTCAGTC
22mer-93	acttacAGGCTCCAATAGTGGT
22mer-94	tacttacAGGCTCCAATAGTGG
22mer-95	atacttacAGGCTCCAATAGTG
22mer-96	tatacttacAGGCTCCAATAGT
22mer-97	gtatacttacAGGCTCCAATAG
22mer-98	agtatacttacAGGCTCCAATA
22mer-99	cagtatacttacAGGCTCCAAT
22mer-100	ccagtatacttacAGGCTCCAA
22mer-101	tccagtatacttacAGGCTCCA
22mer-102	atccagtatacttacAGGCTCC
22mer-103	gatccagtatacttacAGGCTC

22mer-104	ggatccagtatacttacAGGCT

***Table 14C**: 23-mer 5μM PMO tested.*

Oligonucleotide Name	Nucleic Acid Sequence (Reverse complement 5' – 3')
23mer-n1	AGCTCAGATCTTCTAACTTCCTc
23mer-n2	GCTCAGATCTTCTAACTTCCTct
23mer-n3	CTCAGATCTTCTAACTTCCTctt
23mer-n4	TCAGATCTTCTAACTTCCTcttt
23mer-n5	CAGATCTTCTAACTTCCTcttta
23mer-0	GAGCTCAGATCTTCTAACTTCCT
23mer-1	AGAGCTCAGATCTTCTAACTTCC
23mer-2	CAGAGCTCAGATCTTCTAACTTC
23mer-3	TCAGAGCTCAGATCTTCTAACTT
23mer-4	CTCAGAGCTCAGATCTTCTAACT
23mer-5	ACTCAGAGCTCAGATCTTCTAAC
23mer-6	CACTCAGAGCTCAGATCTTCTAA
23mer-7	CCACTCAGAGCTCAGATCTTCTA
23mer-8	TCCACTCAGAGCTCAGATCTTCT
23mer-9	TTCCACTCAGAGCTCAGATCTTC
23mer-10	CTTCCACTCAGAGCTCAGATCTT
23mer-11	CCTTCCACTCAGAGCTCAGATCT
23mer-12	GCCTTCCACTCAGAGCTCAGATC
23mer-13	CGCCTTCCACTCAGAGCTCAGAT
23mer-14	CCGCCTTCCACTCAGAGCTCAGA
23mer-15	ACCGCCTTCCACTCAGAGCTCAG
23mer-16	TACCGCCTTCCACTCAGAGCTCA
23mer-17	TTACCGCCTTCCACTCAGAGCTC
23mer-18	TTTACCGCCTTCCACTCAGAGCT
23mer-19	GTTTACCGCCTTCCACTCAGAGC
23mer-20	GGTTTACCGCCTTCCACTCAGAG
23mer-21	CGGTTTACCGCCTTCCACTCAGA
23mer-22	ACGGTTTACCGCCTTCCACTCAG
23mer-23	AACGGTTTACCGCCTTCCACTCA
23mer-24	AAACGGTTTACCGCCTTCCACTC
23mer-25	TAAACGGTTTACCGCCTTCCACT
23mer-26	GTAAACGGTTTACCGCCTTCCAC
23mer-59	CTAGGTCAGGCTGCTTTGCCCTC
23mer-60	GCTAGGTCAGGCTGCTTTGCCCT
23mer-61	AGCTAGGTCAGGCTGCTTTGCCC
23mer-62	GAGCTAGGTCAGGCTGCTTTGCC
23mer-63	GGAGCTAGGTCAGGCTGCTTTGC
23mer-64	AGGAGCTAGGTCAGGCTGCTTTG
23mer-65	CAGGAGCTAGGTCAGGCTGCTTT
23mer-66	CCAGGAGCTAGGTCAGGCTGCTT

23mer-67	TCCAGGAGCTAGGTCAGGCTGCT
23mer-68	GTCCAGGAGCTAGGTCAGGCTGC
23mer-69	AGTCCAGGAGCTAGGTCAGGCTG
23mer-70	CAGTCCAGGAGCTAGGTCAGGCT
23mer-71	TCAGTCCAGGAGCTAGGTCAGGC
23mer-72	GTCAGTCCAGGAGCTAGGTCAGG
23mer-73	GGTCAGTCCAGGAGCTAGGTCAG
23mer-74	TGGTCAGTCCAGGAGCTAGGTCA
23mer-75	GTGGTCAGTCCAGGAGCTAGGTC
23mer-76	AGTGGTCAGTCCAGGAGCTAGGT
23mer-77	TAGTGGTCAGTCCAGGAGCTAGG
23mer-78	ATAGTGGTCAGTCCAGGAGCTAG
23mer-79	AATAGTGGTCAGTCCAGGAGCTA
23mer-80	CAATAGTGGTCAGTCCAGGAGCT
23mer-81	CCAATAGTGGTCAGTCCAGGAGC
23mer-82	TCCAATAGTGGTCAGTCCAGGAG
23mer-83	CTCCAATAGTGGTCAGTCCAGGA
23mer-84	GCTCCAATAGTGGTCAGTCCAGG
23mer-87	cAGGCTCCAATAGTGGTCAGTCC
23mer-88	acAGGCTCCAATAGTGGTCAGTC
23mer-92	acttacAGGCTCCAATAGTGGTC
23mer-93	tacttacAGGCTCCAATAGTGGT
23mer-94	atacttacAGGCTCCAATAGTGG
23mer-95	tatacttacAGGCTCCAATAGTG
23mer-96	gtatacttacAGGCTCCAATAGT
23mer-97	agtatacttacAGGCTCCAATAG
23mer-98	cagtatacttacAGGCTCCAATA
23mer-99	ccagtatacttacAGGCTCCAAT
23mer-100	gccagtatacttacAGGCTCCAA
23mer-101	atccagtatacttacAGGCTCCA
23mer-102	gatccagtatacttacAGGCTCC
23mer-103	ggatccagtatacttacAGGCTC
23mer-104	gggatccagtatacttacAGGCT

***Table 14D**: 24-mer 5μM PMO tested*

Oligonucleotide Name	**Nucleic Acid Sequence (Reverse complement 5' – 3')**
24mer-n6	CAGATCTTCTAACTTCCTctttaa
24mer-n7	AGATCTTCTAACTTCCTctttaac
24mer-n8	GATCTTCTAACTTCCTctttaaca
24mer-n9	ATCTTCTAACTTCCTctttaacag
24mer-n4	CTCAGATCTTCTAACTTCCTcttt
24mer-n3	GCTCAGATCTTCTAACTTCCTctt
24mer-n2	AGCTCAGATCTTCTAACTTCCTct

24mer-n1	GAGCTCAGATCTTCTAACTTCCTc
24mer-n0	AGAGCTCAGATCTTCTAACTTCCT
24mer-01	CAGAGCTCAGATCTTCTAACTTCC
24mer-02	TCAGAGCTCAGATCTTCTAACTTC
24mer-03	CTCAGAGCTCAGATCTTCTAACTT
24mer-04	ACTCAGAGCTCAGATCTTCTAACT
24mer-05	CACTCAGAGCTCAGATCTTCTAAC
24mer-06	CCACTCAGAGCTCAGATCTTCTAA
24mer-07	TCCACTCAGAGCTCAGATCTTCTA
24mer-08	TTCCACTCAGAGCTCAGATCTTCT
24mer-09	CTTCCACTCAGAGCTCAGATCTTC
24mer-11	GCCTTCCACTCAGAGCTCAGATCT
24mer-12	CGCCTTCCACTCAGAGCTCAGATC
24mer-13	CCGCCTTCCACTCAGAGCTCAGAT
24mer-14	ACCGCCTTCCACTCAGAGCTCAGA
24mer-15	TACCGCCTTCCACTCAGAGCTCAG
24mer-16	TTACCGCCTTCCACTCAGAGCTCA
24mer-17	TTTACCGCCTTCCACTCAGAGCTC
24mer-18	GTTTACCGCCTTCCACTCAGAGCT
24mer-19	GGTTTACCGCCTTCCACTCAGAGC
24mer-20	CGGTTTACCGCCTTCCACTCAGAG
24mer-21	ACGGTTTACCGCCTTCCACTCAGA
24mer-22	AACGGTTTACCGCCTTCCACTCAG
24mer-56	AGGTCAGGCTGCTTTGCCCTCAGC
24mer-57	TAGGTCAGGCTGCTTTGCCCTCAG
24mer-58	CTAGGTCAGGCTGCTTTGCCCTCA
24mer-59	GCTAGGTCAGGCTGCTTTGCCCTC
24mer-60	AGCTAGGTCAGGCTGCTTTGCCCT
24mer-61	GAGCTAGGTCAGGCTGCTTTGCCC
24mer-62	GGAGCTAGGTCAGGCTGCTTTGCC
24mer-63	AGGAGCTAGGTCAGGCTGCTTTGC
24mer-64	CAGGAGCTAGGTCAGGCTGCTTTG
24mer-65	CCAGGAGCTAGGTCAGGCTGCTTT
24mer-66	TCCAGGAGCTAGGTCAGGCTGCTT
24mer-67	GTCCAGGAGCTAGGTCAGGCTGCT
24mer-68	AGTCCAGGAGCTAGGTCAGGCTGC
24mer-69	CAGTCCAGGAGCTAGGTCAGGCTG
24mer-71	GTCAGTCCAGGAGCTAGGTCAGGC
24mer-72	GGTCAGTCCAGGAGCTAGGTCAGG
24mer-73	TGGTCAGTCCAGGAGCTAGGTCAG
24mer-74	GTGGTCAGTCCAGGAGCTAGGTCA
24mer-75	AGTGGTCAGTCCAGGAGCTAGGTC
24mer-76	TAGTGGTCAGTCCAGGAGCTAGGT
24mer-77	ATAGTGGTCAGTCCAGGAGCTAGG
24mer-78	AATAGTGGTCAGTCCAGGAGCTAG

24mer-79	CAATAGTGGTCAGTCCAGGAGCTA
24mer-80	CCAATAGTGGTCAGTCCAGGAGCT
24mer-81	TCCAATAGTGGTCAGTCCAGGAGC
24mer-82	CTCCAATAGTGGTCAGTCCAGGAG
24mer-86	cAGGCTCCAATAGTGGTCAGTCCA
24mer-87	acAGGCTCCAATAGTGGTCAGTCC
24mer-88	tacAGGCTCCAATAGTGGTCAGTC
24mer-89	ttacAGGCTCCAATAGTGGTCAGT
24mer-91	acttacAGGCTCCAATAGTGGTCA
24mer-92	tacttacAGGCTCCAATAGTGGTC
24mer-93	atacttacAGGCTCCAATAGTGGT
24mer-94	tatacttacAGGCTCCAATAGTGG
24mer-96	agtatacttacAGGCTCCAATAGT
24mer-97	cagtatacttacAGGCTCCAATAG
24mer-98	ccagtatacttacAGGCTCCAATA
24mer-99	tccagtatacttacAGGCTCCAAT
24mer-101	gatccagtatacttacAGGCTCCA

[0569] RNA from the cells was extracted using QIAcube following manufacturer protocols. The concentration of the total RNA extracted was determined using a NanoDrop 8000 (Thermo Fisher Scinetific). One-step RT-PCR was performed using extracted total RNA, and the dystrophin products were analyzed using a Labchip assay (5k Labchip Perkin Elmer). The polynucleotide level "A" of the band with exon 50 skipping and the polynucleotide level "B" of the band without exon 50 skipping were measured using ImageJ. Based on these measurement values of "A" and "B", the skipping efficiency was determined by the following equation: % of Exon 50 Skipping =A/(A+B)×100.

[0570] **Results: FIGS. 3A-B** show percent exon 50 skipping in CRL-2061™ cells treated with 5μM of 21mer PMO using the oligonucleotides listed in **Table 14A**. **FIGS. 4A-B** show percent exon 50 skipping in CRL-2061™ cells treated with 5μM of 22mer PMO using the oligonucleotides listed in **Table 14B**. **FIGS. 5A-B** show percent exon 50 skipping in CRL-2061™ cells treated with 5μM of 23mer PMO using the oligonucleotides listed in **Table 14C**. **FIGS. 6A-B** show percent exon 50 skipping in CRL-2061™ cells treated with 5μM of 24mer PMO using the oligonucleotides listed in **Table 14D**. No exon skipping was observed in the absence of treatment with the oligonucleotide.

[0571] **Results: Tables 15A-15D** below show the PMOs with the highest percent of exon 50 skipping.

Table 15A. *21-mer oligonucleotides that hybridize to exon 50 as shown in FIGS. 3A-B.*

Oligonucleotide Name	Nucleic Acid Sequence (Reverse complement 5' – 3')
21mer-09	CCACTCAGAGCTCAGATCTTC
21mer-10	TCCACTCAGAGCTCAGATCTT
21mer-16	CCGCCTTCCACTCAGAGCTCA
21mer-17	ACCGCCTTCCACTCAGAGCTC
21mer-59	AGGTCAGGCTGCTTTGCCCTC
21mer-60	TAGGTCAGGCTGCTTTGCCCT
21mer-66	AGGAGCTAGGTCAGGCTGCTT
21mer-67	CAGGAGCTAGGTCAGGCTGCT
21mer-77	GTGGTCAGTCCAGGAGCTAGG

Table 15B. *22-mer oligonucleotides that hybridize to exon 50 as shown in FIGS. 4A-B.*

Oligonucleotide Name	Nucleic Acid Sequence (Reverse complement 5' – 3')
22mer-10	TTCCACTCAGAGCTCAGATCTT
22mer-15	CCGCCTTCCACTCAGAGCTCAG
22mer-16	ACCGCCTTCCACTCAGAGCTCA
22mer-17	TACCGCCTTCCACTCAGAGCTC
22mer-59	TAGGTCAGGCTGCTTTGCCCTC
22mer-60	CTAGGTCAGGCTGCTTTGCCCT
22mer-61	GCTAGGTCAGGCTGCTTTGCCC
22mer-64	GGAGCTAGGTCAGGCTGCTTTG
22mer-65	AGGAGCTAGGTCAGGCTGCTTT
22mer-74	GGTCAGTCCAGGAGCTAGGTCA
22mer-75	TGGTCAGTCCAGGAGCTAGGTC
22mer-84	CTCCAATAGTGGTCAGTCCAGG
22mer-85	GCTCCAATAGTGGTCAGTCCAG

Table 15C. *23-mer oligonucleotides that hybridize to exon 50 as shown in FIGS. 5A-B.*

Oligonucleotide Name	Nucleic Acid Sequence (Reverse complement 5' – 3')
23mer-n5	CAGATCTTCTAACTTCCTCTTTA
23mer-9	TTCCACTCAGAGCTCAGATCTTC
23mer-10	CTTCCACTCAGAGCTCAGATCTT
23mer-15	ACCGCCTTCCACTCAGAGCTCAG
23mer-16	TACCGCCTTCCACTCAGAGCTCA
23mer-22	ACGGTTTACCGCCTTCCACTCAG
23mer-75	GTGGTCAGTCCAGGAGCTAGGTC
23mer-76	AGTGGTCAGTCCAGGAGCTAGGT
23mer-88	acAGGCTCCAATAGTGGTCAGTC
23mer-104	gggatccagtatacttacAGGCT

Table 15D. *24-mer oligonucleotides that hybridize to exon 50 as shown in FIGS. 6A-B.*

Oligonucleotide Name	Nucleic Acid Sequence (Reverse complement 5' – 3')
24mer-14	ACCGCCTTCCACTCAGAGCTCAGA
24mer-15	TACCGCCTTCCACTCAGAGCTCAG
24mer-16	TTACCGCCTTCCACTCAGAGCTCA
24mer-56	AGGTCAGGCTGCTTTGCCCTCAGC
24mer-57	TAGGTCAGGCTGCTTTGCCCTCAG
24mer-74	GTGGTCAGTCCAGGAGCTAGGTCA
24mer-75	AGTGGTCAGTCCAGGAGCTAGGTC
24mer-76	TAGTGGTCAGTCCAGGAGCTAGGT
24mer-88	tacAGGCTCCAATAGTGGTCAGTC
24mer-101	gatccagtatacttacAGGCTCCA

Example 3. ***In vitro*** **screening of PMO sequences at 1 μM or 3 μM.**

[0572] CRL-2061™ cells were treated with 1 μM, or 3 μM of the PMOs with the highest percent exon 50 skipping at 5 μM (shown in **Tables 15A-15D**) following the same protocol described above. The PMO sequences identified as having the highest percent exon 50 skipping are shown in **Table 16.**

[0573] **Results: FIGS. 7A-7D** show the percent exon 50 skipping in CRL-2061™ cells treated with 1 μM and 3 μM of PMO using the oligonucleotides listed in **Tables 15A-15D**. **FIG. 7A** shows percent exon 50 skipping in CRL-2061™ cells treated with 1 μM and 3 μM of 21mer PMO using the oligonucleotides listed in **Table 15A**. **FIG. 7B** shows percent exon 50 skipping in CRL-2061™ cells treated with 1 μM and 3 μM of 22mer PMO using the oligonucleotides listed in **Table 15B**. **FIG. 7C** shows percent exon 50 skipping in CRL-2061™ cells treated with 1 μM and 3 μM of 23mer PMO using the oligonucleotides listed in **Table 15C**. **FIG. 7D** shows percent exon 50 skipping in CRL-2061™ cells treated with 1 μM and 3 μM of 24mer PMO using the oligonucleotides listed in **Table 15D**. A maximum of approximately 70% exon skipping is observed for each of the 22mer, 22mer, 23mer, and 24mer PMO.

Table 16. *PMOs That Hybridize to Exon 50.*

Oligonucleotide Name	Reverse Complement Nucleic Acid Sequence (5' – 3')
24-mer-75	AGTGGTCAGTCCAGGAGCTAGGTC
21-mer-77	GTGGTCAGTCCAGGAGCTAGG
22-mer-74	GGTCAGTCCAGGAGCTAGGTCA

24-mer-76	TAGTGGTCAGTCCAGGAGCTAGGT
22-mer-85	GCTCCAATAGTGGTCAGTCCAG
24-mer-14	ACCGCCTTCCACTCAGAGCTCAGA
24-mer-16	TTACCGCCTTCCACTCAGAGCTCA
22-mer-64	GGAGCTAGGTCAGGCTGCTTTG
23-mer-75	GTGGTCAGTCCAGGAGCTAGGTC
22-mer-65	AGGAGCTAGGTCAGGCTGCTTT

Example 4. Exon 50 Skipping Analysis of EEV-PMOs using hDMD mice.

[0574] **Purpose:** This study employs hDMD mice to study the percent exon 50 skipping that occurs in gastrocnemius, diaphragm, and heart tissue. (hDMD mice express full-length human dystrophin mRNA.) The delivery of oligonucleotide can alter the splicing and result in a shortened dystrophin mRNA after exon 50 skipping. Each of the PMOs shown in **Table 16** above were individually conjuted to the EEV having the sequence: Ac-PKKKRKV-PEG_2-K(cyclo[FGFGRGRQ])-PEG_{12}-OH and administered intravenously (IV) to hDMD mice at doses of 15 mpk or 30 mpk; (mpk: mg of compound / kg of body weight). Each EEV-PMO conjugate is listed in **Table 17** (e.g., EEV-PMO21mer-77, EEV-PMO22mer-74, EEV-PMO22mer-85, etc.).

[0575] **Methods:** As summarized in **Table 17**, in a first group of hDMD mice, the EEV-PMOs were administered intravenously (IV) to ten groups of five male hDMD mice at a dose of 15 mpk or 30 mpk. The mice were euthanized at one-week post-injection.

***Table 17**: EEV-PMO treatment of hDMD mice, harvested at one-week post injection.*

Groups	Animal Number (n)	Treatment	Dose	Harvest
1	5	Saline	NA	7 days Post-dosing. Tibialis anterior, gastrocnemius, triceps, diaphragm and heart
2	5	EEV-PMO21mer-77		
3	5	EEV-PMO22mer-74		
4	5	EEV-PMO22mer-85		

5	5	EEV-PMO22mer-64	15 mg/kg Once IV	
6	5	EEV-PMO23mer-75		
7	5	EEV-PMO22mer-65		
8	5	EEV-PMO24mer-14		
9	5	EEV-PMO24mer-16		
10	5	EEV-PMO24mer-75		
11	5	EEV-PMO24mer-76		
12	5	EEV-PMO21mer-77		
13	5	EEV-PMO22mer-74		
14	5	EEV-PMO22mer-85		
15	5	EEV-PMO22mer-64		
16	5	EEV-PMO23mer-75		
17	5	EEV-PMO22mer-65		
18	5	EEV-PMO24mer-14		

19	5	EEV-PMO24mer-16		
20	5	EEV-PMO24mer-75		
21	5	EEV-PMO24mer-76		

[0576] As summarized in **Table 18**, in a second group of mice, the EEV-PMOs were administered intravenously (IV) to ten groups of five male hDMD mice at a dose of 30 mpk. The mice were euthanized at one-week post-injection.

Table 18: EEV-PMO treatment of hDMD mice, harvested at one-week post injection.

Groups	Animal Number (n)	Treatment	Dose	Harvest
1	5	Saline	NA/Once IV	7 days Post-dosing. Tibialis anterior, gastrocnemius, triceps, diaphragm and heart
2	5	EEV-PMO21mer-77	30 mg/kg Once IV	
3	5	EEV-PMO22mer-74		
4	5	EEV-PMO22mer-85		
5	5	EEV-PMO22mer-64		
6	5	EEV-PMO23mer-75		
7	5	EEV-PMO22mer-65		
8	5	EEV-PMO24mer-14		

9	5	EEV-PMO24mer-16		
10	5	EEV-PMO24mer-75		
11	5	EEV-PMO24mer-76		

[0577] Gastrocnemius, diaphragm, and heart tissues were homogenized using 1mL of Qiazol Lysis Reagent (Cat. No. / ID: 79306) and total RNA was extracted using a Qiacube Connect RNAse Extraction Kit (Cat. No. / ID: 74106) with an additional DNAse digestion step (Cat. No. / ID: 79254). All samples underwent a One-Step Reverse Transcription droplet digital Polymerase Chain Reaction (RT-ddPCR) to determine the copies of wild-type and skipped transcript thereby determining the efficiency of the splicing correction (Bio-Rad Cat. No. #1864022). During the One Step RT-ddPCR process, the samples were first reverse-transcribed into cDNA using the following primers: forward primer 5′- GGGCAGCATTTGTACAAGGAAA -3′ and reverse primer 5′- CACAGGTTGTGTCACCAGAGTA-3′. This resulted in two transcripts: a wild-type transcript containing exon 50 which is targeted by HEX probe: 5' -/5HEX/CCTGACCTA /ZEN/GCTCCTGGACTGAC /3IABkFQ/-3', and a skip transcript containing a junction between exons 49 and 51, which is targeted by FAM probe 5' -/56-FAM/CAGCCAGTG/ZEN/ AAGCTCCTACTCAGA /3IABkFQ/ -3'. After undergoing PCR, the samples were visualized on Bio-Rad's QX200 and subsequently analyzed for exon-50 skipping efficacy; exon skipping was calculated using the following equation:

$$\%\ exon\ skipping = \frac{\frac{copies}{uL}\ of\ skipped\ transcript\ (FAM)}{\frac{copies}{uL}\ of\ skipped\ transcript\ (FAM) + \frac{copies}{uL}\ of\ wildtype\ transcript\ (HEX)} * 100$$

[0578] **Results: FIGS. 8A** and **9A** show exon skipping in gastrocnemius tissue of hDMD mice administered 15 mpk or 30 mpk, respectively, EEV-PMO with oligonucleotides shown in **Table 16** above via IV injection. **FIGS. 8B** and **9B** show exon skipping in diaphragm tissue of hDMD mice administered 15 mpk or 30 mpk, respectively. **FIGS. 8C** and **9C** show exon skipping in heart tissue of hDMD mice administered 15 mpk or 30 mpk, respectively. Overall, the trend of exon skipping efficacy of EEV-PMOs are consistent between two rounds of studies in all 3

tissues. Strong dose-dependent exon skipping was observed for EEV-PMO21mer-74, EEV-PMO22mer-85, EEV-PMO24mer-14, and EEV-PMO24mer-16.

Example 5. *In silico* off-target analysis.

[0579] **Purpose:** This study utilizes the bioinformatics program EMBOSS-FUZZNUC ("FUZZNUC") to search primary and mature human RNAs sequences to identify any hybridization-dependent mismatches (mm) with the oligonucleotides shown in **Table 16**. FUZZNUC is publicly accessible at emboss.sourceforge.net/apps/release/6.3/emboss/apps/fuzznuc.html.

[0580] **Methods:** In FUZZNUC, the oligonucleotides shown in **Table 16** and their reverse complement were used to search hybridization-dependent mismatches in human RNAs including protein coding genes, noncoding RNAs such as tRNAs, rRNAs, microRNAs, Lincs, and mitochondrial RNAs. Up to 5 mm were analyzed to identify primary and mature RNA hits using FUZZNUC against GRCh38.83 human genome and RefSeq gene annotation. Off-target hits were scored using the number of mismatches identified.

[0581] **Results: FIG. 10** is a table showing the number of mismatches (mm) in primary and mature RNA for each of the oligonucleotides shown in **Table 16**. The top hit for each oligonucleotide has 0 mm in primary and mature RNA. No oligonucleotides have non-DMD 1 or 2 mm hits in primary or mature RNA. The same three oligonucleotides (21mer-77, 22mer-74, and 22mer-85) have non-DMD 3 mm hits in mature RNA and non-DMD 2 mm hits in primary RNAs.

Example 6. Exon 50 Skipping Analysis using a single dose treatment of EEV-PMO24-mer-14 in hDMD mice.

[0582] **Purpose:** This study used hDMD mice to analyze the percent exon 50 skipping that occurs in gastrocnemius, triceps, tibialis anterior, heart, and diaphragm tissue after a single dose of EEV-PMO24mer-14. (hDMD mice express full-length human dystrophin mRNA.) The delivery of oligonucleotide can alter the splicing and result in a shortened dystrophin mRNA after exon 50 skipping. The EEV-PMO24mer-14 was administered intravenously (IV) to hDMD mice at doses of 15 mpk, 30 mpk, or 60mpk; (mpk: mg of compound / kg of body weight). A dose of 7.5 mpk was also tested, yet no significant difference compared to the saline control was observed (data not shown). The nucleic acid sequence of the 24-mer-14 oligonucleotide used is

ACCGCCTTCCACTCAGAGCTCAGA. The EEV used in the EEV-PMO24mer-14 is Ac-PKKKRKV-PEG_2-K(cyclo[FGFGRGRQ])-PEG_{12}-OH.

[0583] **Methods:** As summarized in **Table 19**, in a first group of mice, the EEV-PMO24mer-14 was administered intravenously (IV) to four groups of six male hDMD mice at doses of 15 mpk, 30 mpk, or 60 mpk. The mice were euthanized at one-week post-injection.

***Table 19**: EEV-PMO24mer-14 treatment of hDMD mice, harvested at one-week post injection.*

Group	Genotype	Sex/Age	N (number of mice)	Dose (mg/kg)	Harvest (weeks post-dose)	Tissues
1	hDMD	Male 5-10 weeks	6	vehicle	1 week	Gastroc, Tibialis Anterior, Triceps, Heart, Diaphragm
2			6	15		
3			6	30		
4			6	60		

[0584] As summarized in **Table 20**, In a second group of mice, the EEV-PMO24mer-14 was administered intravenously (IV) to four groups of six male hDMD mice at doses of 15 mpk, 30 mpk, or 60 mpk. The mice were euthanized at six-weeks post-injection.

***Table 20**: EEV-PMO24mer-14 treatment of hDMD mice, harvested at six-weeks post injection.*

Group	Genotype	Sex/Age	No. of mice	Dose (mg/kg)	Harvest (weeks post-dose)	Tissues
1	hDMD	Male 5-10 weeks	6	vehicle	6 weeks	Gastroc, Tibialis Anterior, Triceps, Heart, Diaphragm
2			6	15		
3			6	30		
4			6	60		

[0585] Once euthanized, gastrocnemius, triceps, tibialis anterior, heart, and diaphragm tissue were collected from each group. Tissues were transferred to ceramic bead tubes filled with 1 mL of QIAzol Lysis Reagent. The tissues were homogenized using the Omni Bead Ruptor Elite (Omni International) set to the preset "Heart" 30 second program. Tubes were allowed to cool for five minutes, and then 0.3 mL of chloroform was added to each tube. The tubes were shaken vigorously and then centrifuged at 12,000 rpm for 15 minutes to separate the phases. 350 µL of

the upper aqueous phase which contained RNA was carefully removed from each sample and purified using the QIAcube HT Automated Nucleic acid Purification System (QIAGEN). RNA samples were quantified using Nanodrop Eight (Thermo-Fisher).

[0586] All samples underwent a One-Step Reverse Transcription droplet digital Polymerase Chain Reaction (RT-ddPCR) to determine the copies of wild-type and skipped transcript thereby determining the efficiency of the splicing correction. During the One Step RT-ddPCR process, the samples were first reverse-transcribed into cDNA using the following primers: forward primer (targets part of Exon 49) 5′- GGGCAGCATTTGTACAAGGAAA -3′ and reverse primer 5′- CACAGGTTGTGTCACCAGAGTA -3′ (targets part of Exon 51). This resulted in two transcripts: a wild-type transcript containing exon 50 which is targeted by HEX probe: 5' -/5HEX/CCTGACCTA /ZEN/GCTCCTGGACTGAC /3IABkFQ/-3 , and a skip transcript containing a junction between exons 49 and 51, which is targeted by FAM probe 5' -/56-FAM/CAGCCAGTG/ZEN/ AAGCTCCTACTCAGA /3IABkFQ/ -3.

[0587] The droplets were generated using the Bio-Rad Automated Droplet Generator followed by PCR amplification on the Bio-Rad C1000 Touch™ Thermal Cycler. PCR-positive droplets were detected and quantified using the Bio-Rad QX600 AutoDG Droplet Digital PCR system and analyzed with the QX Manager Software to determine the concentration of the exon 50 skipped and un-skipped transcripts. The Exon 50 skipping was determined as a percentage of skipped product divided by the sum of skipped and un-skipped (full-length) products, according to the following equation:

$$\%\ exon\ skipping = \frac{\frac{copies}{uL}\ of\ skipped\ transcript\ (FAM)}{\frac{copies}{uL}\ of\ skipped\ transcript\ (FAM) + \frac{copies}{uL}\ of\ wildtype\ transcript\ (HEX)} * 100$$

[0588] The ddPCR program is provided in **Table 21**:

***Table 21**: ddPCR Analysis Program*

Cycling Step	Number of Cycles	Time	Temperature (°C)	Ramp Rate
Reverse Transcription	1x	60 minutes	43	2 °C/second

Enzyme Activation	1x	10 minutes	95	
Denaturation, Annealing, & Extension	50x	30 seconds	95	
		60 seconds	58	
		120 seconds	72	
Enzyme Deactivation	1	10 minutes	98	
Droplet Stabilization	1	30 minutes	4	
Hold (Optional)	1	∞	4	

[0589] *In vitro* transcribed (IVT) RNA samples containing a known ratio of exon 50 skipped and un-skipped transcripts of the dystrophin gene were included in each assay plate as quality control QC) standards.

[0590] **Results: FIGS. 11A-11E** show percent exon skipping in different tissues of hDMD mice administered 15 mpk, 30 mpk, or 60 mpk of EEV-PMO24mer-14 via IV injection with the indicated tissues harvested at one- or six-weeks post-injection. **FIG. 11A** shows percent exon 50 skipping in gastrocnemius tissue. **FIG. 11B** shows percent exon 50 skipping in tricep tissue. **FIG. 11C** shows percent exon 50 skipping in tibialis anterior tissue. **FIG. 11D** shows percent exon 50 skipping in heart tissue. **FIG. 11E** shows percent exon 50 skipping in diaphragm tissue.

[0591] A single dose of EEV-PMO24mer-14 at one-week post-injection exhibits significant exon 50 skipping in skeletal muscles as well as a dose response (**FIGS. 11A-11C**). At six-weeks post-injection, exon 50 skipping decreased by approximately 25% at 30 mpk and 60 mpk doses and decreased by approximately 8% at the 15 mpk doses (skipping 3-5x higher than saline control) **(FIGS. 11A-11C)**. A single dose of EEV-PMO24mer-14 at one-week post-injection exhibits significant exon 50 skipping at 30 mpk and 60 mpk in the diaphragm (**FIG. 11E**). At six-weeks post-injection, exon 50 skipping decreases by 9% and 38% in heart (**FIG. 11D**) and diaphragm (**FIG. 11E**) respectively at 60 mpk. Exon skipping decreased to 1.38% (3% drop) at 15 mpk and 3.67% (14% drop) at 30 mpk in diaphragm (**FIG. 11E**). Overall, the trend of exon 50 skipping efficacy shows a more rapid decrease in diaphragm (**FIG. 11E**) and heart (**FIG. 11D**) tissue than in skeletal muscle tissue.

Example 7. Exon 50 Skipping Analysis using a repeat dose treatment of EEV-PMO24-mer-14 in hDMD mice.

[0592] **Purpose:** This study used hDMD mice to analyze the percent exon 50 skipping that occurs in gastrocnemius, triceps, tibialis anterior, heart, and diaphragm tissue after repeated doses of EEV-PMO24mer-14. (hDMD mice express full-length human dystrophin mRNA.)

[0593] **Experimental Design and Treatment:** The study utilized a Tg (DMD)72Thoen/J transgenic mouse model carrying full-length human dystrophin (hDMD). An overview of the experimental mouse study design is provided in **Table 22**. Two cohorts of hDMD mice received three repeat IV bolus doses of EEV-PMO24mer-14 six weeks apart at each of 7.5 mg/kg, 15 mg/kg, 30 mg/kg, and 60 mg/kg. The nucleic acid sequence of the EEV-PMO24mer-14 oligonucleotide is: ACCGCCTTCCACTCAGAGCTCAGA. The EEV used in the EEV-PMO24mer-14 is Ac-PKKKRKV-PEG_2-K(cyclo[FGFGRGRQ])-PEG_{12}-OH.

[0594] Six mice from the six-week group died due to reasons unrelated to the study; four were from the 15 mpk group and two were from the 60 mpk (mg/kg) group.

Table 22. *Repeated Dose Study Design*

Group	Genotype	Sex/Age at injection	No. of mice	Three Doses (mg/kg) six weeks apart	Harvest (weeks post-dose)	Tissues
1	hDMD	Male 5-10 weeks	8	Vehicle	1 & 6 weeks	Gastroc, Tibialis anterior, Triceps, Diaphragm, Heart
2			8	7.5		
3			8	15		
4			8	30		
5			8	60		

[0595] **Exon 50 skipping analysis:** Mice were euthanized at one- or six-weeks post-injection, and gastrocnemius, triceps, tibialis anterior, heart, and diaphragm were collected. Tissues were transferred to ceramic bead tubes filled with 1 mL of QIAzol Lysis Reagent. The tissues were homogenized using the Omni Bead Ruptor Elite (Omni International) set to the preset "Heart" 30 second program. Tubes were allowed to cool for five minutes, and then 0.3 mL of chloroform was added to each tube. The tubes were shaken vigorously and then centrifuged at 12000 rpm for 15 minutes to separate the phases. 350 µL of the upper aqueous phase, which contained RNA, was carefully removed from each sample and purified using the QIAcube HT Automated

Nucleic acid Purification System (QIAGEN). RNA samples were quantified using Nanodrop Eight (Thermo-Fisher).

[0596] For droplet digital polymerase chain reaction (ddPCR), 25-75 ng RNA was used in combination with the Bio-Rad One Step RT-ddPCR Advanced kits for Probe. The droplets were generated using the Bio-Rad Automated Droplet Generator followed by PCR amplification on the Bio-Rad C1000 Touch™ Thermal Cycler. PCR-positive droplets were detected and quantified using the Bio-Rad QX600 AutoDG Droplet Digital PCR system and analyzed with the QX Manager Software to determine the concentration of the exon 50 skipped and un-skipped transcripts. Exon 50 skipping was determined as a percentage of skipped product divided by the sum of skipped and un-skipped (full-length) products. The primer and probe sequences are listed in **Table 23**. The ddPCR analysis program is the same as that in Example 6 and listed in **Table 21**.

Table 23. *Primer and Probe Sequences*

Identification	Target	Sequence
Forward Primer	Part of Exon 49	GGGCAGCATTTGTACAAGGAAA
Reverse Primer	Part of Exon 51	CACAGGTTGTGTCACCAGAGTA
HEX Probe	Exon 50 binding probe	CCTGACCTAGCTCCTGGACTGAC
FAM Probe	Exon 49/51 junction binding probe	CAGCCAGTGAAGCTCCTACTCAGA

Abbreviations: FAM= 6-carboxyfluorescein; HEX=Hexachlorofluorescein

[0597] *In vitro* transcribed (IVT) RNA samples containing a known ratio of Exon 50 skipped and un-skipped transcripts of the dystrophin gene were included in each assay plate as quality control (QC) standards. The ratio of exon 50 skipped and un-skipped transcripts from 0% skipped/100% un-skipped to 100% skipped/un-skipped was determined. The assay was considered valid if the coefficient variation (CV) of IVTs fell within predefined specifications.

[0598] **Results:** At one week post-injection, significant exon 50 skipping was observed in all tissues at 60 mg/kg, as well as in all tissues except the heart at 30 mg/kg and 15 mg/kg (**FIGS.**

12A-12E). Also at one week post-injection, significant exon 50 skipping was observed in tibialis anterior at 7.5 mg/kg (**FIG. 12C**). At six weeks post-injection, significant exon 50 skipping was observed in all tissues at 60 mg/kg, as well as in all tissues except heart at 30 mg/kg (**FIGS. 13A-13E**). Also at six weeks post-injection, significant exon 50 skipping was observed in tibialis anterior at 15 mg/kg (**FIG. 13C**). Statistics for **FIGS. 12A-12E** and **FIGS. 13A-13E**: One-way ANOVA with Dunnett's multiple comparison test to saline vehicle group, * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$, **** = $p \leq 0.0001$.

Example 8. Analysis of duration of effect of EEV-PMO24-mer-14 using a single dose in hDMD mice.

[0599] **Purpose:** This study used hDMD mice to analyze duration of effect of EEV-PMO24mer-14 in hDMD mice at from 1 to 12 weeks after a single injection.

[0600] **Experimental Design and Treatment:** The study utilized a Tg (DMD)72Thoen/J transgenic mouse model carrying full-length human dystrophin (hDMD). An overview of the experimental mouse study design is provided in **Table 24**. Two cohorts of mice were dosed at separate times. The animals euthanized at 6, 8, and 12 weeks post-injection were dosed first, and animals euthanized at 1, 2, and 4 weeks post-injection were recruited in the study at a later time point. Each of the two cohorts of hDMD mice received one single IV dose of EEV-PMO24mer-14 at each of 15 mg/kg, 30 mg/kg, and 60 mg/kg. The nucleic acid sequence of the EEV-PMO24mer-14 oligonucleotide is: ACCGCCTTCCACTCAGAGCTCAGA. The EEV used in the EEV-PMO24mer-14 is Ac-PKKKRKV-PEG_2-K(cyclo[FGFGRGRQ])-PEG_{12}-OH.

[0601] Mice were euthanized at 1, 2, 4, 6, 8, or 12 weeks post-injection, and gastrocnemius, triceps, tibialis anterior, heart, and diaphragm were collected. Tissues were transferred to ceramic bead tubes filled with 1 mL of QIAzol Lysis Reagent. The tissues were homogenized using the Omni Bead Ruptor Elite (Omni International) set to the preset "Heart" 30 second program. Tubes were allowed to cool for five minutes and 0.3 mL of chloroform was added to each tube. The tubes were shaken vigorously and then centrifuged at 12000 rpm for 15 minutes to separate the phases. 350 µL of the upper aqueous phase which contained RNA was carefully removed from each sample and purified using the QIAcube HT Automated Nucleic acid Purification System (QIAGEN). RNA samples were quantified using Nanodrop Eight (Thermo-Fisher).

[0602] For droplet digital polymerase chain reaction (ddPCR), 25-75 ng RNA was used in combination with the Bio-Rad One Step RT-ddPCR Advanced kits for Probe. The droplets were

generated using the Bio-Rad Automated Droplet Generator followed by PCR amplification on the Bio-Rad C1000 Touch™ Thermal Cycler. PCR-positive droplets were detected and quantified using the Bio-Rad QX600 AutoDG Droplet Digital PCR system and analyzed with the QX Manager Software to determine the concentration of the exon 50 skipped and un-skipped transcripts. Exon 50 skipping was determined as a percentage of skipped product divided by the sum of skipped and un-skipped (full-length) products. The primer and probe sequences are the same as those used in Example 7 and listed in **Table 23**. The ddPCR analysis program is the same as that in Example 6 and listed in **Table 21**.

Table 24. *Duration of Effect Study Design*

Group	Genotype	Sex/Age at injection	No. of mice	Dose (mg/kg)	Harvest (weeks post-dose)	Tissues
1	hDMD	Male 5-10 weeks	8	Vehicle	1, 2, 4, 6, 8, and 12 weeks	Gastroc, Tibialis anterior, Triceps, Diaphragm, Heart
2			8	15		
3			8	30		
4			8	60		

[0603] **Results:** Significant exon 50 skipping was observed at 60 mg/kg up to 12 weeks in all tissues except heart (**FIGS. 14A-14E**). Also observed up to 12 weeks was significant exon 50 skipping at 30 mg/kg in tibialis anterior (**FIG. 14B**) and up to 8 weeks in gastrocnemius (**FIG. 14C**) and triceps (**FIG. 14A**). Significant exon 50 skipping was observed at 30 mg/kg up to six weeks in diaphragm (**FIG. 14D**) and up to 2 weeks in heart (**FIG. 14E**). Significant exon 50 skipping was observed at 15 mg/kg up to 2 weeks in triceps, tibialis anterior, & gastrocnemius and up to 1 week in diaphragm. Statistics for **FIGS. 14A-14E**: One-way ANOVA with Dunnett's multiple comparison test to saline vehicle group, * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$, **** = $p \leq 0.0001$.

Example 9. Analysis of early timepoint engagement of EEV-PMO24-mer-14 using a single dose in hDMD mice.

[0604] **Purpose:** This study used hDMD mice to analyze the target engagement of EEV-PMO24mer-14 in hDMD mice at various early timepoints (e.g., from 4 to 168 hours) after a single injection.

[0605] **Experimental Design and Treatment:** The study utilized a Tg (DMD)72Thoen/J transgenic mouse model carrying full-length human dystrophin (hDMD). An overview of the experimental mouse study design is provided in **Table 25**. Two cohorts of hDMD mice received a single IV doses of EEV-PMO24mer-14 at each of 15 mg/kg, 30 mg/kg, and 60 mg/kg. The nucleic acid sequence of the EEV-PMO24mer-14 oligonucleotide is: ACCGCCTTCCACTCAGAGCTCAGA. The EEV used in the EEV-PMO24mer-14 is Ac-PKKKRKV-PEG_2-K(cyclo[FGFGRGRQ])-PEG_{12}-OH.

[0606] Mice were euthanized at 4, 8, 16, 24, 48, 96, or 168-hours post-injection, and gastrocnemius, triceps, tibialis anterior, heart, and diaphragm were collected. Tissues were transferred to ceramic bead tubes filled with 1 mL of QIAzol Lysis Reagent. The tissues were homogenized using the Omni Bead Ruptor Elite (Omni International) set to the preset "Heart" 30 second program. Tubes were allowed to cool for five minutes and 0.3 mL of chloroform was added to each tube. The tubes were shaken vigorously and then centrifuged at 12000 rpm for 15 minutes to separate the phases. 350 µL of the upper aqueous phase which contained RNA was carefully removed from each sample and purified using the QIAcube HT Automated Nucleic acid Purification System (QIAGEN). RNA samples were quantified using Nanodrop Eight (Thermo-Fisher).

[0607] For droplet digital polymerase chain reaction (ddPCR), 25-75 ng RNA was used in combination with the Bio-Rad One Step RT-ddPCR Advanced kits for Probe. The droplets were generated using the Bio-Rad Automated Droplet Generator followed by PCR amplification on the Bio-Rad C1000 Touch™ Thermal Cycler. PCR-positive droplets were detected and quantified using the Bio-Rad QX600 AutoDG Droplet Digital PCR system and analyzed with the QX Manager Software to determine the concentration of the exon 50 skipped and un-skipped transcripts. Exon 50 skipping was determined as a percentage of skipped product divided by the sum of skipped and un-skipped (full-length) products. The primer and probe sequences are the

same as those used in Example 7 and listed in **Table 23**. The ddPCR analysis program is the same as that in Example 6 and listed in **Table 21.**

Table 25. *Early Timepoint Study Design*

Group	Genotype	Sex/Age at injection	No. of mice	Three Doses (mg/kg) six weeks apart	Harvest (hours post-dose)	Tissues
1	hDMD	Male 5-10 weeks	5	Vehicle	4, 8, 16, 24, 48, 96, and 168 hours	Gastroc, Tibialis anterior, Triceps, Diaphragm, Heart
2			5	15		
3			5	30		
4			5	60		

[0608] *In vitro* transcribed (IVT) RNA samples containing a known ratio of Exon 50 skipped and un-skipped transcripts of the dystrophin gene were included in each assay plate as quality control (QC) standards. The ratio of exon 50 skipped and un-skipped transcripts from 0% skipped/100% un-skipped to 100% skipped/un-skipped was determined. The assay was considered valid if the coefficient variation (CV) of IVTs fell within predefined specifications.

[0609] **Results**: Human *DMD* Exon 50 skipping reaches its peak at 48 hours after a single injection of EEV-PMO24mer-14 in triceps (**FIG. 15A**), tibialis anterior (**FIG. 15B**), gastrocnemius (**FIG. 15C**), and diaphragm (**FIG. 15D**), while exon 50 skipping reaches its peak in heart (**FIG. 15E**) eight hours after a single injection. Statistics for **FIGS. 15A-15E**: One-way ANOVA with Dunnett's multiple comparison test to saline vehicle group, * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$, **** = $p \leq 0.0001$.

Example 10. *In vitro* screening of *DMD* exon 50 skipping and dystrophin protein expression in induced pluripotent stem cells (iPSCs) differentiated to cardiomyocytes

[0610] **Purpose**: The *in vitro* efficacy of EEV-PMO24mer-14 was evaluated in DMDΔ51 patient-induced pluripotent cell (iPSC) derived cardiomyocytes (CMs) originating from a DMD patient harboring an exon 51 deletion (DMDΔ51) in the dystrophin gene (*DMD*) amenable to exon 50 skipping. The observed *DMD* exon 50 skipping and dystrophin protein expression were significant and dose dependent in DMDΔ51 CMs treated with EEV-PMO24mer-14.

[0611] **Methods**: Fibroblasts from one normal donor and one DMD patient having an exon 51 sequence deletion (DMDΔ51) in the dystrophin gene were reprogrammed to iPSCs using the CytoTune iPS 2.0 Sendai Reprogramming Kit (ThermoFisher). The iPSCs were then differentiated to cardiomyocytes using the STEMdiff™ Ventricular Cardiomyocyte Differentiation Kit (STEMCELL Technologies).

[0612] Cardiomyocytes were thawed in STEMdiff Cardiomyocyte Support Medium, plated in thawing medium supplemented with 10 μM Y-27632 (rho kinase inhibitor) for 24 hours, and recovered in STEMdiff Cardiomyocyte Maintenance Medium for 4 days prior to use in an assay. Collagen-coated plates were coated with 100 μg/mL Matrigel (Corning), and cardiomyocytes were plated at 100% confluency in maintenance medium. DMDΔ51 cardiomyocytes were exposed to the test compound, EEV-PMO24mer-14, at 0.1 μM, 0.3 μM, 1 μM, 3 μM, and 10 μM for 24 hours and analyzed five days following test compound washout.

[0613] The nucleic acid sequence of the EEV-PMO24mer-14 test compound is ACCGCCTTCCACTCAGAGCTCAGA. The EEV used in the EEV-PMO24mer-14 test compound is Ac-PKKKRKV-PEG_2-K(cyclo[FGFGRGRQ])-PEG_{12}-OH. The test compound was washed out with test compuond-free maintenance medium after a 24-hour exposure and samples were analyzed 5 days later.

[0614] Samples were collected to isolate ribonucleic acid (RNA) and to assess *DMD* exon 50 skipping by droplet digital polymerase chain reaction (ddPCR). Dystrophin protein expression levels were determined by Simple Western Jess (SWJ), or by fixing cells with 100% ice-cold acetone to measure expression levels by immunofluorescence (IF). Four independent experiments were performed, each containing four biological replicates, for a total of 16 biological replicates run across 4 experiments. Means from each of the 4 independent experiments were pooled to generate the final set of data.

[0615] **Results**: DMDΔ51 cardiomyocytes (CMs) exposed to EEV-PMO24mer-14 showed significant and dose dependent DMD exon 50 skipping at all concentrations tested ($p < 0.0001$) (**FIG. 16**), ranging between 57.3% with 0.1 μM treatment, and 97.8% at 10 μM treatment. *DMD* exon 50 skipping was measured by ddPCR and determined as a percentage of the exon 50 skipped product concentration over the concentration sum of skipped and full-length products.

[0616] Dystrophin protein restoration was dose-dependent and significant with exposure to EEV-PMO24mer-14 at all concentrations tested when assessed by Simple Western Jess (**FIG.**

17) and measured as dystrophin signal intensity mean (**FIG. 18**) or percent dystrophin positive cardiomyocyte area by immunofluorescence (IF) (**FIG. 19**). Dystrophin protein restoration levels ranged between 6.2% and 12.1% of normal when assessed by SWJ. Dystrophin positive signal intensity means ranged between 19.0% and 31.0% of normal when measured by IF. Additionally, IF was used to determine the total cardiomyocyte area showing dystrophin protein expression (dystrophin positive cardiomyocyte area) as a measure of dystrophin protein distribution throughout the muscle cells. Significantly increased levels of cardiomyocyte area expressing dystrophin were observed, ranging between 50.3% and 71.3% with exposure to test-article.

[0617] Each data point in **FIGS. 16-19** represents the mean of 4 biological replicates performed in an independent experiment. For SWJ, dystrophin was normalized to total protein and presented as a percent relative to untreated normal cells (**FIG. 17**). CMs were fixed and probed for dystrophin and cardiac troponin, and counterstained with Hoechst 33342 to visualize nuclei. cardiomyocytes were defined by cardiac troponin to determine dystrophin protein expression. Baseline levels of dystrophin signal intensity measured in the untreated DMDΔ51 cardiomyocytes were subtracted from the normal and treated DMDΔ51 cardiomyocytes, and the untreated DMDΔ51 group was set to 0%. Data are presented as a percent relative to untreated normal cardiomyocytes which were set to 100%. Dystrophin-positive cardiomyocyte area was calculated as a percent of the sum of dystrophin positive area divided by the sum of cardiac troponin positive area. Statistical significance was determined by ordinary one-way ANOVA and Dunnett's multiple comparison test. Reported p-values are relative to the untreated DMDΔ51 group. All data are shown as mean ± SD (standard deviation); *$p<0.05$, ***$p< 0.001$; ****$p<0.0001$.

Example 11. *In vitro* screening of *DMD* exon 50 skipping and dystrophin protein expression in inducible directly reprogrammable myotubes (iDRMs)

[0618] **Purpose**: The *in vitro* efficacy of EEV-PMO24mer-14 was evaluated in induced directly reprogrammable myotubes (iDRMs) derived from DMD patient fibroblasts harboring a deletion of exons 51-55 (DMDΔ51-55) in the dystrophin gene (*DMD*) amenable to exon 50 skipping. The observed *DMD* exon 50 skipping and dystrophin protein restoration were significant and dose dependent in DMDΔ51-55 iDRMs treated with EEV-PMO24mer-14.

[0619] The nucleic acid sequence used in the EEV-PMO24mer-14 test compound is ACCGCCTTCCACTCAGAGCTCAGA. The EEV used in the EEV-PMO24mer-14 test compound is Ac-PKKKRKV-PEG_2-K(cyclo[FGFGRGRQ])-PEG_{12}-OH. The iDRM cells were generated by the Institute of Myology (Paris, France).

[0620] **Methods**: One normal iDRM cell line and one DMDΔ51-55 iDRM line were used to evaluate the test compound, EEV-PMO24mer-14, for target engagement and efficacy.The iDRMs express a tetracycline-on construct which induces overexpression of MyoD in the presence of doxocycline to trigger transdifferentiation of fibroblasts to myotubes. iDRMs were thawed, recovered, and passaged one time prior to use in an assay. Collagen-coated plates were coated with a thin layer of Matrigel (Corning), and iDRMs were plated at 100% confluency in growth medium. Transdifferentiation was triggered by addition of Dulbecco's modified Eagle medium (DMEM) supplemented with 10 μg/mL insulin, 2% horse serum, and 2 μg/mL doxycycline to cultures. iDRMs were transdifferentiated for 48 hours prior to test compound addition. The test compound EEV-PMO24mer-14 was serially diluted in differentiation medium and added to cells to reach final concentrations of 0 μM, 1 μM, 3 μM, 10 μM, and 30 μM. The test compound was washed out with test compound-free differentiation medium after a 24-hour exposure and samples were analyzed 4 days later, after seven days of differentiation.

[0621] Samples were collected to isolate ribonucleic acid (RNA) and to assess *DMD* exon 50 skipping by droplet digital polymerase chain reaction (ddPCR). Dystrophin protein expression levels were determined by Simple Western Jess (SWJ), or by fixing cells with 100% ice-cold acetone to measure expression levels by immunofluorescence (IF). Four independent experiments were performed, each containing four biological replicates, for a total of 16 biological replicates run across 4 experiments. Means from each of the 4 independent experiments were pooled to generate the final set of data.

[0622] DMDΔ51-55 iDRMs exposed to EEV-PMO24mer-14 showed significant and dose-dependent *DMD* exon 50 skipping at all concentrations tested ($p < 0.0001$) (**FIG. 20**) . Additionally, relative dystrophin transcript expression was significantly increased in DMDΔ51-55 iDRMS treated with EEV-PMO24mer-14 at 10 and 30 μM (**FIG. 21**). Without being bound by any particular theory, it is thought that stabilization of the dystrophin mRNA may result from correcting the reading frame with *DMD* exon 50 skipping. Each data point in **FIG. 19** and **FIG. 20** represents the mean of 4 biological replicates performed in an independent experiment. *DMD*

exon 50 skipping was measured by ddPCR and determined as a percentage of the exon 50 skipped product concentration over the concentration sum of skipped and full-length products. Relative DMD expression was measured by ddPCR, normalized to HPRT1, and reported as a percentage of the normal group which was set to 100%. Statistical significance was determined by ordinary one-way ANOVA and Dunnett's multiple comparison test. Reported p-values are relative to the untreated DMDΔ51-55 group. All data are shown as mean ± SD; **p< 0.01; ****p<0.0001.

[0623] Without treatment, full length dystrophin protein was present in normal cells, whereas DMDΔ51-55 iDRMs did not express full-length dystrophin as reported by by SWJ (data not shown) . However, the DP71 isoform of dystrophin was observed in both normal and untreated DMDΔ51-55 iDRMs. Without being bound by any particular theory, it is thought that the DP71 isoform may be originating from residual fibroblasts which failed to undergo reprogramming (data not shown), since mature skeletal muscle does not express DP71 (See Muntoni F, et al., Lancet Neurol. 2003 Dec;2(12):731-40).

[0624] Dystrophin protein restoration was dose-dependent and significant with EEV-PMO24mer-14 treatment at 10 and 30 μM when assessed by SWJ ($p < 0.0001$) (**FIG. 22**) and measured as dystrophin signal intensity mean (**FIG. 23**) or percent dystrophin positive myotube area by IF ($p < 0.01$) (**FIG. 24**) as reported by immunofluorescence. At baseline, the dystrophin positive myotube area was approximately 24.3% in the DMDΔ51-55 iDRMs. Without being bound by any particular theory, it is thought that this may be attributed to the anti-dystrophin antibody used recognizing DP71. Each data point represents the mean of 4 biological replicates performed in an independent experiment. For SWJ, dystrophin was normalized to total protein and presented as a percent relative to untreated normal cells (A). iDRMs were fixed and probed for dystrophin and α-actinin, and counterstained with Hoechst 33342 to visualize nuclei. Myotubes were defined by α-actinin to determine dystrophin protein expression. Baseline levels of dystrophin signal intensity measured in the untreated DMDΔ51-55 iDRMs were subtracted from the normal and treated DMDΔ51-55 iDRMs, and the untreated DMDΔ51-55 group was set to 0%. Data are presented as a percent relative to untreated normal iDRMs which were set to 100%. Dystrophin-positive myotube area was calculated as a percent of the sum of dystrophin positive area divided by the sum of α-actinin positive area. Statistical significance was determined by ordinary one-way ANOVA and Dunnett's multiple comparison test. Reported p-

values are relative to the untreated DMDΔ51-55 group. All data are shown as mean ± SD; **p< 0.01; ****p<0.0001.

Example 12. *In vitro* screening of *DMD* exon 50 skipping and dystrophin protein expression in AdMyoD-induced patient-derived DMDΔ51 skeletal myotubes

[0625] **Experimental design**: Patient-derived DMDΔ51 fibroblasts were thawed from liquid nitrogen cryogenic vial storage (approximately 500K cells/vial) in a 37 °C water bath and were recovered with 15 mL pre-warmed growth medium in a 50-mL conical tube. The growth medium was prepared by adding 75 mL of fetal bovine serume (FBS) to 425 mL MEM/Glutamax to make MEM/Glutamax/15% FBS growth medium. The cell suspension was then transferred to a T-75 flask and grown at 37 °C and 5% CO_2 with saturating humidity. For each experiment, the cells were expanded by tripling the media volume every passage until the total number of cells was approximately equal to 7 million.

[0626] Expanded cells were plated on Matrigel coated wells at 78,125 cells/cm^2 and grown overnight in growth media at 37°C and 5% CO_2 with saturating humidity. The plated cells were then transduced by adding recombinant MyoD adenovirus at a multiplicity of infection (MOI) of 200. After 24 hours incubation (37°C and 5% CO_2 with saturating humidity), the adenovirus was washed out with DPBS, an additional layer of Matrigel was added, and the transduced cells were incubated for 48 hours in differentiation medium. The differentiation medium was prepared by adding 10 mL of horse serum to 490 mL DMEM/Glutamax medium to make DMEM/Glutamax/2% horse serum differentiation medium.

[0627] The differentiated cells were then treated with EEV-PMO24mer-14 at 0 μM, 1 μM, 3 μM, 10 μM, and 20 μM in differentiation media for 24 hours before the compound was washed out with DPBS. The nucleic acid sequence of the EEV-PMO24mer-14 oligonucleotide is ACCGCCTTCCACTCAGAGCTCAGA. The EEV used in the EEV-PMO24mer-14 is Ac-PKKKRKV-PEG_2-K(cyclo[FGFGRGRQ])-PEG_{12}-OH. Differentiation media was added back to the wells and the treated cells were grown at 37°C and 5% CO_2 with saturating humidity for 4 additional days before further analysis.

[0628] **Exon 50 skiping analysis by ddPCR:** RNA was extracted from the 24-mer-14 treated cells using Illustra RNAspin 96 kit. Exon skipping was determined by running ddPCR with probes specific for Exon 50 (unskipped) and the Exon 49/52 junction (skipped). The percentage

of exon skipping was determined by the copies per µL of skipped dystrophin transcript divided by the sum of skipped and unskipped dystrophin transcripts multiplied by 100. The ddPCR primer and probe sequences are shown in **Table 26**. The ddPCR thermocycler times and temperatures are shown in **Table 27**.

[0629] **Protein Expression analysis by Simple Western Jess**: Dystrophin protein expression levels were determined by Simple Western Jess capillary electrophoresis and normalized to total protein. Protein was isolated from the 24-mer-14 cells using 1x radioimmunoprecipitation assay (RIPA) buffer supplemented with protease inhibitors. The total protein in each cell lysate was quantified by Bicinchoninic acid (BCA) assay and a total of 600 ng of isolated protein was analyzed from each replicate. Dystrophin was quantified by peak integration of Ab154168 (rabbit anti-dystrophin) visualized by an anti-rabbit-HRP secondary antibody.

Table 26. *Probe and primer sequences for ddPCR*

Name	**Sequence**
Forward Primer	**AGCAGTTCAAGCTAAACAACCG**
Reverse Primer	**TTTCAAATTTTGGGCAGCGGTA**
HEX Probe (unskipped)	**CCTGACCTAGCTCCTGGACTGAC**
FAM Probe (skipped)	**CTCAGCCAGTGAAGGCAACAATGCAGG**

Table 27. *Thermocycler times and temperatures for ddPCR*

Cycling Step	**Number of Cycles**	**Time**	**Temperature (°C)**	**Ramp Rate**
Reverse Transcription	1x	60 minutes	43	1C/Sec
Enzyme Activation	1x	10 minutes	95	
Denaturation, Annealing, & Extension	70x	30 seconds	95	
		150 seconds	61	
		120 seconds	72	
Enzyme Deactivation	1	10 minutes	98	
Droplet Stabilization	1	30 minutes	4	

Hold (Optional)	1	∞	4	

[0630] **Results:** 24-mer-14 treatment promoted exon 50 skipping in a dose-dependent manner (**FIG. 25**). Averages of exon 50 skipping of 98.3% and 99.4% were observed for 10μM and 20μM of 24-mer-14, respectively. Dystrophin protein expression was increased in a dose-dependent manner in DMDΔ51 skeletal myotubes treated with 24-mer-14 (**FIG. 26**). The highest dystrophin protein restoration was achieved at a dose of 20 μM of EEV-PMO24mer-14 test compound treated on Day 2 after myotube differentiation. The relative dystrophin protein expression shown in **FIG. 26** is summarized in **Table 28**.

Table 28. *Dystrophin Protein Expression by Simple Western Jess*

MyotubeType	**EEV-PMO24mer-14 Concentration (μM)**	**Dystrophin/Total Protein Mean ± SD**	**p-value**
DMDΔ51	0	0.00335 ± 0.00121	NA
	1	0.0179 ± 0.00289	NS
	3	0.0475 ± 0.00689	0.006
	10	0.109 ± 0.0186	<0.0001
	20	0.163 ± 0.0307	<0.0001

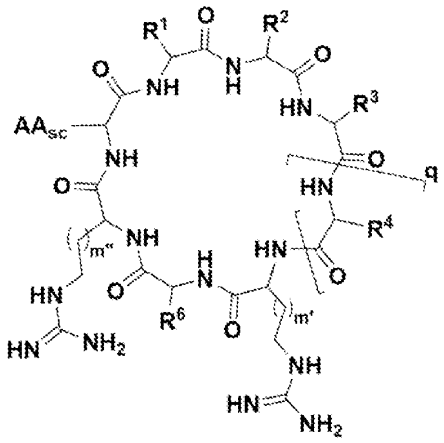
[0631] Each data point in **FIG. 25** and **FIG. 26** represents the mean of 4 biological replicates performed in an independent experiment.

CLAIMS

1. An EEV-cargo conjugate comprising:

(a) a cyclic cell penetrating peptide (cCPP) wherein the cCPP has a structure of Formula (2):

Formula (2):



(2), or a protonated form thereof,

wherein:

R^1, R^2, and R^3 can each independently be H or an amino acid residue having a side chain comprising an aryl or heteroaryl group;

at least two of R^1, R^2, and R^3 are, independently, an aryl or heteroaryl side chain of an amino acid;

R^4 and R^6 are independently H or an amino acid side chain;

AA_{SC} is an amino acid side chain;

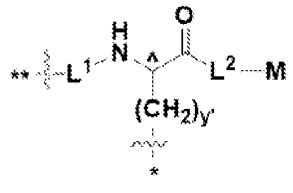
q is 1, 2, 3 or 4; and

m′ and m″ are each, independently, an integer from 0 to 3;

(b) a linear exocyclic peptide (EP) comprising from 2 to 10 amino acid residues;

(c) a linker of Formula (A′):

Formula (A'):



(A′)

wherein:

** is a point of attachment to the linear exocyclic peptide (EP);

* is a point of attachment to the cyclic cell penetrating peptide (cCPP);

L^1 and L^2, are, independently, a linker arm;

^ indicates L- or D-stereochemistry;

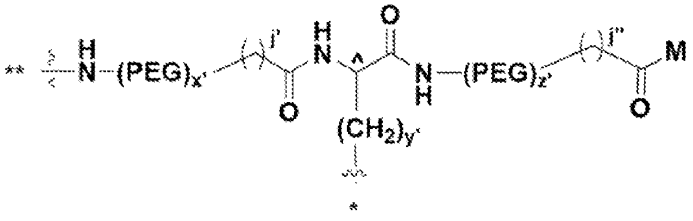
y′ is an integer from 1 to 5; and

M is a reactive handle comprising a functional group that reacts with a corresponding functional group on a cargo to form a bonding group (M′); and

(d) a cargo that comprises an oligonucleotide that binds to exon 50 in a pre-mRNA transcript of human dystrophin gene (*DMD*) to cause exon skipping, wherein the oligonucleotide binds to or comprises a nucleic acid sequence shown in **Tables 11A-11D, 12A-12D,** or **13,** comprises a reverse complement thereof, or a sequence with at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% nucleic acid sequence identity thereto.

2. The EEV-cargo conjugate of claim 1, wherein the linker comprises Formula (B′):

Formula (B'):



(B′),

wherein:

x′ is an integer from 0 to 12;

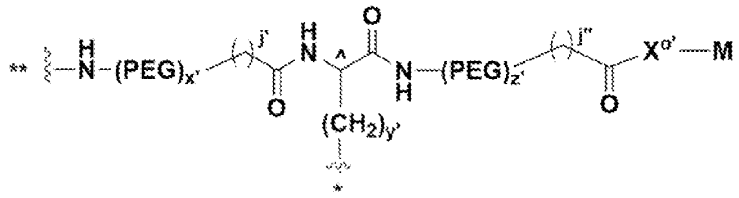
j′ is 0, 1, or 2, wherein j′ is 0 when x′ is 0;

z′ is an integer from 0 to 12; and

j″ is 0, 1, or 2, wherein j″ is 0 when z′ is 0.

3. The EEV-cargo conjugate of claim 1, wherein the linker comprises Formula (C′):

Formula (C′):



(C′),

x′ is an integer from 1 to 12;

j′ is 0, 1, or 2, wherein j′ is 0 when x′ is 0;

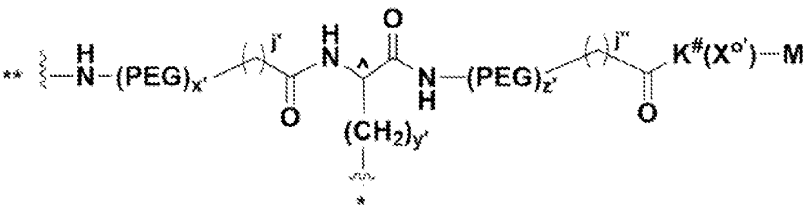
z′ is an integer from 0 to 12;

j″ is 0, 1, or 2, wherein j″ is 0 when z′ is 0; and

$X^{o\prime}$ is a hydrophobic component.

4. The EEV-cargo conjugate of claim 1, wherein the linker comprises Formula (D′):

Formula (D′):



(D′);

x′ is an integer from 1 to 12;

j′ is 0, 1, or 2, wherein j′ is 0 when x′ is 0;

z′ is an integer from 0 to 12;

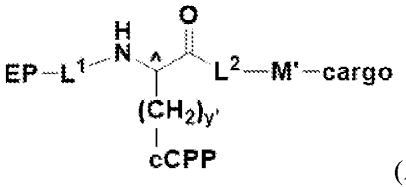
j″ is 0, 1, or 2, wherein j″ is 0 when z′ is 0;

$X^{o\prime}$ is a hydrophobic component; and

$K^{\#}$ is D-lysine or L-lysine residue.

5. The EEV-cargo conjugate of claim 1, comprising Formula (A-1):

Formula (A-1):



(A-1),

wherein

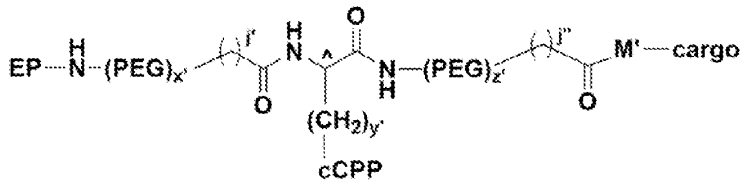
EP is a linear exocyclic peptide;

cCPP is a cyclic cell penetrating peptide; and

M′ is a bonding group.

6. The EEV-cargo conjugate of claim 5, comprising Formula (B-1):

Formula (B-1):



(B-1),

wherein

x′ is an integer from 0 to 12;

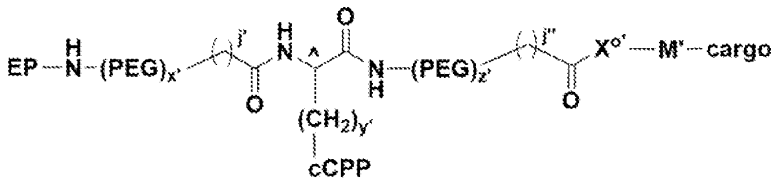
j′ is 0, 1, or 2, wherein j′ is 0 when x′ is 0;

z′ is an integer from 0 to 12; and

j″ is 0, 1, or 2, wherein j″ is 0 when z′ is 0.

7. The EEV-cargo conjugate of claim 5, comprising Formula (C-1):

Formula (C-1):



(C-1),

wherein

x′ is an integer from 0 to 12;

j′ is 0, 1, or 2, wherein j′ is 0 when x′ is 0;

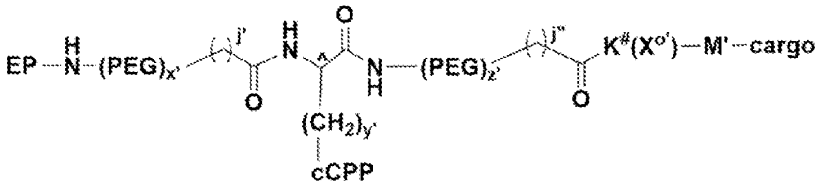
z′ is an integer from 0 to 12;

j″ is 0, 1, or 2, wherein j″ is 0 when z′ is 0; and

$X^{o\prime}$ is a hydrophobic group.

8. The EEV-cargo conjugate of claim 5, comprising Formula (D-1):

Formula (D-1):



(D-1),

wherein

x′ is an integer from 0 to 12;

j′ is 0, 1, or 2, wherein j′ is 0 when x′ is 0;

z′ is an integer from 0 to 12;

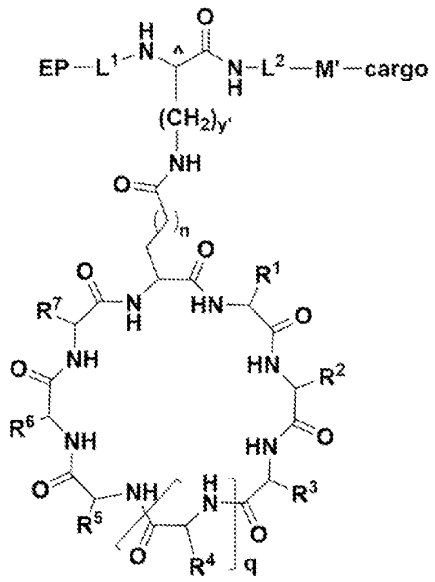
j″ is 0, 1, or 2, wherein j″ is 0 when z′ is 0;

$X^{o\prime}$ is a hydrophobic group; and

$K^{\#}$ is D-lysine or L-lysine residue.

9. The compound of claim 1, comprising Formula (A-3):

Formula (A-3):



(A-3)

wherein:

R^1, R^2, and R^3 are, independently, H or an aryl or heteroaryl side chain of an amino acid residue;

at least two of R^1, R^2, and R^3 are an aryl or heteroaryl side chain of an amino acid residue; and

R^4, R^5, R^6, R^7 are, independently, H or an amino acid residue side chain.

10. The EEV-cargo conjugate of claim 9, wherein

R^1, R^2, and R^3 are each independently a side chain comprising an aryl or heteroaryl group;

at least two of R^1, R^2, and R^3 are a side chain of phenylalanine;

at least one of R^1, R^2, and R^3 is a side chain of naphthylalanine;

R^4, R^5, R^6, and R^7 are, independently, H or an amino acid side chain; and

q is 1, 2, 3, or 4.

11. The EEV-cargo conjugate of claim 9, wherein

R^1, R^2, and R^3 are $-CH_2Ph$;

R^4, R^5, R^6, and R^7 are, independently, H or an amino acid side chain.

12. The EEV-cargo conjugate of claim 9, wherein
one of R^1, R^2, and R^3 is H;
two of R^1, R^2, and R^3 are CH_2Ph; and
R^4, R^5, R^6, and R^7 are, independently, H or an amino acid side chain.

13. The EEV-cargo conjugate of claim 9, wherein
one of R^1, R^2, and R^3 is H;
two of R^1, R^2, and R^3 are CH_2Ph; and
R^4, R^5, R^6, and R^7 are, independently, H or an amino acid side chain of Arginine.

14. The EEV-cargo conjugate of claim 9, wherein the cCPP has a sequence selected from: fNalRrRrQ, Ff-Nal-Cit-r-Cit-r-Q, and Ff-Nal-GrGrQ.

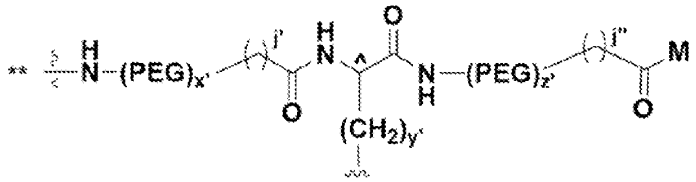
15. The EEV-cargo conjugate of claim 9, wherein the cCPP has a sequence of FfFGRGRQ.

16. The EEV-cargo conjugate of claim 9, wherein the cCPP has a sequence of FGFGRGRQ or GfFGrGrQ.

17. The EEV-cargo conjugate of claim 1, wheren AA_{SC} comprises glutamine (Q).

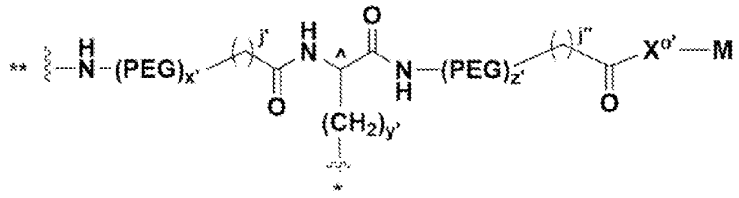
18. The EEV-cargo conjugate of claim 9, wherein the linker is selected from:

Formula (B'):



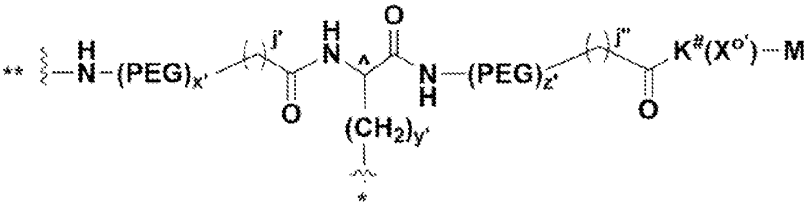
(B′);

Formula (C'):



(C′); and

Formula (D'):



(D′);

wherein:

x′ is an integer from 1 to 12;

j′ is 0, 1, or 2, wherein j′ is 0 when x′ is 0;

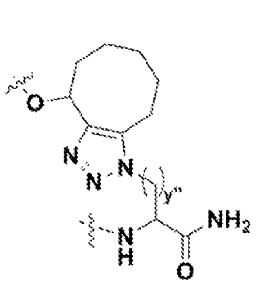
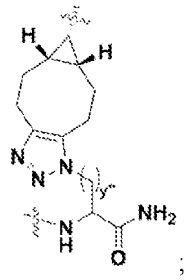
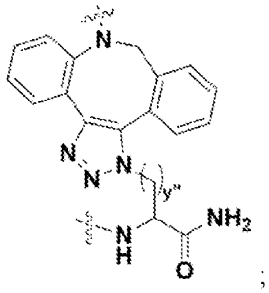
z′ is an integer from 0 to 12;

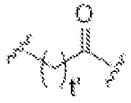
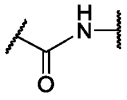
j″ is 0, 1, or 2, wherein j″ is 0 when z′ is 0;

$X^{o'}$ is a hydrophobic component; and

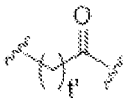
$K^{\#}$ is D-lysine or L-lysine residue.

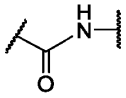
19. The EEV-cargo conjugate of any of the preceding claims, wherein M′ comprises:

-NH-, -C(O)-, -O-,  ;  ;  ;

 , or  , wherein y″ is an integer from 1 to 4 and t′ is an integer from 0 to 10.

20. The EEV-cargo conjugate of claim 19, wherein M comprises -C(O)-.

21. The EEV-cargo conjugate of claim 19, wherein M comprises  , or

 , wherein t′ is an integer from 0 to 10.

22. The EEV-cargo conjugate of any of claims 2-4, 6-8, and 19-21, wherein z′ is 0, 2, 4, 8, or 12.

23. The EEV-cargo conjugate of claim 22, wherein z′ is 2 or 12.

24. The EEV-cargo conjugate of claim 22, wherein z′ is 2.

25. The EEV-cargo conjugate of claim 22, wherein z′ is 12.

26. The EEV-cargo conjugate of any of claims 2-4, 6-8, 17, and 22-25, wherein x′ is 0, 2, 4, 8, or 12.

27. The EEV-cargo conjugate of claim 26, wherein x′ is 0 or 2.

28. The EEV-cargo conjugate of claim 26, wherein x′ is 0.

29. The EEV-cargo conjugate of claim 26, wherein x′ is 2.

30. The EEV-cargo conjugate of any of claims 3-4, 7-8, 19-21, and 22-25, wherein X^{o}′ is selected from: L-2-naphtylalanine (L-Nal), D-2-naphtylalanine (d-nal), 3-(4′,4-biphenyl)-L-alanine (L-Bip), or 3-(4′,4-biphenyl)-D-alanine (d-bip).

31. The EEV-cargo conjugate of claim 30, wherein X^{o}′ is L-2-naphtylalanine (L-Nal) or D-2-naphtylalanine (d-nal).

32. The EEV-cargo conjugate of claim 30, wherein X^{o}′ is 3-(4′,4-biphenyl)-L-alanine (L-Bip) or 3-(4′,4-biphenyl)-D-alanine (d-bip).

33. The EEV-cargo conjugate of claim 30, wherein X^{o}′ is L-2-naphtylalanine (L-Nal)

34. The EEV-cargo conjugate of claim 30, wherein X^{o}′ is D-2-naphtylalanine (d-nal).

35. The EEV-cargo conjugate of claim 30, wherein X^{o}′ is 3-(4′,4-biphenyl)-L-alanine (L-Bip).

36. The EEV-cargo conjugate of claim 30, wherein X^{o}′ is 3-(4′,4-biphenyl)-D-alanine (d-bip).

37. The EEV-cargo conjugate of any of the preceding claims, wherein the linker is covalently bound to the 5' end, the 3’ end, or the backbone, of the oligonucleotide cargo.

38. The EEV-cargo conjugate of any of the preceding claims, wherein the EP comprises from 2 to 10 amino acid residues, wherein at least 1 amino acid residue comprises a side chain comprising a guanidine group, a terminal amine, an imidazole, or a protonated form thereof.

39. The EEV-cargo conjugate of claim 38, wherein the EP comprises from 2 to 8 amino acid residues.

40. The EEV-cargo conjugate of claim 38, wherein the EP comprises from 2 to 6 amino acid residues.

41. The EEV-cargo conjugate of any of claims 38-40, wherein the EP comprises 1, 2, 3, or 4 arginine residues.

42. The EEV-cargo conjugate of any of claims 38-40, wherein the EP comprises 1, 2, 3, or 4 lysine residues.

43. The EEV-cargo conjugate of any of claims 38-40, wherein the EP comprises 1 or 2 uncharged hydrophobic amino acid residues.

44. The EEV-cargo conjugate of claim 43, wherein the uncharged hydrophobic amino acid residue is selected from valine, proline, β-alanine, glycine or a combination thereof.

45. The EEV-cargo conjugate of any one of claims 1-40, wherein the EP comprises one of the following sequences: PKKKRKV; KR; RR; KKK; KGK; KBK; KBR; KRK; KRR; RKK; RRR; KKKK; KKRK; KRKK; KRRK; RKKR; RRRR; KGKK; KKGK; KKKKK; KKKRK; KBKBK; KKKRKV; PGKKRKV; PKGKRKV; PKKGRKV; PKKKGKV; PKKKRGV; or PKKKRKG.

46. The EEV-cargo conjugate of any one of claims 1-40, wherein the EP has the structure: Ac-PKKKRKV.

47. The EEV-cargo conjugate of claim 1, selected from:

Ac-PKKKRKV-K(cyclo[Ff-Nal-RrRrQ])-PEG_{12}-M′-cargo
Ac-PKKKRKV-K(cyclo[Ff-Nal-GrGrQ])-PEG_{12}-M′-cargo

48. The EEV-cargo conjugate of claim 1, selected from:

Ac-PKKKRKV-PEG_2-K(cyclo[Ff-Nal-Cit-r-Cit-rQ])-PEG_{12}-M′-cargo
Ac-PKKKRKV-PEG_2-K(cyclo[Ff-Nal-GrGrQ])-PEG_{12}-M′-cargo
Ac-PKKKRKV-PEG_2-K(cyclo[FGFGRGRQ])-PEG_{12}-M′-cargo
Ac-PKKKRKV-PEG_2-K(cyclo[GfFGrGrQ])-PEG_{12}-M′-cargo

49. The EEV-cargo conjugate of claim 1, selected from:

Ac-PKKKRKV-PEG_2-K(cyclo[FGFGRGRQ])-PEG_2-M′-cargo
Ac-PKKKRKV-PEG_2-K(cyclo[GfFGrGrQ])-PEG_2-M′-cargo
Ac-PKKKRKV-PEG_2-K(cyclo[FfFGRGRQ])-PEG_2-M′-cargo

50. The EEV-cargo conjugate of claim 1, comprising:

Ac-PKKKRKV-PEG_2-K(cyclo[βhF-F-Nal-SRSRQ])-PEG_{12}-M′-cargo

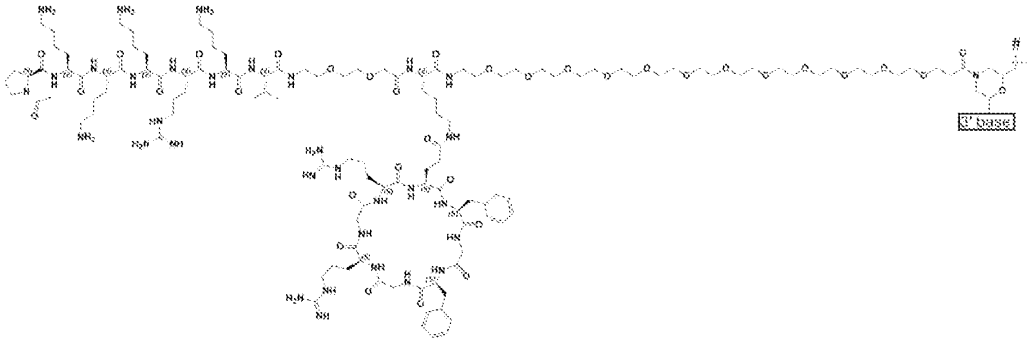
51. The EEV-cargo conjugate of claim 1, selected from:

Ac-PKKKRKV-PEG_2-K(cyclo[FGFGRGRQ])-PEG_{12}-K(Bip)-M′-cargo
Ac-PKKKRKV-PEG_2-K(cyclo[FGFGRGRQ])-PEG_{12}-K(Nal)-M′-cargo

52. The EEV-cargo conjugate of claim 1, selected from:

Ac-PKKKRKV-PEG_2-K(cyclo[FGFGRGRQ])-PEG_{12}-Nal-M′-cargo
Ac-PKKKRKV-PEG_2-K(cyclo[FGFGRGRQ])-PEG_{12}-Bip-M′-cargo

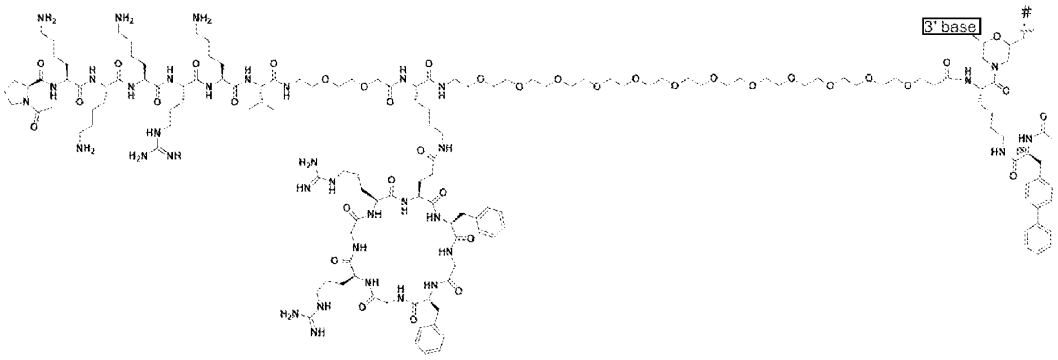
53. The EEV-cargo conjugate of claim 1, having the structure



,

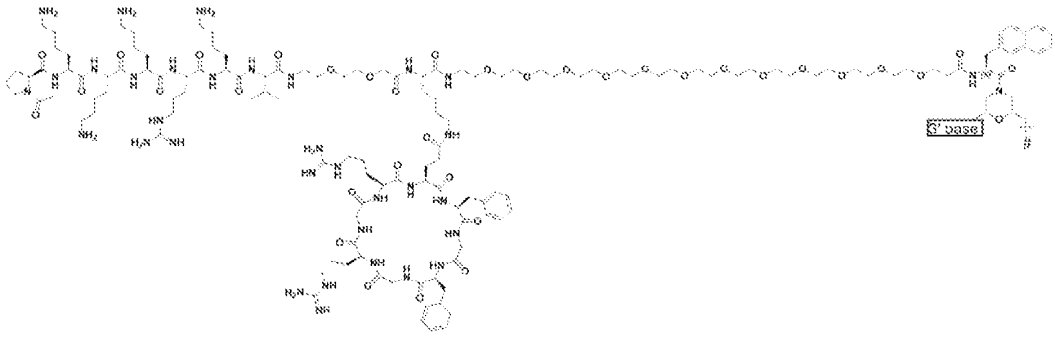
wherein # is a phosphorodiamidate morpholino oligonucleotide (PMO) and 3' base is a nucleobase at the 3' end of the PMO.

54. The EEV-cargo conjugate of claim 1, having the structure



, wherein # is a phosphorodiamidate morpholino oligonucleotide (PMO) and 3' base is a nucleobase at the 3' end of the PMO.

55. The EEV-cargo conjugate of claim 1, having the structure



,

wherein # is a phosphorodiamidate morpholino oligonucleotide (PMO) and 3' base is a nucleobase at the 3' end of the PMO.

56. The EEV-cargo conjugate of any of claims 1-55, wherein the cargo is a therapeutic oligonucleotide.

57. The EEV-cargo conjugate of any of claims 1-56, wherein the oligonucleotide is an antisense oligonucleotide.

58. The EEV-cargo conjugate of of any of claims 1-57, wherein the oligonucleotide comprises at least one modified nucleotide or nucleic acid selected from a phosphorothioate (PS) nucleotide, a phosphorodiamidate morpholino oligonucleotide (PMO), a locked nucleic acid (LNA), a peptide nucleic acid (PNA), a nucleotide comprising a 2'-O-methyl (2'-OMe) modified backbone, a 2'O-methoxy-ethyl (2'-MOE) nucleotide, a 2',4' constrained ethyl (cEt) nucleotide, and a 2'-deoxy-2'-fluoro-beta-D-arabinonucleic acid (2'F-ANA).

59. The EEV-cargo conjugate of of any of claims 1-58, wherein the oligonucleotide comprises at least one a phosphorodiamidate morpholino oligonucleotide (PMO).

60. The EEV-cargo conjugate of of any of claims 1-59, wherein the oligonucleotide comprises a phosphorodiamidate morpholino oligonucleotide (PMO).

61. The EEV-cargo conjugate of any of claims 1-60, wherein the oligonucleotide comprises from 15 to 30 nucleotides.

62. The EEV-cargo conjugate of any of claims 1-61, wherein the oligonucleotide comprises from 20 to 30 nucleotides.

63. The EEV-cargo conjugate of any of claims 1-62, wherein the oligonucleotide comprises from 20 to 25 nucleotides.

64. The EEV-cargo conjugate of any of claims 1-63, wherein the oligonucleotide binds to or comprises the nucleic acid sequence shown in **Tables 11A-11D, 12A-12D**, or **13**.

65. The EEV-cargo conjugate of any of claims 1-63, wherein the oligonucleotide binds to or comprises the nucleic acid sequence shown in **Tables 11A-11D**.

66. The EEV-cargo conjugate of any of claims 1-63, wherein the oligonucleotide binds to or comprises the nucleic acid sequence shown in **Tables 12A-12D**.

67. The EEV-cargo conjugate of any of claims 1-63, wherein the oligonucleotide binds to or comprises the nucleic acid sequence shown in **Table 13**.

68. The EEV-cargo conjugate of any of claims 1-63, wherein the oligonucleotide comprises a sequence comprising: 5'-XX ACC GCC XXC CAC XCA GAG CXC AGA-3', wherein X = U or T, the reverse complement thereof, or a sequence with at least 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% o4 100% sequence identity thereto.

69. The EEV-cargo conjugate of any of claims 1-63, wherein the oligonucleotide comprises a sequence comprising at least 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, or 26 contiguous nucleic acids of a base sequence comprising: 5'-XX ACC GCC XXC CAC XCA GAG CXC AGA-3', wherein X = U or T.

70. The EEV-cargo conjugate of any of claims 1-63, wherein the oligonucleotide comprises a sequence selected from:

5'-AGTGGTCAGTCCAGGAGCTAGGTC-3',
5'-GTGGTCAGTCCAGGAGCTAGG-3',
5'-GGTCAGTCCAGGAGCTAGGTCA-3',
5'-TAGTGGTCAGTCCAGGAGCTAGGT-3',
5'-GCTCCAATAGTGGTCAGTCCAG-3',
5'-ACCGCCTTCCACTCAGAGCTCAGA-3',
5'-TTACCGCCTTCCACTCAGAGCTCA,-3'
5'-GGAGCTAGGTCAGGCTGCTTTG-3',
5'-GTGGTCAGTCCAGGAGCTAGGTC-3', and
5'-AGGAGCTAGGTCAGGCTGCTTT-3'.

71. The EEV-cargo conjugate of any of claims 1-63, wherein the oligonucleotide comprises: 5'-AGTGGTCAGTCCAGGAGCTAGGTC-3'.

72. The EEV-cargo conjugate of any of claims 1-63, wherein the oligonucleotide comprises: 5'-GTGGTCAGTCCAGGAGCTAGG-3'.

73. The EEV-cargo conjugate of any of claims 1-63, wherein the oligonucleotide comprises: 5'-GGTCAGTCCAGGAGCTAGGTCA-3'.

74. The EEV-cargo conjugate of any of claims 1-63, wherein the oligonucleotide comprises: 5'-TAGTGGTCAGTCCAGGAGCTAGGT-3'.

75. The EEV-cargo conjugate of any of claims 1-63, wherein the oligonucleotide comprises: 5'-GCTCCAATAGTGGTCAGTCCAG-3'.

76. The EEV-cargo conjugate of any of claims 1-63, wherein the oligonucleotide comprises: 5'-ACCGCCTTCCACTCAGAGCTCAGA-3'.

77. The EEV-cargo conjugate of any of claims 1-63, wherein the oligonucleotide comprises: 5'-TTACCGCCTTCCACTCAGAGCTCA,-3'.

78. The EEV-cargo conjugate of any of claims 1-63, wherein the oligonucleotide comprises: 5'-GGAGCTAGGTCAGGCTGCTTTG-3'.

79. The EEV-cargo conjugate of any of claims 1-63, wherein the oligonucleotide comprises: 5'-GTGGTCAGTCCAGGAGCTAGGTC-3'.

80. The EEV-cargo conjugate of any of claims 1-63, wherein the oligonucleotide comprises: 5'-AGGAGCTAGGTCAGGCTGCTTT-3'.

81. A pharmaceutical composition comprising the EEV-cargo conjugate of any one of claims 1-80 and a pharmaceutically acceptable carrier.

82. A method of treating DMD comprising administering the pharmaceutical composition of claim 81 to a patient in need thereof.

83. The method of claim 82, wherein administering comprises parenteral administration.

84. The method of claim 83, wherein parenteral administration is selected from: subcutaneous, intradermal, intravenous, intramuscular, intraperitoneal, intrasternal, and intrathecal administration.