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(71) Applicant: **UNIVERSITY OF MASSACHUSETTS** [US/US]; One Beacon Street, 31st Floor, Boston, MA 02108 (US).

(72) Inventors: **KHVOROVA, Anastasia**; 10 Rocklawn Road, Westborough, MA 01581 (US). **ARONIN, Neil**; 19 Whittier Road, Newtonville, MA 02460 (US). **ALTERMAN, Julia**; 125 Hamilton Street, Apt. 3, Worcester, MA 01604 (US).

(74) Agents: **VELEMA, James, H.** et al.; Lathrop & Gage LLP, 28 State Street, Boston, MA 01209 (US).

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(54) Title: OLIGONUCLEOTIDE COMPOUNDS FOR TARGETING HUNTINGTIN MRNA

(57) Abstract: This disclosure relates to novel huntingtin targets. Novel oligonucleotides for the treatment of Huntington's disease are also provided.

OLIGONUCLEOTIDE COMPOUNDS FOR TARGETING HUNTINGTIN mRNA

Related Applications

[001] This application claims priority to U.S. Provisional Patent Application No. 62/289,274, filed January 31, 2016, and U.S. Provisional Patent Application No. 62/142,731, filed April 3, 2015. The entire contents of these applications are herein incorporated by reference.

Statement Regarding Federally Sponsored Research or Development

[002] This invention was made with government support under grant numbers NS038194 and TR000888 awarded by the National Institutes of Health. The Government has certain rights in the invention.

Field of the Invention

[003] This disclosure relates to novel huntingtin targets and novel oligonucleotides for the treatment of Huntington's disease.

Background

[004] Neurological disorders including Huntington's disease, Parkinson's disease and Alzheimer's disease represent a major unmet medical need. In some cases, these diseases are monogenic, making them ideal targets for oligonucleotide therapeutic intervention, e.g., RNA interference (RNAi). RNAi is a fundamental mechanism involving short double stranded RNA fragments that can be used to reprogram cellular machinery and silence and degrade targeted mRNA on demand. This technology is clinically advanced and has revolutionized the field of human functional genetics.

[005] Many different technologies have been explored for mRNA knockdown both as therapeutics and as tools for functional study, including viral based delivery of short hairpin RNAs (shRNAs), antisense oligonucleotides (ASOs), and naked or slightly modified siRNAs (Sah, D. W. Y. & Aronin, N. Oligonucleotide therapeutic approaches for Huntington disease. *J. Clin. Invest.* 121, 500–507 (2011); DiFiglia, M. *et al.* Therapeutic silencing of mutant huntingtin with siRNA attenuates striatal and cortical neuropathology and behavioral deficits. *Proceedings of the National Academy of Sciences of the United States of America* 104, 17204–17209 (2007)).

[006] ASOs have also shown to be a promising approach. This technology exhibits efficient delivery to cells without a delivery vehicle and has been administered to brain for the treatment of Huntington's disease for successful knockdown in both rodent and non-human primate brains (Mantha, N., Das, S. K. & Das, N. G. RNAi-based therapies for Huntington's disease: delivery challenges and opportunities. *Therapeutic delivery* 3, 1061–1076 (2012); Kordasiewicz, H. B. *et al.* Sustained Therapeutic Reversal of Huntington's Disease by Transient Repression of Huntingtin Synthesis. *NEURON* 74, 1031–1044 (2012)). Unfortunately, current studies show that a 700 µg cumulative dose administrated over two weeks is required to see just 50% silencing (Kordasiewicz, *Supra*).

[007] Unmodified siRNA ("naked siRNA") has been difficult to deliver to more sensitive cell lines and *in vivo* to tissue in the past. Although transfection reagents such as Lipofectamine can be used, there is a very narrow window within which it is efficacious and non-toxic, and it must be optimized independently for different batches of neurons to determine siRNA to lipid ratios necessary for comparable levels of silencing (Bell, H., Kimber, W. L., Li, M. & Whittle, I. R. Liposomal transfection efficiency and toxicity on glioma cell lines: in vitro and in vivo studies. *NeuroReport* 9, 793–798 (1998); Dass, C. R. Cytotoxicity issues pertinent to lipoplex-mediated gene therapy in-vivo. *Journal of Pharmacy and Pharmacology* 1–9 (2010); Masotti, A. *et al.* Comparison of different commercially available cationic liposome–DNA lipoplexes: Parameters influencing toxicity and transfection efficiency. *Colloids and Surfaces B: Biointerfaces* 68, 136–144 (2009); Zou, L. L. *et al.* Liposome-mediated NGF gene transfection following neuronal injury: potential therapeutic applications. *Gene Ther* 6, 994–1005 (1999)). Hydrophobically modified siRNAs have also been used as an alternative for cellular and brain delivery (Sah, Supra; Soutschek, J. *et al.* Therapeutic silencing of an endogenous gene by systemic administration of modified siRNAs. *Nature* 432, 173–178 (2004); Cheng, K., Ye, Z., Guntaka, R. V. & Mahato, R. I. Enhanced hepatic uptake and bioactivity of type alpha1(I) collagen gene promoter-specific triplex-forming oligonucleotides after conjugation with cholesterol. *Journal of Pharmacology and Experimental Therapeutics* 317, 797–805 (2006); Byrne, M. *et al.* Novel Hydrophobically Modified Asymmetric RNAi Compounds (sd-rxRNA) Demonstrate Robust Efficacy in the Eye. *Journal of Ocular Pharmacology and Therapeutics* 29, 855–864 (2013)), and some of these compounds have even made it to clinic, but ensuring both chemical stability and minimal toxicity while maximizing delivery remains a difficult task. Current hurdles in RNAi technology limit its ability to be used for both functional genomics studies

and therapeutics, providing an opportunity for improvement to their design as it applies to the area of neuroscience both *in vitro* and *in vivo*.

Summary

[008] Accordingly, provided herein are novel huntingtin target sequences. Also provided herein are novel RNA molecules (e.g., siRNAs) that target the novel huntingtin target sequences. Said novel RNA molecules (e.g., siRNAs) demonstrate efficacy and potency in both primary neurons *in vitro*, and *in vivo* in mouse brain subsequent to a single, low dose injection.

[009] In one aspect, an RNA molecule is provided that is between 15 and 30 bases in length or between 15 and 35 bases in length, comprising a region of complementarity which is substantially complementary to 5' CAGUAAAGAGAUUAA 3' (SEQ ID NO:1).

[010] In certain embodiments, the RNA molecule is single stranded (ss) RNA or double stranded (ds) RNA. In certain embodiments, the dsRNA comprises a sense strand and an antisense strand, wherein the antisense strand comprises the region of complementarity which is substantially complementary to 5' CAGUAAAGAGAUUAA 3' (SEQ ID NO:1).

[011] In certain embodiments, the dsRNA is between 30 and 35 base pairs in length. In certain embodiments the region of complementarity is complementary to at least 10, 11, 12 or 13 contiguous nucleotides of SEQ ID NO:1. In certain embodiments, the region of complementarity contains no more than 3 mismatches with SEQ ID NO:1. In certain embodiments, the region of complementarity is fully complementary to SEQ ID NO:1.

[012] In certain embodiments, the dsRNA is blunt-ended. In certain embodiments, the dsRNA comprises at least one single stranded nucleotide overhang. In certain embodiments, the dsRNA comprises naturally occurring nucleotides.

[013] In certain embodiments, the dsRNA comprises at least one modified nucleotide. In certain embodiments, the modified nucleotide is chosen from the group of: a 2'-O-methyl modified nucleotide, a nucleotide comprising a 5'phosphorothioate group, and a terminal nucleotide linked to a cholesteryl derivative or dodecanoic acid bisdecylamide group. In certain embodiments, the modified nucleotide is chosen from the group of: a 2'-deoxy-2'-fluoro modified nucleotide, a 2'-deoxy-modified nucleotide, a locked nucleotide, an abasic nucleotide, 2'-amino-modified nucleotide, 2'-alkyl-modified nucleotide, morpholino nucleotide, a phosphoramidate, and a non-natural base comprising nucleotide. In certain

embodiments, the dsRNA comprises at least one 2'-O-methyl modified nucleotide and at least one nucleotide comprising a 5'phosphorothioate group.

[014] In certain embodiments, the RNA molecule comprises a 5' end, a 3' end and has complementarity to a target, wherein: (1) the RNA molecule comprises alternating 2'-methoxy-ribonucleotides and 2'-fluoro-ribonucleotides; (2) the nucleotides at positions 2 and 14 from the 5' end are not 2'-methoxy-ribonucleotides; (3) the nucleotides are connected via phosphodiester or phosphorothioate linkages; and (4) the nucleotides at positions 1-6 from the 3' end, or positions 1-7 from the 3' end, are connected to adjacent nucleotides via phosphorothioate linkages.

[015] In certain embodiments, the dsRNA has a 5' end, a 3' end and complementarity to a target, and comprises a first oligonucleotide and a second oligonucleotide, wherein: (1) the first oligonucleotide comprises a sequence set forth as SEQ ID NO:1; (2) a portion of the first oligonucleotide is complementary to a portion of the second oligonucleotide; (3) the second oligonucleotide comprises alternating 2'-methoxy-ribonucleotides and 2'-fluoro-ribonucleotides; (4) the nucleotides at positions 2 and 14 from the 3' end of the second oligonucleotide are 2'-methoxy-ribonucleotides; and (5) the nucleotides of the second oligonucleotide are connected via phosphodiester or phosphorothioate linkages.

[016] In certain embodiments, the second oligonucleotide is linked to a hydrophobic molecule at the 3' end of the second oligonucleotide. In certain embodiments, the linkage between the second oligonucleotide and the hydrophobic molecule comprises polyethylene glycol or triethylene glycol. In certain embodiments, the nucleotides at positions 1 and 2 from the 3' end of second oligonucleotide are connected to adjacent nucleotides via phosphorothioate linkages. In certain embodiments, the nucleotides at positions 1 and 2 from the 3' end of second oligonucleotide, and the nucleotides at positions 1 and 2 from the 5' end of second oligonucleotide, are connected to adjacent ribonucleotides via phosphorothioate linkages.

[017] In certain aspects, a pharmaceutical composition for inhibiting the expression of the HTT gene in an organism, comprising a dsRNA and a pharmaceutically acceptable carrier is provided. The dsRNA comprises a sense strand and an antisense strand. The dsRNA is between 15 and 35 base pairs in length and the antisense strand comprises a region

of complementarity which is substantially complementary to 5' CAGUAAAGAGAUUAA 3' (SEQ ID NO:1).

[018] In certain embodiments, the dsRNA comprises a cholesterol moiety.

[019] In certain aspects, a method for inhibiting expression of *HTT* gene in a cell is provided. The method includes the steps of introducing into the cell a double-stranded ribonucleic acid (dsRNA) comprising a sense strand and an antisense strand, the dsRNA is between 15 and 35 base pairs in length and the antisense strand comprises a region of complementarity which is substantially complementary to 5' CAGUAAAGAGAUUAA 3' (SEQ ID NO:1), and maintaining the cell produced in step (a) for a time sufficient to obtain degradation of the mRNA transcript of the *HTT* gene, thereby inhibiting expression of the *HTT* gene in the cell.

[020] In certain aspects, a method of treating or managing Huntington's disease comprising administering to a patient in need of such treatment or management a therapeutically effective amount of a dsRNA is provided. The dsRNA comprises a sense strand and an antisense strand, and is between 15 and 35 base pairs in length, and the antisense strand comprises a region of complementarity which is substantially complementary to 5' CAGUAAAGAGAUUAA 3' (SEQ ID NO:1).

[021] In certain embodiments, the dsRNA is administered to the brain of the patient. In certain embodiments, the dsRNA is administered by any of intrastriatal, intracerebroventricular and/or intrathecal infusion and/or pump. In certain embodiments, administering the dsRNA to the brain causes a decrease in HTT gene mRNA in the striatum. In certain embodiments, administering the dsRNA to the brain causes a decrease in *HTT* gene mRNA in the cortex.

[022] In certain aspects, a vector for inhibiting the expression of *HTT* gene in a cell is provided. The vector comprising a regulatory sequence operably linked to a nucleotide sequence that encodes an RNA molecule substantially complementary to 5' CAGUAAAGAGAUUAA 3' (SEQ ID NO:1), wherein said RNA molecule is between 15 and 35 bases in length, and wherein said RNA molecule, upon contact with a cell expressing said *HTT* gene, inhibits the expression of said *HTT* gene by at least 20%.

[023] In certain embodiments, the RNA molecule is ssRNA or dsRNA. In certain embodiments, the dsRNA comprises a sense strand and an antisense strand, wherein the

antisense strand comprises the region of complementarity which is substantially complementary to 5' CAGUAAAGAGAUUAA 3' (SEQ ID NO:1).

[024] In certain aspects, a cell comprising a vector for inhibiting the expression of *HTT* gene in a cell is provided. The vector comprising a regulatory sequence operably linked to a nucleotide sequence that encodes an RNA molecule substantially complementary to 5' CAGUAAAGAGAUUAA 3' (SEQ ID NO:1), wherein said RNA molecule is between 15 and 35 bases in length, and wherein said RNA molecule, upon contact with a cell expressing said *HTT* gene, inhibits the expression of said *HTT* gene by at least 20%.

[025] In certain embodiments, the RNA molecule is ssRNA or dsRNA. In certain embodiments, the dsRNA comprises a sense strand and an antisense strand, wherein the antisense strand comprises the region of complementarity which is substantially complementary to 5' CAGUAAAGAGAUUAA 3' (SEQ ID NO:1).

[026] In one aspect, an RNA molecule is provided that is between 15 and 35 bases in length, comprising a region of complementarity which is substantially complementary to 5' AUAUCAGUAAAGAGA 3' (SEQ ID NO:2) or 5' CUCAGGAUUUAAAAU 3' (SEQ ID NO:3).

[027] In certain embodiments, the RNA molecule is single stranded (ss) RNA or double stranded (ds) RNA. In certain embodiments, the dsRNA comprises a sense strand and an antisense strand, wherein the antisense strand comprises the region of complementarity which is substantially complementary to 5' CAGUAAAGAGAUUAA 3' (SEQ ID NO:1).

[028] In certain embodiments, the dsRNA is between 30 and 35 base pairs in length. In certain embodiments the region of complementarity is complementary to at least 10, 11, 12 or 13 contiguous nucleotides of SEQ ID NO:2 or 3. In certain embodiments, the region of complementarity contains no more than 3 mismatches with SEQ ID NO:1. In certain embodiments, the region of complementarity is fully complementary to SEQ ID NO:2 or 3.

[029] In certain embodiments, the dsRNA is blunt-ended. In certain embodiments, the dsRNA comprises at least one single stranded nucleotide overhang. In certain embodiments, the dsRNA comprises naturally occurring nucleotides.

[030] In certain embodiments, the dsRNA comprises at least one modified nucleotide. In certain embodiments, the modified nucleotide is chosen from the group of: a 2'-O-methyl modified nucleotide, a nucleotide comprising a 5'phosphorothioate group, and a terminal nucleotide linked to a cholesteryl derivative or dodecanoic acid bisdecylamide

group. In certain embodiments, the modified nucleotide is chosen from the group of: a 2'-deoxy-2'-fluoro modified nucleotide, a 2'-deoxy-modified nucleotide, a locked nucleotide, an abasic nucleotide, 2'-amino-modified nucleotide, 2'-alkyl-modified nucleotide, morpholino nucleotide, a phosphoramidate, and a non-natural base comprising nucleotide. In certain embodiments, the dsRNA comprises at least one 2'-O-methyl modified nucleotide and at least one nucleotide comprising a 5'phosphorothioate group.

[031] In certain embodiments, the RNA molecule comprises a 5' end, a 3' end and has complementarity to a target, wherein: (1) the RNA molecule comprises alternating 2'-methoxy-ribonucleotides and 2'-fluoro-ribonucleotides; (2) the nucleotides at positions 2 and 14 from the 5' end are not 2'-methoxy-ribonucleotides; (3) the nucleotides are connected via phosphodiester or phosphorothioate linkages; and (4) the nucleotides at positions 1-6 from the 3' end, or positions 1-7 from the 3' end, are connected to adjacent nucleotides via phosphorothioate linkages.

[032] In certain embodiments, the dsRNA has a 5' end, a 3' end and complementarity to a target, and comprises a first oligonucleotide and a second oligonucleotide, wherein: (1) the first oligonucleotide comprises a sequence set forth as SEQ ID NO:1; (2) a portion of the first oligonucleotide is complementary to a portion of the second oligonucleotide; (3) the second oligonucleotide comprises alternating 2'-methoxy-ribonucleotides and 2'-fluoro-ribonucleotides; (4) the nucleotides at positions 2 and 14 from the 3' end of the second oligonucleotide are 2'-methoxy-ribonucleotides; and (5) the nucleotides of the second oligonucleotide are connected via phosphodiester or phosphorothioate linkages.

[033] In certain embodiments, the second oligonucleotide is linked to a hydrophobic molecule at the 3' end of the second oligonucleotide. In certain embodiments, the linkage between the second oligonucleotide and the hydrophobic molecule comprises polyethylene glycol or triethylene glycol. In certain embodiments, the nucleotides at positions 1 and 2 from the 3' end of second oligonucleotide are connected to adjacent nucleotides via phosphorothioate linkages. In certain embodiments, the nucleotides at positions 1 and 2 from the 3' end of second oligonucleotide, and the nucleotides at positions 1 and 2 from the 5' end of second oligonucleotide, are connected to adjacent ribonucleotides via phosphorothioate linkages.

[034] In certain aspects, a pharmaceutical composition for inhibiting the expression of the HTT gene in an organism, comprising a dsRNA and a pharmaceutically acceptable carrier is provided. The dsRNA comprises a sense strand and an antisense strand. The dsRNA is between 15 and 35 base pairs in length and the antisense strand comprises a region of complementarity which is substantially complementary to 5' AUAUCAGUAAAGAGA 3' (SEQ ID NO:2) or 5' CUCAGGAUUUAAAAU 3' (SEQ ID NO:3).

[035] In certain embodiments, the dsRNA comprises a cholesterol moiety.

[036] In certain aspects, a method for inhibiting expression of *HTT* gene in a cell is provided. The method includes the steps of introducing into the cell a double-stranded ribonucleic acid (dsRNA) comprising a sense strand and an antisense strand, the dsRNA is between 15 and 35 base pairs in length and the antisense strand comprises a region of complementarity which is substantially complementary to 5' AUAUCAGUAAAGAGA 3' (SEQ ID NO:2) or 5' CUCAGGAUUUAAAAU 3' (SEQ ID NO:3), and maintaining the cell produced in step (a) for a time sufficient to obtain degradation of the mRNA transcript of the *HTT* gene, thereby inhibiting expression of the *HTT* gene in the cell.

[037] In certain aspects, a method of treating or managing Huntington's disease comprising administering to a patient in need of such treatment or management a therapeutically effective amount of a dsRNA is provided. The dsRNA comprises a sense strand and an antisense strand, and is between 15 and 35 base pairs in length, and the antisense strand comprises a region of complementarity which is substantially complementary to 5' AUAUCAGUAAAGAGA 3' (SEQ ID NO:2) or 5' CUCAGGAUUUAAAAU 3' (SEQ ID NO:3).

[038] In certain embodiments, the dsRNA is administered to the brain of the patient. In certain embodiments, the dsRNA is administered by intrastriatal infusion. In certain embodiments, administering the dsRNA to the brain causes a decrease in *HTT* gene mRNA in the striatum. In certain embodiments, administering the dsRNA to the brain causes a decrease in *HTT* gene mRNA in the cortex.

[039] In certain aspects, a vector for inhibiting the expression of *HTT* gene in a cell is provided. The vector comprising a regulatory sequence operably linked to a nucleotide sequence that encodes an RNA molecule substantially complementary to 5' AUAUCAGUAAAGAGA 3' (SEQ ID NO:2) or 5' CUCAGGAUUUAAAAU 3' (SEQ ID NO:3), wherein said RNA molecule is between 15 and 35 bases in length, and wherein said

RNA molecule, upon contact with a cell expressing said *HTT* gene, inhibits the expression of said *HTT* gene by at least 20%.

[040] In certain embodiments, the RNA molecule is ssRNA or dsRNA. In certain embodiments, the dsRNA comprises a sense strand and an antisense strand, wherein the antisense strand comprises the region of complementarity which is substantially complementary to 5' AUAUCAGUAAAGAGA 3' (SEQ ID NO:2) or 5' CUCAGGAUUUAAAAU 3' (SEQ ID NO:3).

[041] In certain aspects, a cell comprising a vector for inhibiting the expression of *HTT* gene in a cell is provided. The vector comprising a regulatory sequence operably linked to a nucleotide sequence that encodes an RNA molecule substantially complementary to 5' AUAUCAGUAAAGAGA 3' (SEQ ID NO:2) or 5' CUCAGGAUUUAAAAU 3' (SEQ ID NO:3), wherein said RNA molecule is between 15 and 35 bases in length, and wherein said RNA molecule, upon contact with a cell expressing said *HTT* gene, inhibits the expression of said *HTT* gene by at least 20%.

[042] In certain embodiments, the RNA molecule is ssRNA or dsRNA. In certain embodiments, the dsRNA comprises a sense strand and an antisense strand, wherein the antisense strand comprises the region of complementarity which is substantially complementary to 5' AUAUCAGUAAAGAGA 3' (SEQ ID NO:2) or 5' CUCAGGAUUUAAAAU 3' (SEQ ID NO:3).

[043] In certain aspects, an RNA molecule that is between 15 and 35 bases in length is provided. The RNA molecule comprises a region of complementarity which is substantially complementary to 5' CAGUAAAGAGAUUAA 3' (SEQ ID NO:1), 5' AUAUCAGUAAAGAGA 3' (SEQ ID NO:2) or 5' CUCAGGAUUUAAAAU 3' (SEQ ID NO:3), and the RNA molecule targets a 3' untranslated region (UTR) of *HTT* gene short mRNA.

[044] The 3' UTR of the *HTT* gene short mRNA is as follows:

AGCGCCAUGGUGGGAGAGACUGUGAGGCGGCAGCUGGGGCCGGAGCCUUUGGAAGUCUGCGC
CCUUGUGCCCUGCCUCCACCGAGCCAGCUUGGUCCCUAUGGGCUUCCGCACAUGCCGCGGGC
GGCCAGGCAACGUGCGUGUCUCUGCCAUGUGGCAGAAGUGCUCUUUGUGGCAGUGGCCAGGC
AGGGAGUGUCUGCAGUCCUGGUGGGGCUGAGCCUGAGGCCUUCCAGAAAGCAGGAGCAGCUG
UGCUGCACCCCAUGUGGGUGACCAGGUCCUUUCUCCUGAUAGUCACCUGCUGGUUGUUGCCA
GGUUGCAGCUGCUCUUGCAUCUGGGCCAGAAGUCCUCCCUCCUGCAGGCUGGCUGUUGGCCC

CUCUGCUGUCCUGCAGUAGAAGGUGCCGUGAGCAGGCUUUGGGAACACUGGCCUGGGUCUCC
CUGGUGGGGUGUGCAUGCCACGCCCCGUGUCUGGAUGCACAGAUGCCAUGGCCUGUGCUGGG
CCAGUGGCUGGGGGUGCUAGACACCCGGCACCAUUCUCCCUUCUCUCUUUUCUUCUCAGGAU
UUAAAAUUUAAUUAUAUCAGUAAAGAGAUUAAUUUUAACGUAACUCUUUCUAUGCCCGUGUA
(SEQ ID NO:4)

[045] In certain embodiments, the RNA molecule is ssRNA or dsRNA. In certain embodiments, the dsRNA comprises a sense strand and an antisense strand, wherein the antisense strand comprises the region of complementarity which is substantially complementary to 5' CAGUAAAGAGAUUAA 3' (SEQ ID NO:1), 5' AUAUCAGUAAAGAGA 3' (SEQ ID NO:2) or 5' CUCAGGAUUUAAAAU 3' (SEQ ID NO:3).

[046] In certain aspects, a dsRNA molecule that is between 15 and 35 bases in length, comprising a region of complementarity which is substantially complementary to 5' CAGUAAAGAGAUUAA 3' (SEQ ID NO:1), 5' AUAUCAGUAAAGAGA 3' (SEQ ID NO:2) or 5' CUCAGGAUUUAAAAU 3' (SEQ ID NO:3), wherein the RNA molecule targets an *HTT* mRNA and comprises at least one modified nucleotide is provided. In certain embodiments, the modified nucleotide is a terminal nucleotide linked to a phosphatidylcholine derivative.

[047] In certain aspects, a di-branched RNA compound comprising two RNA molecules that are between 15 and 35 bases in length, comprising a region of complementarity which is substantially complementary to 5' CAGUAAAGAGAUUAA 3' (SEQ ID NO:1), 5' AUAUCAGUAAAGAGA 3' (SEQ ID NO:2) or 5' CUCAGGAUUUAAAAU 3' (SEQ ID NO:3), wherein the two RNA molecules are connected to one another by one or more moieties independently selected from a linker, a spacer and a branching point, is provided.

[048] In any of the aspects described herein, the RNA molecule is an antisense molecule (e.g., ASO) or a GAPMER molecule. In certain embodiments, the antisense molecule enhances degradation of the region of complementarity. In certain embodiments, the degradation is nuclease degradation (e.g., RNase H).

Brief Description of the Drawings

[049] The foregoing and other features and advantages of the present invention will be more fully understood from the following detailed description of illustrative embodiments

taken in conjunction with the accompanying drawings. The patent or application file contains at least one drawing executed in color. Copies of this patent or patent application publication with color drawing(s) will be provided by the Office upon request and payment of the necessary fee.

[050] ***Figures 1A-B*** depict hydrophobic siRNA structural and chemical composition and efficient internalization in primary cortical neurons. A) Schematic of the hydrophobically modified and stabilized siRNAs (hsiRNAs) B) Cy3-HTT10150 hsiRNA (red), 0.5 μM, was added to primary cortical neurons. Imaged on Zeiss confocal microscope, 63X, nuclei stained with Hoechst dye (blue).

[051] ***Figures 2A-C*** depict a systematic screening of unformulated hsiRNAs targeting huntingtin mRNA plotted as a line graph (A) or a bar graphs (B) and (C). A panel of 94 hsiRNAs were added to HeLa cells at 1.5 μM. Level of huntingtin mRNA was measured using QUANTIGENE (Affymetrix) at 72 hours normalized to housekeeping gene, PPIB (cyclophilin B), and presented as percent of untreated control (n=3, mean +/- SD). UNT – untreated cells, NTC – non-targeting control. Active compounds (red) were selected for further analysis.

[052] ***Figures 3A-C*** depict concentration-dependent silencing of huntingtin mRNA by HTT10150, in both passive (A) and lipid-mediated delivery (B). Chemical modifications enable passive uptake without negative impact on siRNA RISC (RNA Induced Silencing Complex) entry. HeLa cells were incubated with modified (containing both hydrophobic and base chemical modifications) or unmodified HTT10150 at concentrations shown in the absence (A) and presence (B) of RNAIMAX. Level of huntingtin mRNA was measured using QUANTIGENE (Affymetrix) at 72 hours normalized to housekeeping gene, PPIB (cyclophillin B), and presented as percent of untreated control (n=3, mean +/- SD). UNT – untreated cells. IC50 values calculated as described herein. (C) Is a table summarizing these results.

[053] ***Figures 4A-B*** graphically depict concentration-dependent silencing of huntingtin mRNA and protein by HTT10150 in primary neurons (passive uptake). Primary neurons were incubated with HTT10150 at concentrations shown. Level of huntingtin mRNA was measured using QUANTIGENE (Affymetrix) normalized to housekeeping gene, PPIB (cyclophillin B), and presented as percent of untreated control (n=3, mean +/- SD). UNT – untreated cells. A) In primary cortical and striatal neurons, 1 week. B) Huntingtin

protein levels after one week incubation with HTT10150 were detected by western blot and normalized to β-Tubulin.

[054] ***Figures 5A-H*** depict a single intrastriatal injection of HTT10150 is localized to neurons and fiber tracts ipsilateral to the injection site after 24 hours. 1 nmol CY3-HTT10150 (Red) was unilaterally injected into the striatum of WT (FVBNj) mice. Brains were collected after 24 hours, paraffin imbedded and sectioned and sectioned. (A) Tiled image of coronal brain section (16X). Majority of HTT10150 was localized at site of injection with sharp gradient of diffusion. (B) Tiled image of sagittal brain section (16X), injected side. (C) Image of coronal brain section (40X), non-injected side. (D) Image of coronal brain section (40X), injected side. (E, G) NueN stained neurons from non-injected side (60X). (F, H) NueN stained neurons from injected side (60X).

[055] ***Figure 6*** graphically depicts evaluation of HTT10150 efficacy *in vivo*. HTT10150 was unilaterally injected into the striatum of WT (FVB) mice (2 µl). Mice were sacrificed at 120 hours. Brains were sliced into 300µm sections and six - 2mm punch biopsies of the striatum were collected from both Ipsilateral and Contralateral sides. Level of huntingtin mRNA was measured using QUANTIGENE (Affymetrix) normalized to housekeeping gene, PPIB (cyclophillin B), and presented as percent of untreated control (n=24, mean +/- SEM, 8 animals, 3 biopsies per region).

[056] ***Figures 7A-E*** depict that HTT10150 shows no toxicity in DARPP-32 positive neurons around the site of injection. HTT10150 was unilaterally injected into the striatum of WT (FVB) mice. Brains were collected after 5 days fixed, sectioned, and stained with antibodies against DARPP-32 (A-D). Representative image of striatum after injection of ACSF, full brain scan and 60X magnification (A, B) or 12.5µg HTT10150, full brain scan and 60X magnification (C, D). Quantification of DARPP-32 positive neurons (E) (n=3 animals, mean +/- SD).

[057] ***Figure 8*** depicts target sequences, modified oligonucleotides and their efficacy according to certain embodiments.

[058] ***Figure 9*** depicts efficient uptake and internalization of hsiRNA in primary cortical neurons over time. Cy3-HTT10150 hsiRNA (red), 0.5 µM, was added to primary cortical neurons. Imaged on Zeiss confocal microscope, 63X, nuclei stained with Hoechst dye (blue).

[059] ***Figures 10A-B*** graphically depict concentration-dependent silencing of huntingtin mRNA by HTT10150 in HeLa cells. Level of huntingtin mRNA was measured using QUANTIGENE (Affymetrix) at 72 hours normalized to housekeeping gene, PPIB (cyclophillin B), and presented as percent of untreated control (n=3, mean +/- SD). UNT – untreated cells, NTC – non-targeting control. A) Dose response of 16 active sequences in passive uptake (no formulation). B) Dose response of eight selected sequences in lipid-mediated uptake (using Invitrogen LIPOFECTAMINE RNAIMAX Transfection Reagent). Dose response data was fitted using GraphPad Prism 6.03.

[060] ***Figures 11A-B*** graphically depict huntingtin mRNA levels. A) Cell viability was tested using ALAMAR BLUE (Life Technologies) after incubation of HTT10150 and NTC with primary cortical neurons for 72 hours and one week. B) Primary cortical neurons were incubated with three HTT hsiRNA sequences HTT10150, HTT10146, and HTT1215 at concentrations shown. Level of huntingtin mRNA was measured using QUANTIGENE (Affymetrix) normalized to housekeeping gene, PPIB (cyclophillin B), and presented as percent of untreated control (n=3, mean +/- SD). UNT – untreated cells.

[061] ***Figures 12A-B*** graphically depict concentration-dependent silencing of huntingtin mRNA by HTT10150 in primary neurons (passive uptake). Primary neurons were incubated with HTT10150 at concentrations shown. Level of huntingtin mRNA was measured using QUANTIGENE (Affymetrix) normalized to housekeeping gene, PPIB (cyclophillin B), and presented as percent of untreated control (n=3, mean +/- SD). UNT – untreated cells. A) For 72 hours and 1 week. B) For 1, 2 and 3 weeks.

[062] ***Figure 13*** graphically depicts efficacy of hsiRNA against cyclophilin B (PPIB) in primary cortical neurons. Primary neurons were incubated with hsiRNA targeting PPIB at concentrations shown. Level of PPIB mRNA was measured using QUANTIGENE (Affymetrix) normalized to housekeeping gene, HTT and presented as percent of untreated control (n=3, mean +/- SD). UNT – untreated cells for 1 week.

[063] ***Figure 14*** depicts representative Western blots of Htt reduction in primary cortical neurons. Primary cortical neurons were cultured from five individual pups (#1-5) and incubated with HTT10150 at concentrations shown for one week. Huntingtin protein levels were detected by Western blot using antibody AB1.

[064] ***Figures 15A-B*** graphically depict evaluation of HTT10150 efficacy *in vivo*. A) HTT10150 was unilaterally injected into the striatum of WT (FVB) mice (2 μl). Mice

were sacrificed at 120 hours. Brains were sliced into 300μm sections and six 2 mm punch biopsies of the striatum were collected from both ipsilateral and contralateral sides. Level of huntingtin mRNA was measured using QUANTIGENE (Affymetrix) normalized to housekeeping gene, PPIB (cyclophilin B), and presented as percent of untreated control (n=8 animals, mean +/- SD). B) Quantification of huntingtin protein silencing by Western blot.

[065] ***Figure 16*** graphically depicts evaluation of HTT10150 cytotoxicity *in vivo*. DARPP32 neuronal marker was minimally affected by HTT10150 injection, indicating no major impact on neuronal health. HTT10150 was unilaterally injected into the striatum of wild-type (FVB) mice at doses shown. Mice were sacrificed at 120 hours. Brains were sliced into 300μm sections and six punch biopsies (2mm) of the striatum were collected from both ipsilateral and contralateral sides. Level of DARPP32 mRNA expression was measured using QUANTIGENE (Affymetrix) normalized to housekeeping gene, PPIB (cyclophilin B), and presented as percent of untreated control (n=24, mean +/- SD).

[066] ***Figures 17A-C*** depict that HTT10150 showed a two-fold increase in microglial activation at the site of injection. HTT10150 was unilaterally injected into the striatum of WT (FVB) mice. Brains were collected after 6 hours (b) and 5 days (a and c) fixed, sectioned, and stained with antibodies against IBA-1. (A) Representative images of activated (black arrowhead) and resting (open arrowhead) after injection of 1 nmol HTT10150 and ACSF 5 days post injection. 40X magnification. (B) Quantification of activated and resting microglia 6hrs post-injection of ACSF (n=6) and 1nmol HTT10150 (n=3). (c) Quantification of activated and resting microglia 5 days post-injection of ACSF (n=4) and 1nmol HTT10150 (n=3).

[067] ***Figures 18A-C*** depict that HTT10150 showed limited toxicity at the site of injection at the 25 μg dose. HTT10150 was unilaterally injected into the striatum of WT (FVB) mice. Brains were collected after 5 days fixed, sectioned, and stained with antibodies against DARPP-32. Representative image of striatum after injection of 25 μg, full brain scan (A), 10X magnification at injections site (B), 20X magnification at injection site (C), and 60X magnification.

[068] ***Figure 19*** depicts that HTT10150 showed no toxicity to Darpp32 positive neurons at lower concentrations. HTT10150 was unilaterally injected into the striatum of WT (FVB) mice. Brains were collected after 5 days fixed, sectioned, and stained with

antibodies against DARPP-32. Representative image of striatum after injection of 25 µg, 12.5 µg, and ACSF (20X magnification) ipsilateral and contralateral to the site of injection.

[069] ***Figures 20A-B*** depict that HTT10150 caused a slight increase in total resting microglia 5 days post injection. HTT10150 was unilaterally injected into the striatum of WT (FVB) mice. Brains were collected after 6 hours and 5 days fixed, sectioned, and stained with antibodies against IBA-1. Quantification of total microglia 6hrs (A) and 5 days (B) post-injection of ACSF (n=6, A) (n=4, B) and 12.5 µg HTT10150 (n=3, A, B).

[070] ***Figure 21*** depicts additional target sequences along with chemical modifications and structural scaffolds according to certain embodiments of the invention.

[071] ***Figure 22*** depicts hsiRNA^{HTT} efficacy in primary cortical neurons (cell viability) after one week using QUANTIGENE and ALAMAR BLUE. NTC = non-targeting control.

[072] ***Figure 23*** depicts *HTT* hsiRNA efficacy in wild-type primary striatal neurons and primary cortical neurons after one week using QUANTIGENE. NTC = non-targeting control.

[073] ***Figure 24*** depicts HTT hsiRNA efficacy in primary neurons (duration of effect) from one to three weeks post-treatment via passive uptake. HTT expression was normalized to PPIB. Data is shown is an approximate percentage of non-targeting control. UNT = untreated.

[074] ***Figure 25*** graphically depicts that hsiRNA^{HTT} but not LNA-GAPMER exhibits a silencing plateau in cortical neurons after 72 hours using QUANTIGENE. N = 3.

[075] ***Figure 26*** shows intracellular localization of *htt* and *ppib* in primary cortical neurons using RNA-SCOPE. *Htt* mRNA, red; *ppib* mRNA, green; nuclei (DAPI), blue.

[076] ***Figure 27*** validates in neurons an *htt* detection probe set, affirming specificity.

[077] ***Figure 28*** validates in neurons an *htt* detection probe set, showing that the signal is not intron-specific (validated for intron 60-61).

[078] ***Figure 29*** depicts that *htt* mRNA nuclear localization is specific to neurons only. Left panel depicts primary neurons; *ppib* mRNA, green; *htt* mRNA, red, nuclei, blue.

[079] ***Figure 30*** depicts that hsiRNA^{HTT} treatment of cortical neurons preferentially eliminates cytoplasmic htt mRNA. *Ppib* mRNA, green; *htt* mRNA, red; nuclei, blue. Top panel: non-treated. Bottom panel, treated with 1.5 μM hsiRNA^{HTT} for three days.

[080] ***Figure 31*** graphically depicts that hsiRNA^{HTT} treatment of cortical neurons preferentially eliminates cytoplasmic *htt* mRNA.

[081] ***Figure 32*** depicts a Western blot showing HTT protein silencing in wild-type primary cortical neurons. hsiRNA htt-10150; NTC = non-targeting control, 1 week.

[082] ***Figure 33*** graphically depicts the results of HTT10150 direct injection. No effects on neuronal viability were observed.

[083] ***Figure 34*** depicts toxicity adjacent to the injection site following cholesterol-hsiRNA administration.

[084] ***Figures 35A-C*** show that partially modified hsiRNAs exhibit a short duration of effect and no systemic exposure.

[085] ***Figures 36A-C*** depict full metabolic stabilization of hsiRNAs.

[086] ***Figures 37A-C*** show that full metabolic stabilization does not interfere with RISC entry of hsiRNAs.

[087] ***Figures 38A-38E*** depict fully metabolically stabilized hsiRNA (FM-hsiRNA) enhancement of local delivery and distribution.

[088] ***Figures 39A-B*** depict enhanced potency and duration of effect mediated by FM-hsiRNA.

[089] ***Figure 40*** characterizes neuroactive, naturally occurring lipids as hsiRNA bioconjugates.

[090] ***Figure 41*** depicts that hsiRNA hydrophobicity directly correlates with brain distribution and retention. Intrastriatal injection, 12.5 μg (0.5 mg/kg), t = 24 hours, FVB/NJ mice (n = 2).

[091] ***Figure 42*** depicts docosahexaenoic acid (DHA) hsiRNA synthesis.

[092] ***Figure 43*** depicts internalization of DHA-hsiRNA and chol-hsiRNA into primary cortical neurons. Uptake: 0.5 μM Cy3-DHA-hsiRNA (red), DAPI (blue).

[093] ***Figure 44*** depicts co-localization of DHA-hsiRNA with neurons and astrocytes. Intrastriatal injection, 12.5 μg (0.5 mg/kg), t = 24 hours, FVB/NJ mice (n = 2).

[094] ***Figure 45*** depicts localization of DHA-hsiRNA to the perinuclear region in striatal neurons, while chol-hsiRNA is undetectable. Intrastriatal injection, 12.5 μg (0.5 mg/kg), t = 24 hours, FVB/NJ mice (n = 2).

[095] ***Figure 46*** depicts co-localization of DHA-hsiRNA with neurons and astrocytes in the cortex following a single intrastriatal injection. Intrastriatal injection, 12.5 μg (0.5 mg/kg), t = 24 hours, FVB/NJ mice (n = 2).

[096] ***Figure 47*** depicts localization of DHA-hsiRNA to the perinuclear region in cortical neurons, while chol-hsiRNA is undetectable.

[097] ***Figure 48*** depicts robust silencing efficiency of DHA-hsiRNA in the striatum and cortex. Intrastriatal injection, 6-25 μg (0.25-1 mg/kg), t = 5 days, FVB/NJ mice (n = 8).

[098] ***Figure 49*** depicts the duration of effect and recovery in the striatum following a single intrastriatal dose of DHA-hsiRNA.

[099] ***Figure 50*** depicts a pilot safety study showing that DHA-siRNA does not affect striatal neuronal integrity at greater than 20-fold over the efficacious dose.

[0100] ***Figure 51*** depicts a pilot safety study showing that DHA-siRNA causes minimal striatal microglial activation at greater than 20-fold over the efficacious dose.

[0101] ***Figure 52*** depicts perinuclear localization caused by oligonucleotide chemistry.

[0102] ***Figure 53*** depicts intra-nuclear foci distribution caused by oligonucleotide chemistry.

[0103] ***Figure 54*** shows that the degree of *htt* mRNA striatal silencing is effected by oligonucleotide cellular localization.

[0104] ***Figure 55*** depicts targeted glial delivery.

[0105] ***Figure 56*** depicts targeted neuronal delivery.

[0106] ***Figure 57*** shows that DHA-hsiRNA efficiently distributes throughout the brain and silences genes in both the striatum and the cortex. Intrastriatal injection, 12.5 μg (0.5 mg/kg), t = 24 hours, FVB/NJ mice (n = 2).

[0107] ***Figure 58*** shows hsiRNA efficacy in wild-type primary hippocampal neurons and Q140 primary hippocampal neurons. 16% gel.

[0108] ***Figure 59*** graphically depicts hsiRNA efficacy in wild-type primary hippocampal neurons and Q140 primary hippocampal neurons.

[0109] ***Figure 60*** shows hsiRNA efficacy in wild-type primary hippocampal neurons and Q140 primary hippocampal neurons. 7.5% gel.

[0110] ***Figure 61*** shows that each of PC-DHA-hsiRNA and chol-hsiRNA silence mutant and wild-type *htt* mRNA.

[0111] ***Figure 62*** describes three classes of hsiRNA chemistries: DHA-hsiRNA, PC-DHA-hsiRNA and chol-hsiRNA.

[0112] ***Figures 63A-B*** graphically depict enhanced potency of PC-DHA-hsiRNA relative to DHA-hsiRNA in cortical primary neurons. 1 week, analyzed by QUANTIGENE, data normalized to PPIB.

[0113] ***Figure 64*** illustrates that chol-hsiRNA has a more effective chemistry for gene modulation in primary cortical neurons relative to PC-DHA-hsiRNA and DHA-hsiRNA. 1 week, analyzed by QUANTIGENE, data normalized to PPIB.

[0114] ***Figure 65*** shows that PC-DHA-hsiRNA shows better brain retention and wider distribution that DHA-hsiRNA. Intrastriatal injections at either 2 or 10 nmol, N = 2, brains collected at 48 hours.

[0115] ***Figure 66*** shows approximately 80% silencing in mouse striatum after a single IS injection PC-DHA-hsiRNA.

[0116] ***Figure 67*** shows approximately 60% silencing in mouse cortex after a single IS injection PC-DHA-hsiRNA.

[0117] ***Figure 68*** depicts di-hsiRNA brain distribution after an CSF bolus injection (250 μg), 48 hours.

[0118] ***Figure 69*** depicts distribution of di-hsiRNA after a single IS injection.

[0119] ***Figure 70*** depicts effect of branching on brain distribution.

[0120] ***Figure 71*** depicts a study design to assay *in vivo* gene silencing after single IS injections of di-hsiRNA.

[0121] ***Figure 72*** depicts neuronal delivery of di-hsiRNA.

[0122] ***Figure 73*** depicts efficacy of di-hsiRNA in the striatum and cortex. IS injection, 2 nmol di-hsiRNA, 1 week, QuantiGene 2.0.

[0123] ***Figure 74*** depicts uniform spinal cord distribution of di-hsiRNA.

[0124] ***Figure 75*** depicts *htt* mRNA silencing in the spinal cord after administration of a di-hsiRNA^{HTT} bolus. IT, 3 nmol, one week, QuantiGene.

[0125] ***Figure 76*** depicts di-hsiRNA-mediated in vitro silencing in HeLa cells and primary cortical neurons.

[0126] ***Figure 77*** depicts biodistribution of di-hsiRNA. Intrastriatal injection of 2 nmol of Di-siRNA oligo (4 nmol of corresponding antisense strand). N=2 mice per conjugate. Brains collected 48 hours later and stained with DAPI (nuclei, blue) and NeuN (neuronal marker, green). Image is representative. Red-oligo.

[0127] ***Figure 78*** depicts biodistribution of di-hsiRNA. Intrastriatal injection of 2 nmol of Di-siRNA oligo (4 nmol of corresponding antisense strand). N=2 mice per conjugate. Brains collected 48 hours later and stained with DAPI (nuclei, blue) and NeuN (neuronal marker, green). Image is representative. Red-oligo.

[0128] ***Figure 79*** depicts brain distribution of di-hsiRNA, TEG-azide, TEG and vitamin D after 48 hours. 2 nmole injected IS, N=2 mice per conjugate, brains collected 48 hours later.

[0129] ***Figure 80*** depicts the efficacy of vitamin D synthesis on *htt* mRNA expression.

[0130] ***Figure 81*** depicts a chemical Formula of a compound provided herein.

[0131] ***Figure 82*** depicts examples of internucleotide linkages of R^3.

[0132] ***Figure 83*** depicts an embodiment of the chemical Formula of Figure 81.

[0133] ***Figure 84*** depicts a chemical Formula of a compound provided herein.

[0134] ***Figure 85*** depicts a chemical Formula of a compound provided herein.

[0135] ***Figure 86*** depicts an embodiment of the Y moiety of Figure 84 or Figure 85.

[0136] ***Figure 87*** depicts a chemical Formula of a compound provided herein.

[0137] ***Figure 88*** depicts an embodiment of the chemical Formula of Figure 87.

[0138] ***Figure 89*** depicts a chemical Formula of a compound provided herein.

[0139] ***Figure 90*** depicts an embodiment of the chemical Formula of Figure 89.

[0140] ***Figures 91A-D*** depict the development of fully metabolically stabilized hsiRNAs (FM-hsiRNAs). (A) Schematics of partially and fully modified hsiRNAs. (B) hsiRNA and FM-hsiRNA have equal ability to enter RISC (HeLa, 72 hours). (C) Metabolically stable 5'-E-VP is as active as 5'-P. (D) 5'-E-VP enables sustained delivery to distant tissues (7 days post injection, PNA assay).

[0141] ***Figure 92*** depicts that the evolution of chemistry enabled wide distribution of hsiRNA in mouse brain after a bolus CSF (ICV) infusion. Images of sagittal sections (left panels) from 48 hours after ICV injection with 250 μg Cy3-labled hsiRNA variants (right panels). Images taken with Leica tiling array microscope at 10× and at identical laser intensity. Nuclei (blue); Cy3-hsiRNA (red). Chol-hsiRNA mainly stayed around the injected ventricle with marginal distribution to the distal sides of the brain. DHA-hsiRNA shows better distribution. PC-DHA and Di-hsiRNAs shows most diffuse distribution with clear delivery to cortex, striatum, and even cerebellum. Scale bar = 900 μm.

[0142] ***Figure 93*** depicts a synthetic protocol for PC-DHA-functionalized solid support.

[0143] ***Figure 94*** depicts a synthetic protocol for DI-functionalized solid support.

[0144] ***Figures 95A-C*** depict di-hsiRNA discovery. (A) Chemical composition of the four bi-products from calciferol-hsiRNA synthesis (analytical HPLC of the crude synthesis). (B) Efficacy of bi-products in HeLa cells, 72 hours, QuantiGene®. All compounds were equally active. (C) A single, unilateral intrastriatal injection (25 μg) of each Cy3-hsiRNA bi-product, 48 hours. Only di-hsiRNAs showed broad distribution with preferential neuronal uptake.

[0145] ***Figure 96*** depicts an hsiANTIDOTE antisense oligonucleotide carrying high affinity modification (LNA) designed to be fully complementary to the hsiRNA antisense strand seed region.

[0146] ***Figure 97*** depicts a cholesterol and endocytic peptide (proton sponge) conjugated hsiRNA.

[0147] ***Figure 98*** depicts a solution-phase synthetic protocol for a GM1-conjugated hsiRNA.

[0148] ***Figure 99*** depicts chemical structures for DHA-conjugates (g1DHA) and PC-DHA hsiRNA conjugates (g2DHA).

[0149] ***Figure 100*** depicts a solid-phase synthetic protocol for PC-DHA hsiRNA conjugates.

[0150] ***Figure 101*** depicts a solution-phase synthetic protocol for PC-DHA hsiRNA conjugates.

[0151] ***Figures 102A-D*** depict that full metabolic stabilization was essential for conjugate mediated siRNA delivery and duration of effect *in vivo*. (A, B) Compared to hsiRNA (A), FM-hsiRNA (B) showed significantly enhanced distribution and retention in tissues after intravenous (IV) and CSF (ICV) administration. Wild-type pregnant mice (E15) were injected with 10 mg/kg IV or 60 μg, ICV. Tissues were imaged at 10× on a Leica tiling fluorescent microscope at identical laser intensity. HsiRNAs (red); nuclei (blue). Scale bar = 900 μm. (C) Intact guide strand in tissues quantified 5 days after IV injection (n=3, mean ± SEM). (D) FM-hsiRNAs silence Htt mRNA in mouse striatum one month after injection (12 μg, intrastriatal). Partially modified hsiRNAs silence for less than two weeks.

[0152] ***Figures 103A-C*** depict PC-DHA-hsiRNAs efficacy and safety in mouse brain *in vivo*. (A) *Htt* mRNA levels in striatum and cortex 1 week after injecting 25 or 50 μg DHA-hsiRNA. ***P < 0.0001 relative to both aCSF and NTC. (B) No detectable innate immune activation occurred at dose levels 20-fold higher than the effective dose (data shown for total microglia for DHA-hsiRNA). (C) Normal neuronal viability based on DARP32 levels. Note the toxic dose (red bar) for chol-hsiRNA.

[0153] ***Figures 104A-C*** show that di-hsiRNA exhibited wide distribution and efficacy in mouse brain. (A) Robust and uniform distribution of Cy3-Di-hsiRNA throughout the brain, visually and histologically, with clear neuronal uptake 48 hours after ICV injection (250 μg, CSF, both sides), scale bar = 100 μm. (B) *Htt* mRNA silencing in cortex and striatum 7 days after single intrastriatal injection (25 μg). (C) hsiRNA accumulation in tissues 7 days after injection (PNA assay).

[0154] ***Figures 105A-B*** show that di-hsiRNAs exhibited wide distribution and efficacy in the mouse spinal cord after a bolus lumbar intrathecal injection. (A) Chol-hsiRNAs showed a steep gradient of diffusion from outside to inside of spinal cord, but Di-hsiRNAs distribute widely throughout the spinal cord. Animals were injected intrathecally

with 75 μg Cy3-Chol-hsiRNA or Cy3-Di-hsiRNA. Scale bar = 100 μm. (B) Robust *Htt* mRNA silencing was observed in all regions of spinal cord (7 days post-injection, n=6).

[0155] ***Figure 106*** depicts a PNA (Peptide Nucleic Acid)-based assay for detection of hsiRNA guide strand in mouse tissues. Tissues were lysed, debris separated by precipitation, and the PNA-guide strand duplex purified by HPLC (DNAPac P100, 50% water 50% acetonitrile, salt gradient 0-1M $NaClO_4$).

[0156] ***Figure 107*** depicts targeting of the kidney by PC-DHA-hsiRNA.

[0157] ***Figure 108*** depicts GM1-hsiRNA internalization and GM1-hsiRNA-mediated *htt* mRNA silencing.

[0158] ***Figure 109*** depicts GM1-hsiRNA brain distribution.

[0159] ***Figures 110A-G*** show that systemically-administered fully modified (FM) hsiRNA exhibits dramatically enhanced tissue distribution and efficacy *in vivo*. (a) Tissue distribution of Cy3-hsiRNA and Cy3-FM-hsiRNAsFLT1 (red) 10 mg/kg IV injection. Nuclei stained with DAPI (blue). All images were acquired at identical settings. (b-e) Guide strand quantification by PNA hybridization-based assay (b) 10 mg/kg, IV, 24 hours (c) 10 mg/kg, SC, 24 hours (d) 2x20 mg/kg, IV, 120 hours, (n= 7) (e) 2x15 mg/kg, IV, 120 hours, (n=12). (f, g) Quantification of sFLT1 mRNA silencing after (f) 2x20 mg/kg, C57B6 mice, (n=3, PBS; n=7, FM-hsiRNAsFLT), (g) 2x15 mg/kg, CD1 mice. (n=12, for PBS; n=6, NTC; n=12, FM-hsiRNAsFLT1). mRNA levels were measured 120 hours after injection with QuantiGene® (Affymetrix) assay, normalized to housekeeping gene FLT1, and presented as percent of PBS treated control. All error bars represent mean ± SD. ***, $P<0.001$; ****, $P<0.0001$.

[0160] ***Figures 111A-G*** show that fully modified hsiRNAs are broadly distributed throughout the brain and demonstrate higher potency and longer duration of silencing upon local administration. hsiRNAHTT (a) and FM-hsiRNAHTT(b, c, d, e) were injected ICV, distribution through the sagittal section of the brain after 48 hours is shown. Nuclei stained with DAPI (blue). Cy3-hsiRNA (red). (f, g) hsiRNAHTT and FM-hsiRNAHTT were unilaterally injected into the striatum and level of HTT mRNA was measured using QuantiGene® (Affymetrix) after (f) 5 days or (g) 7, 14 and 28 days, normalized to housekeeping gene, PPIB, and presented as percent of untreated control (n=8 mice, mean ± SD). NTC = non-targeting control; CSF = artificial cerebrospinal fluid All error bars represent mean ± SD. ** , $P<0.01$; ***, $P<0.001$; ****, $P<0.0001$.

Detailed Description of Certain Exemplary Embodiments

[0161] Novel huntingtin target sequences are provided. Also provided are novel siRNAs that target the novel huntingtin target sequences of the invention.

[0162] Generally, nomenclature used in connection with cell and tissue culture, molecular biology, immunology, microbiology, genetics and protein and nucleic acid chemistry and hybridization described herein are those well-known and commonly used in the art. The methods and techniques provided herein are generally performed according to conventional methods well known in the art and as described in various general and more specific references that are cited and discussed throughout the present specification unless otherwise indicated. Enzymatic reactions and purification techniques are performed according to manufacturer's specifications, as commonly accomplished in the art or as described herein. The nomenclature used in connection with, and the laboratory procedures and techniques of, analytical chemistry, synthetic organic chemistry, and medicinal and pharmaceutical chemistry described herein are those well-known and commonly used in the art. Standard techniques are used for chemical syntheses, chemical analyses, pharmaceutical preparation, formulation, and delivery, and treatment of patients.

[0163] Unless otherwise defined herein, scientific and technical terms used herein have the meanings that are commonly understood by those of ordinary skill in the art. In the event of any latent ambiguity, definitions provided herein take precedent over any dictionary or extrinsic definition. Unless otherwise required by context, singular terms shall include pluralities and plural terms shall include the singular. The use of "or" means "and/or" unless stated otherwise. The use of the term "including," as well as other forms, such as "includes" and "included," is not limiting.

[0164] So that the invention may be more readily understood, certain terms are first defined.

[0165] The term "nucleoside" refers to a molecule having a purine or pyrimidine base covalently linked to a ribose or deoxyribose sugar. Exemplary nucleosides include adenosine, guanosine, cytidine, uridine and thymidine. Additional exemplary nucleosides include inosine, 1-methyl inosine, pseudouridine, 5,6-dihydrouridine, ribothymidine, 2N-methylguanosine and 2,2N,N-dimethylguanosine (also referred to as "rare" nucleosides). The term "nucleotide" refers to a nucleoside having one or more phosphate groups joined in

ester linkages to the sugar moiety. Exemplary nucleotides include nucleoside monophosphates, diphosphates and triphosphates. The terms “polynucleotide” and “nucleic acid molecule” are used interchangeably herein and refer to a polymer of nucleotides joined together by a phosphodiester or phosphorothioate linkage between 5' and 3' carbon atoms.

[0166] The term “RNA” or “RNA molecule” or “ribonucleic acid molecule” refers to a polymer of ribonucleotides (e.g., 2, 3, 4, 5, 10, 15, 20, 25, 30, or more ribonucleotides). The term “DNA” or “DNA molecule” or deoxyribonucleic acid molecule" refers to a polymer of deoxyribonucleotides. DNA and RNA can be synthesized naturally (e.g., by DNA replication or transcription of DNA, respectively). RNA can be post-transcriptionally modified. DNA and RNA can also be chemically synthesized. DNA and RNA can be single-stranded (i.e., ssRNA and ssDNA, respectively) or multi-stranded (e.g., double stranded, i.e., dsRNA and dsDNA, respectively). "mRNA" or "messenger RNA" is single-stranded RNA that specifies the amino acid sequence of one or more polypeptide chains. This information is translated during protein synthesis when ribosomes bind to the mRNA.

[0167] As used herein, the term "small interfering RNA" ("siRNA") (also referred to in the art as "short interfering RNAs") refers to an RNA (or RNA analog) comprising between about 10-50 nucleotides (or nucleotide analogs) which is capable of directing or mediating RNA interference. Preferably, a siRNA comprises between about 15-30 nucleotides or nucleotide analogs, more preferably between about 16-25 nucleotides (or nucleotide analogs), even more preferably between about 18-23 nucleotides (or nucleotide analogs), and even more preferably between about 19-22 nucleotides (or nucleotide analogs) (e.g., 19, 20, 21 or 22 nucleotides or nucleotide analogs). The term "short" siRNA refers to a siRNA comprising about 21 nucleotides (or nucleotide analogs), for example, 19, 20, 21 or 22 nucleotides. The term "long" siRNA refers to a siRNA comprising about 24-25 nucleotides, for example, 23, 24, 25 or 26 nucleotides. Short siRNAs may, in some instances, include fewer than 19 nucleotides, e.g., 16, 17 or 18 nucleotides, provided that the shorter siRNA retains the ability to mediate RNAi. Likewise, long siRNAs may, in some instances, include more than 26 nucleotides, provided that the longer siRNA retains the ability to mediate RNAi absent further processing, e.g., enzymatic processing, to a short siRNA.

[0168] The term "nucleotide analog" or "altered nucleotide" or "modified nucleotide" refers to a non-standard nucleotide, including non-naturally occurring ribonucleotides or deoxyribonucleotides. Exemplary nucleotide analogs are modified at any position so as to alter certain chemical properties of the nucleotide yet retain the ability of the nucleotide

analog to perform its intended function. Examples of positions of the nucleotide which may be derivatized include the 5 position, e.g., 5-(2-amino)propyl uridine, 5-bromo uridine, 5-propyne uridine, 5-propenyl uridine, etc.; the 6 position, e.g., 6-(2-amino)propyl uridine; the 8-position for adenosine and/or guanosines, e.g., 8-bromo guanosine, 8-chloro guanosine, 8-fluoroguanosine, etc. Nucleotide analogs also include deaza nucleotides, e.g., 7-deaza-adenosine; O- and N-modified (e.g., alkylated, e.g., N6-methyl adenosine, or as otherwise known in the art) nucleotides; and other heterocyclically modified nucleotide analogs such as those described in Herdewijn, Antisense Nucleic Acid Drug Dev., 2000 Aug. 10(4):297-310.

[0169] Nucleotide analogs may also comprise modifications to the sugar portion of the nucleotides. For example the 2' OH-group may be replaced by a group selected from H, OR, R, F, Cl, Br, I, SH, SR, NH_2, NHR, NR_2, COOR, or OR, wherein R is substituted or unsubstituted C_1-C_6 alkyl, alkenyl, alkynyl, aryl, etc. Other possible modifications include those described in U.S. Pat. Nos. 5,858,988, and 6,291,438.

[0170] The phosphate group of the nucleotide may also be modified, e.g., by substituting one or more of the oxygens of the phosphate group with sulfur (e.g., phosphorothioates), or by making other substitutions which allow the nucleotide to perform its intended function such as described in, for example, Eckstein, Antisense Nucleic Acid Drug Dev. 2000 Apr. 10(2):117-21, Rusckowski et al. Antisense Nucleic Acid Drug Dev. 2000 Oct. 10(5):333-45, Stein, Antisense Nucleic Acid Drug Dev. 2001 Oct. 11(5): 317-25, Vorobjev et al. Antisense Nucleic Acid Drug Dev. 2001 Apr. 11(2):77-85, and U.S. Pat. No. 5,684,143. Certain of the above-referenced modifications (e.g., phosphate group modifications) preferably decrease the rate of hydrolysis of, for example, polynucleotides comprising said analogs *in vivo* or *in vitro*.

[0171] The term "oligonucleotide" refers to a short polymer of nucleotides and/or nucleotide analogs. The term "RNA analog" refers to an polynucleotide (e.g., a chemically synthesized polynucleotide) having at least one altered or modified nucleotide as compared to a corresponding unaltered or unmodified RNA but retaining the same or similar nature or function as the corresponding unaltered or unmodified RNA. As discussed above, the oligonucleotides may be linked with linkages which result in a lower rate of hydrolysis of the RNA analog as compared to an RNA molecule with phosphodiester linkages. For example, the nucleotides of the analog may comprise methylenediol, ethylene diol, oxymethylthio, oxyethylthio, oxycarbonyloxy, phosphorodiamidate, phosphoroamidate, and/or phosphorothioate linkages. Preferred RNA analogues include sugar- and/or backbone-

modified ribonucleotides and/or deoxyribonucleotides. Such alterations or modifications can further include addition of non-nucleotide material, such as to the end(s) of the RNA or internally (at one or more nucleotides of the RNA). An RNA analog need only be sufficiently similar to natural RNA that it has the ability to mediate (mediates) RNA interference.

[0172] As used herein, the term "RNA interference" ("RNAi") refers to a selective intracellular degradation of RNA. RNAi occurs in cells naturally to remove foreign RNAs (e.g., viral RNAs). Natural RNAi proceeds via fragments cleaved from free dsRNA which direct the degradative mechanism to other similar RNA sequences. Alternatively, RNAi can be initiated by the hand of man, for example, to silence the expression of target genes.

[0173] An RNAi agent, e.g., an RNA silencing agent, having a strand which is "sequence sufficiently complementary to a target mRNA sequence to direct target-specific RNA interference (RNAi)" means that the strand has a sequence sufficient to trigger the destruction of the target mRNA by the RNAi machinery or process.

[0174] As used herein, the term "isolated RNA" (e.g., "isolated siRNA" or "isolated siRNA precursor") refers to RNA molecules which are substantially free of other cellular material, or culture medium when produced by recombinant techniques, or substantially free of chemical precursors or other chemicals when chemically synthesized.

[0175] As used herein, the term "RNA silencing" refers to a group of sequence-specific regulatory mechanisms (e.g. RNA interference (RNAi), transcriptional gene silencing (TGS), post-transcriptional gene silencing (PTGS), quelling, co-suppression, and translational repression) mediated by RNA molecules which result in the inhibition or "silencing" of the expression of a corresponding protein-coding gene. RNA silencing has been observed in many types of organisms, including plants, animals, and fungi.

[0176] The term "discriminatory RNA silencing" refers to the ability of an RNA molecule to substantially inhibit the expression of a "first" or "target" polynucleotide sequence while not substantially inhibiting the expression of a "second" or "non-target" polynucleotide sequence," e.g., when both polynucleotide sequences are present in the same cell. In certain embodiments, the target polynucleotide sequence corresponds to a target gene, while the non-target polynucleotide sequence corresponds to a non-target gene. In other embodiments, the target polynucleotide sequence corresponds to a target allele, while the non-target polynucleotide sequence corresponds to a non-target allele. In certain

embodiments, the target polynucleotide sequence is the DNA sequence encoding the regulatory region (e.g. promoter or enhancer elements) of a target gene. In other embodiments, the target polynucleotide sequence is a target mRNA encoded by a target gene.

[0177] The term "*in vitro*" has its art recognized meaning, e.g., involving purified reagents or extracts, e.g., cell extracts. The term "*in vivo*" also has its art recognized meaning, e.g., involving living cells, e.g., immortalized cells, primary cells, cell lines, and/or cells in an organism.

[0178] As used herein, the term "transgene" refers to any nucleic acid molecule, which is inserted by artifice into a cell, and becomes part of the genome of the organism that develops from the cell. Such a transgene may include a gene that is partly or entirely heterologous (i.e., foreign) to the transgenic organism, or may represent a gene homologous to an endogenous gene of the organism. The term "transgene" also means a nucleic acid molecule that includes one or more selected nucleic acid sequences, e.g., DNAs, that encode one or more engineered RNA precursors, to be expressed in a transgenic organism, e.g., animal, which is partly or entirely heterologous, i.e., foreign, to the transgenic animal, or homologous to an endogenous gene of the transgenic animal, but which is designed to be inserted into the animal's genome at a location which differs from that of the natural gene. A transgene includes one or more promoters and any other DNA, such as introns, necessary for expression of the selected nucleic acid sequence, all operably linked to the selected sequence, and may include an enhancer sequence.

[0179] A gene "involved" in a disease or disorder includes a gene, the normal or aberrant expression or function of which effects or causes the disease or disorder or at least one symptom of said disease or disorder.

[0180] The term "gain-of-function mutation" as used herein, refers to any mutation in a gene in which the protein encoded by said gene (i.e., the mutant protein) acquires a function not normally associated with the protein (i.e., the wild type protein) causes or contributes to a disease or disorder. The gain-of-function mutation can be a deletion, addition, or substitution of a nucleotide or nucleotides in the gene which gives rise to the change in the function of the encoded protein. In one embodiment, the gain-of-function mutation changes the function of the mutant protein or causes interactions with other proteins. In another embodiment, the gain-of-function mutation causes a decrease in or removal of normal wild-type protein, for example, by interaction of the altered, mutant protein with said normal, wild-type protein.

[0181] As used herein, the term "target gene" is a gene whose expression is to be substantially inhibited or "silenced." This silencing can be achieved by RNA silencing, e.g., by cleaving the mRNA of the target gene or translational repression of the target gene. The term "non-target gene" is a gene whose expression is not to be substantially silenced. In one embodiment, the polynucleotide sequences of the target and non-target gene (e.g. mRNA encoded by the target and non-target genes) can differ by one or more nucleotides. In another embodiment, the target and non-target genes can differ by one or more polymorphisms (e.g., Single Nucleotide Polymorphisms or SNPs). In another embodiment, the target and non-target genes can share less than 100% sequence identity. In another embodiment, the non-target gene may be a homologue (e.g. an orthologue or paralogue) of the target gene.

[0182] A "target allele" is an allele (e.g., a SNP allele) whose expression is to be selectively inhibited or "silenced." This silencing can be achieved by RNA silencing, e.g., by cleaving the mRNA of the target gene or target allele by a siRNA. The term "non-target allele" is a allele whose expression is not to be substantially silenced. In certain embodiments, the target and non-target alleles can correspond to the same target gene. In other embodiments, the target allele corresponds to, or is associated with, a target gene, and the non-target allele corresponds to, or is associated with, a non-target gene. In one embodiment, the polynucleotide sequences of the target and non-target alleles can differ by one or more nucleotides. In another embodiment, the target and non-target alleles can differ by one or more allelic polymorphisms (e.g., one or more SNPs). In another embodiment, the target and non-target alleles can share less than 100% sequence identity.

[0183] The term "polymorphism" as used herein, refers to a variation (e.g., one or more deletions, insertions, or substitutions) in a gene sequence that is identified or detected when the same gene sequence from different sources or subjects (but from the same organism) are compared. For example, a polymorphism can be identified when the same gene sequence from different subjects are compared. Identification of such polymorphisms is routine in the art, the methodologies being similar to those used to detect, for example, breast cancer point mutations. Identification can be made, for example, from DNA extracted from a subject's lymphocytes, followed by amplification of polymorphic regions using specific primers to said polymorphic region. Alternatively, the polymorphism can be identified when two alleles of the same gene are compared. In particular embodiments, the polymorphism is a single nucleotide polymorphism (SNP).

[0184] A variation in sequence between two alleles of the same gene within an organism is referred to herein as an "allelic polymorphism." In certain embodiments, the allelic polymorphism corresponds to a SNP allele. For example, the allelic polymorphism may comprise a single nucleotide variation between the two alleles of a SNP. The polymorphism can be at a nucleotide within a coding region but, due to the degeneracy of the genetic code, no change in amino acid sequence is encoded. Alternatively, polymorphic sequences can encode a different amino acid at a particular position, but the change in the amino acid does not affect protein function. Polymorphic regions can also be found in non-encoding regions of the gene. In exemplary embodiments, the polymorphism is found in a coding region of the gene or in an untranslated region (e.g., a 5' UTR or 3' UTR) of the gene.

[0185] As used herein, the term "allelic frequency" is a measure (e.g., proportion or percentage) of the relative frequency of an allele (e.g., a SNP allele) at a single locus in a population of individuals. For example, where a population of individuals carry n loci of a particular chromosomal locus (and the gene occupying the locus) in each of their somatic cells, then the allelic frequency of an allele is the fraction or percentage of loci that the allele occupies within the population. In particular embodiments, the allelic frequency of an allele (e.g., an SNP allele) is at least 10% (e.g., at least 15%, 20%, 25%, 30%, 35%, 40% or more) in a sample population.

[0186] As used herein, the term "sample population" refers to a population of individuals comprising a statistically significant number of individuals. For example, the sample population may comprise 50, 75, 100, 200, 500, 1000 or more individuals. In particular embodiments, the sample population may comprise individuals which share at least on common disease phenotype (e.g., a gain-of-function disorder) or mutation (e.g., a gain-of-function mutation).

[0187] As used herein, the term "heterozygosity" refers to the fraction of individuals within a population that are heterozygous (e.g., contain two or more different alleles) at a particular locus (e.g., at a SNP). Heterozygosity may be calculated for a sample population using methods that are well known to those skilled in the art.

[0188] The term "polyglutamine domain," as used herein, refers to a segment or domain of a protein that consist of a consecutive glutamine residues linked to peptide bonds. In one embodiment the consecutive region includes at least 5 glutamine residues.

[0189] The term "expanded polyglutamine domain" or "expanded polyglutamine segment," as used herein, refers to a segment or domain of a protein that includes at least 35 consecutive glutamine residues linked by peptide bonds. Such expanded segments are found in subjects afflicted with a polyglutamine disorder, as described herein, whether or not the subject has shown to manifest symptoms.

[0190] The term "trinucleotide repeat" or "trinucleotide repeat region" as used herein, refers to a segment of a nucleic acid sequence e.g.,) that consists of consecutive repeats of a particular trinucleotide sequence. In one embodiment, the trinucleotide repeat includes at least 5 consecutive trinucleotide sequences. Exemplary trinucleotide sequences include, but are not limited to, CAG, CGG, GCC, GAA, CTG and/or CGG.

[0191] The term "trinucleotide repeat diseases" as used herein, refers to any disease or disorder characterized by an expanded trinucleotide repeat region located within a gene, the expanded trinucleotide repeat region being causative of the disease or disorder. Examples of trinucleotide repeat diseases include, but are not limited to spino-cerebellar ataxia type 12 spino-cerebellar ataxia type 8, fragile X syndrome, fragile XE mental retardation, Friedreich's ataxia and myotonic dystrophy. Exemplary trinucleotide repeat diseases for treatment according to the present invention are those characterized or caused by an expanded trinucleotide repeat region at the 5' end of the coding region of a gene, the gene encoding a mutant protein which causes or is causative of the disease or disorder. Certain trinucleotide diseases, for example, fragile X syndrome, where the mutation is not associated with a coding region may not be suitable for treatment according to the methodologies of the present invention, as there is no suitable mRNA to be targeted by RNAi. By contrast, disease such as Friedreich's ataxia may be suitable for treatment according to the methodologies of the invention because, although the causative mutation is not within a coding region (i.e., lies within an intron), the mutation may be within, for example, an mRNA precursor (e.g., a pre-spliced mRNA precursor).

[0192] The term "polyglutamine disorder" as used herein, refers to any disease or disorder characterized by an expanded of a (CAG)n repeats at the 5' end of the coding region (thus encoding an expanded polyglutamine region in the encoded protein). In one embodiment, polyglutamine disorders are characterized by a progressive degeneration of nerve cells. Examples of polyglutamine disorders include but are not limited to: Huntington's disease, spino-cerebellar ataxia type 1, spino-cerebellar ataxia type 2, spino-cerebellar ataxia

type 3 (also known as Machado-Joseph disease), and spino-cerebellar ataxia type 6, spino-cerebellar ataxia type 7 and dentatoiubral-pallidoluysian atrophy.

[0193] The phrase "examining the function of a gene in a cell or organism" refers to examining or studying the expression, activity, function or phenotype arising therefrom.

[0194] As used herein, the term "RNA silencing agent" refers to an RNA which is capable of inhibiting or "silencing" the expression of a target gene. In certain embodiments, the RNA silencing agent is capable of preventing complete processing (e.g., the full translation and/or expression) of a mRNA molecule through a post-transcriptional silencing mechanism. RNA silencing agents include small (<50 b.p.), noncoding RNA molecules, for example RNA duplexes comprising paired strands, as well as precursor RNAs from which such small non-coding RNAs can be generated. Exemplary RNA silencing agents include siRNAs, miRNAs, siRNA-like duplexes, antisense oligonucleotides, GAPMER molecules, and dual-function oligonucleotides as well as precursors thereof. In one embodiment, the RNA silencing agent is capable of inducing RNA interference. In another embodiment, the RNA silencing agent is capable of mediating translational repression.

[0195] As used herein, the term "rare nucleotide" refers to a naturally occurring nucleotide that occurs infrequently, including naturally occurring deoxyribonucleotides or ribonucleotides that occur infrequently, e.g., a naturally occurring ribonucleotide that is not guanosine, adenosine, cytosine, or uridine. Examples of rare nucleotides include, but are not limited to, inosine, 1-methyl inosine, pseudouridine, 5,6-dihydrouridine, ribothymidine, ^{2}N-methylguanosine and ^{2,2}N,N-dimethylguanosine.

[0196] The term "engineered," as in an engineered RNA precursor, or an engineered nucleic acid molecule, indicates that the precursor or molecule is not found in nature, in that all or a portion of the nucleic acid sequence of the precursor or molecule is created or selected by a human. Once created or selected, the sequence can be replicated, translated, transcribed, or otherwise processed by mechanisms within a cell. Thus, an RNA precursor produced within a cell from a transgene that includes an engineered nucleic acid molecule is an engineered RNA precursor.

[0197] As used herein, the term "microRNA" ("miRNA"), also referred to in the art as "small temporal RNAs" ("stRNAs"), refers to a small (10-50 nucleotide) RNA which are genetically encoded (e.g., by viral, mammalian, or plant genomes) and are capable of

directing or mediating RNA silencing. An "miRNA disorder" shall refer to a disease or disorder characterized by an aberrant expression or activity of an miRNA.

[0198] As used herein, the term "dual functional oligonucleotide" refers to a RNA silencing agent having the formula T-L-μ, wherein T is an mRNA targeting moiety, L is a linking moiety, and μ is a miRNA recruiting moiety. As used herein, the terms "mRNA targeting moiety," "targeting moiety," "mRNA targeting portion" or "targeting portion" refer to a domain, portion or region of the dual functional oligonucleotide having sufficient size and sufficient complementarity to a portion or region of an mRNA chosen or targeted for silencing (i.e., the moiety has a sequence sufficient to capture the target mRNA). As used herein, the term "linking moiety" or "linking portion" refers to a domain, portion or region of the RNA-silencing agent which covalently joins or links the mRNA.

[0199] As used herein, the term "antisense strand" of an RNA silencing agent, e.g., an siRNA or RNA silencing agent, refers to a strand that is substantially complementary to a section of about 10-50 nucleotides, e.g., about 15-30, 16-25, 18-23 or 19-22 nucleotides of the mRNA of the gene targeted for silencing. The antisense strand or first strand has sequence sufficiently complementary to the desired target mRNA sequence to direct target-specific silencing, e.g., complementarity sufficient to trigger the destruction of the desired target mRNA by the RNAi machinery or process (RNAi interference) or complementarity sufficient to trigger translational repression of the desired target mRNA.

[0200] The term "sense strand" or "second strand" of an RNA silencing agent, e.g., an siRNA or RNA silencing agent, refers to a strand that is complementary to the antisense strand or first strand. Antisense and sense strands can also be referred to as first or second strands, the first or second strand having complementarity to the target sequence and the respective second or first strand having complementarity to said first or second strand. miRNA duplex intermediates or siRNA-like duplexes include a miRNA strand having sufficient complementarity to a section of about 10-50 nucleotides of the mRNA of the gene targeted for silencing and a miRNA* strand having sufficient complementarity to form a duplex with the miRNA strand.

[0201] As used herein, the term "guide strand" refers to a strand of an RNA silencing agent, e.g., an antisense strand of an siRNA duplex or siRNA sequence, that enters into the RISC complex and directs cleavage of the target mRNA.

[0202] As used herein, the term "asymmetry," as in the asymmetry of the duplex region of an RNA silencing agent (e.g., the stem of an shRNA), refers to an inequality of bond strength or base pairing strength between the termini of the RNA silencing agent (e.g., between terminal nucleotides on a first strand or stem portion and terminal nucleotides on an opposing second strand or stem portion), such that the 5' end of one strand of the duplex is more frequently in a transient unpaired, e.g., single-stranded, state than the 5' end of the complementary strand. This structural difference determines that one strand of the duplex is preferentially incorporated into a RISC complex. The strand whose 5' end is less tightly paired to the complementary strand will preferentially be incorporated into RISC and mediate RNAi.

[0203] As used herein, the term "bond strength" or "base pair strength" refers to the strength of the interaction between pairs of nucleotides (or nucleotide analogs) on opposing strands of an oligonucleotide duplex (e.g., an siRNA duplex), due primarily to H-bonding, van der Waals interactions, and the like between said nucleotides (or nucleotide analogs).

[0204] As used herein, the "5' end," as in the 5' end of an antisense strand, refers to the 5' terminal nucleotides, e.g., between one and about 5 nucleotides at the 5' terminus of the antisense strand. As used herein, the "3' end," as in the 3' end of a sense strand, refers to the region, e.g., a region of between one and about 5 nucleotides, that is complementary to the nucleotides of the 5' end of the complementary antisense strand.

[0205] As used herein the term "destabilizing nucleotide" refers to a first nucleotide or nucleotide analog capable of forming a base pair with second nucleotide or nucleotide analog such that the base pair is of lower bond strength than a conventional base pair (i.e., Watson-Crick base pair). In certain embodiments, the destabilizing nucleotide is capable of forming a mismatch base pair with the second nucleotide. In other embodiments, the destabilizing nucleotide is capable of forming a wobble base pair with the second nucleotide. In yet other embodiments, the destabilizing nucleotide is capable of forming an ambiguous base pair with the second nucleotide.

[0206] As used herein, the term "base pair" refers to the interaction between pairs of nucleotides (or nucleotide analogs) on opposing strands of an oligonucleotide duplex (e.g., a duplex formed by a strand of a RNA silencing agent and a target mRNA sequence), due primarily to H-bonding, van der Waals interactions, and the like between said nucleotides (or

nucleotide analogs). As used herein, the term "bond strength" or "base pair strength" refers to the strength of the base pair.

[0207] As used herein, the term "mismatched base pair" refers to a base pair consisting of non-complementary or non-Watson-Crick base pairs, for example, not normal complementary G:C, A:T or A:U base pairs. As used herein the term "ambiguous base pair" (also known as a non-discriminatory base pair) refers to a base pair formed by a universal nucleotide.

[0208] As used herein, term "universal nucleotide" (also known as a "neutral nucleotide") include those nucleotides (e.g. certain destabilizing nucleotides) having a base (a "universal base" or "neutral base") that does not significantly discriminate between bases on a complementary polynucleotide when forming a base pair. Universal nucleotides are predominantly hydrophobic molecules that can pack efficiently into antiparallel duplex nucleic acids (e.g., double-stranded DNA or RNA) due to stacking interactions. The base portion of universal nucleotides typically comprise a nitrogen-containing aromatic heterocyclic moiety.

[0209] As used herein, the terms "sufficient complementarity" or "sufficient degree of complementarity" mean that the RNA silencing agent has a sequence (e.g. in the antisense strand, mRNA targeting moiety or miRNA recruiting moiety) which is sufficient to bind the desired target RNA, respectively, and to trigger the RNA silencing of the target mRNA.

[0210] As used herein, the term "translational repression" refers to a selective inhibition of mRNA translation. Natural translational repression proceeds via miRNAs cleaved from shRNA precursors. Both RNAi and translational repression are mediated by RISC. Both RNAi and translational repression occur naturally or can be initiated by the hand of man, for example, to silence the expression of target genes.

[0211] Various methodologies of the instant invention include step that involves comparing a value, level, feature, characteristic, property, etc. to a "suitable control," referred to interchangeably herein as an "appropriate control." A "suitable control" or "appropriate control" is any control or standard familiar to one of ordinary skill in the art useful for comparison purposes. In one embodiment, a "suitable control" or "appropriate control" is a value, level, feature, characteristic, property, etc. determined prior to performing an RNAi methodology, as described herein. For example, a transcription rate, mRNA level, translation rate, protein level, biological activity, cellular characteristic or property, genotype,

phenotype, etc. can be determined prior to introducing an RNA silencing agent of the invention into a cell or organism. In another embodiment, a "suitable control" or "appropriate control" is a value, level, feature, characteristic, property, etc. determined in a cell or organism, e.g., a control or normal cell or organism, exhibiting, for example, normal traits. In yet another embodiment, a "suitable control" or "appropriate control" is a predefined value, level, feature, characteristic, property, etc.

[0212] Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, suitable methods and materials are described below. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including definitions, will control. In addition, the materials, methods, and example are illustrative only and not intended to be limiting.

[0213] Various aspects of the invention are described in further detail in the following subsections.

I. Polyglutamine Disorders

[0214] Polyglutamine disorders are a class of disease or disorders characterized by a common genetic mutation. In particular, the disease or disorders are characterized by an expanded repeat of the trinucleotide CAG which gives rise, in the encoded protein, to an expanded stretch of glutamine residues. Polyglutamine disorders are similar in that the diseases are characterized by a progressive degeneration of nerve cells. Despite their similarities, polyglutamine disorders occur on different chromosomes and thus occur on entirely different segments of DNA. Examples of polyglutamine disorders include Huntington's disease, Dentatorubropallidoluysian Atrophy, Spinobulbar Muscular atrophy, Spinocerebellar Ataxia Type 1, Spinocerebellar Ataxia Type 2, Spinocerebellar Ataxia Type 3, Spinocerebellar Ataxia Type 6 and Spinocerebellar Ataxia Type 7.

[0215] Polyglutamine disorders of the invention are characterized by, e.g., domains having between about 30 to 35 glutamine residues, between about 35 to 40 glutamine residues, between about 40 to 45 glutamine residues or having about 45 or more glutamine residues. The polyglutamine domain typically contains consecutive glutamine residues (Q n>36).

II. Huntington Disease

[0216] In some embodiments, the RNA silencing agents of the invention are designed to target polymorphisms (e.g. single nucleotide polymorphisms) in the mutant human huntingtin protein (htt) for the treatment of Huntington's disease.

[0217] Huntington's disease, inherited as an autosomal dominant disease, causes impaired cognition and motor disease. Patients can live more than a decade with severe debilitation, before premature death from starvation or infection. The disease begins in the fourth or fifth decade for most cases, but a subset of patients manifest disease in teenage years. The genetic mutation for Huntington's disease is a lengthened CAG repeat in the huntingtin gene. CAG repeats vary in number from 8 to 35 in normal individuals (Kremer et al., 1994). The genetic mutation e.g., an increase in length of the CAG repeats from normal (less than 36 in the huntingtin gene to greater than 36 in the disease) is associated with the synthesis of a mutant Huntingtin protein, which has greater than 36 polyglutamates (Aronin et al., 1995). In general, individuals with 36 or more CAG repeats will develop Huntington's disease. Prototypic for as many as twenty other diseases with a lengthened CAG as the underlying mutation, Huntington's disease still has no effective therapy. A variety of interventions, such as interruption of apoptotic pathways, addition of reagents to boost mitochondrial efficiency, and blockade of NMDA receptors, have shown promise in cell cultures and mouse model of Huntington's disease. However, at best these approaches reveal a short prolongation of cell or animal survival.

[0218] Huntington's disease complies with the central dogma of genetics: a mutant gene serves as a template for production of a mutant mRNA; the mutant mRNA then directs synthesis of a mutant protein (Aronin et al., 1995; DiFiglia et al., 1997). Without intending to be bound by scientific theory, it is thought that mutant huntingtin protein accumulates in selective neurons in the striatum and cortex, disrupts as yet determined cellular activities, and causes neuronal dysfunction and death (Aronin et al., 1999; Laforet et al., 2001). Because a single copy of a mutant gene suffices to cause Huntington's disease, the most parsimonious treatment would render the mutant gene ineffective. Theoretical approaches might include stopping gene transcription of mutant huntingtin, destroying mutant mRNA, and blocking translation. Each has the same outcome: loss of mutant huntingtin.

III. Huntingtin Gene

[0219] The disease gene linked to Huntington's disease is termed Huntingtin or (*htt*). The huntingtin locus is large, spanning 180 kb and consisting of 67 exons. The huntingtin gene is widely expressed and is required for normal development. It is expressed as 2 alternatively polyadenylated forms displaying different relative abundance in various fetal and adult tissues. The larger transcript is approximately 13.7 kb and is expressed predominantly in adult and fetal brain whereas the smaller transcript of approximately 10.3 kb is more widely expressed. The two transcripts differ with respect to their 3' untranslated regions (Lin et al., 1993). Both messages are predicted to encode a 348 kilodalton protein containing 3144 amino acids. The genetic defect leading to Huntington's disease is believed to confer a new property on the mRNA or alter the function of the protein.

[0220] The present invention targets huntingtin (e.g., wild-type and/or mutant huntingtin) using RNA interference (Hutvagner et al., 2002). One strand of double-stranded RNA (siRNA) complements a target sequence within the huntingtin mRNA. After introduction of siRNA into neurons, the siRNA partially unwinds, binds to polymorphic region within the huntingtin mRNA in a site-specific manner, and activates an mRNA nuclease. This nuclease cleaves the huntingtin mRNA, thereby halting translation of the huntingtin (e.g., wild-type and/or mutant huntingtin). Cells rid themselves of partially digested mRNA, thus precluding translation, or cells digest partially translated proteins. In certain embodiments, neurons survive on the wild-type huntingtin from the normal allele, preventing the ravages of mutant huntingtin by eliminating its production.

[0221] In embodiments of the invention, RNA silencing agents of the invention are capable of targeting one or more of the target sequences listed in Figure 8. In certain exemplary embodiments, RNA silencing agents of the invention are capable of targeting one or more of the target sequences at one or more target sequences listed at gene positions selected from the group consisting of 1214, 1218, 1219, 1257, 1894, 1907, 2866, 4041, 4049, 5301, 6016, 6579, 8603, 10125, 10146, 10150, 424, 456, 522, 527, 878, 879, 908, 1024, 1165, 1207, 1212, 1217, 1220, 1223, 1227, 1229, 1260, 1403, 1470, 1901, 1903, 2411, 2412, 2865, 3801, 4040, 4048, 4052, 4055, 4083, 4275, 4372, 4374, 4376, 4425, 4562, 4692, 4721, 5200, 5443, 5515, 8609, 10130, 10134, 10142, 10169, 10182, 10186, 10809, 11116, 11129, 11134, 11147, 11412, 11426, 11443, 11659, 11666, 11677, 11863, 11890, 11927, 11947, 12163, 12218, 12223, 12235, 12279, 12282, 12297, 12309, 12313, 12331, 13136, 13398, 13403, 13423, 13428 of the human *htt* gene (as set forth at Figure 8). In certain exemplary embodiments, RNA silencing agents of the invention are capable of targeting one or more of

the target sequences at one or more target sequences listed at gene positions selected from the group consisting of 5301, 10125, 10146, 10150, 424, 878, 879, 4083, 4275, 4562, 4721, 5200, 10130, 10134, 10142, 11116, 11129, 11134, 11147, 11412, 11426, 11443, 11659, 11666, 11677, 11863, 11890, 11927, 11947, 12163, 12218, 12223, 12235, 12279, 12282, 12297, 12331, 13136, 13423 and 13428 of the human *htt* gene (as set forth at Figure 8). Particularly exemplary target sequences of the human *htt* gene can be found at positions 10150 (5' CAGUAAAGAGAUUAA 3' (SEQ ID NO:1)), 10146 (5' AUAUCAGUAAAGAGA 3' (SEQ ID NO:2)) and 10125 (5' CUCAGGAUUUAAAAU 3' (SEQ ID NO:3)). Genomic sequence for each target sequence can be found in, for example, the publically available database maintained by the NCBI.

[0222] In certain exemplary embodiments, RNA silencing agents of the invention that are capable of targeting one or more of the target sequences at one or more target sequences are set forth in Table 1, below, and in Figure 21 (which also includes exemplary modifications).

GCUGCCGGGA	Accession Number	Position	Targeting region (20 mer)	Targeting Region (30 mer)
HTT	NM_002111 .6	1214	GUCCAGGUUUAUGAACUGAC	AGCUUGUCCAGGUUUAUGAACUGACGUUAC
HTT	NM_002111 .6	1218	AGGUUUAUGAACUGACGUUA	UGUCCAGGUUUAUGAACUGACGUUACAUCA
HTT	NM_002111 .6	1219	GGUUUAUGAACUGACGUUAC	GUCCAGGUUUAUGAACUGACGUUACAUCAU
HTT	NM_002111 .6	1257	ACCACAAUGUUGUGACCGGA	CCAAGACCACAAUGUUGUGACCGGAGCCCU
HTT	NM_002111 .6	1894	UGUGUUAGACGGUACCGACA	GAAAUUGUGUUAGACGGUACCGACAACCAG
HTT	NM_002111 .6	1907	ACCGACAACCAGUAUUUGGG	ACGGUACCGACAACCAGUAUUUGGGCCUGC
HTT	NM_002111 .6	2866	ACGAGUGCUCAAUAAUGUUG	CAAGAACGAGUGCUCAAUAAUGUUGUCAUC
HTT	NM_002111 .6	4041	UGAAAUCCUGCUUUAGUCGA	AUACCUGAAAUCCUGCUUUAGUCGAGAACC
HTT	NM_002111 .6	4049	UGCUUUAGUCGAGAACCAAU	AAUCCUGCUUUAGUCGAGAACCAAUGAUGG
HTT	NM_002111 .6	5301	GGGACAGUACUUCAACGCUA	AGAUGGGGACAGUACUUCAACGCUAGAAGA
HTT	NM_002111 .6	6016	GGCAAUUCAGUCUCGUUGUG	AUCCAGGCAAUUCAGUCUCGUUGUGAAAAC
HTT	NM_002111 .6	6579	GCCUGCUAGCUCCAUGCUUA	CCUAAGCCUGCUAGCUCCAUGCUUAAGCCU
HTT	NM_002111 .6	8603	GCCCACUGCGUGAACAUUCA	GGAUCGCCCACUGCGUGAACAUUCACAGCC
HTT	NM_002111 .6	10125	UUCUUCUCAGGAUUUAAAAU	CUCUUUUCUUCUCAGGAUUUAAAAUUUAAU
HTT	NM_002111 .6	10146	UAAUUAUAUCAGUAAAGAGA	AAAUUUAAUUAUAUCAGUAAAGAGAUUAAU
HTT	NM_002111 .6	10150	UAUAUCAGUAAAGAGAUUAA	UUAAUUAUAUCAGUAAAGAGAUUAAUUUUA
HTT	NM_002111 .6	424	ACUUUCAGCUACCAAGAAAG	AAAGAACUUUCAGCUACCAAGAAAGACCGU
HTT	NM_002111 .6	456	AUUGUCUGACAAUAUGUGAA	GAAUCAUUGUCUGACAAUAUGUGAAAACAU
HTT	NM_002111 .6	522	UUCUGGGCAUCGCUAUGGAA	
HTT	NM_002111 .6	527	GGCAUCGCUAUGGAACUUUU	UUCUGGGCAUCGCUAUGGAACUUUUUCUGC
HTT	NM_002111 .6	878	GCAAAUGACAAUGAAAUUAA	AUUUUGCAAAUGACAAUGAAAUUAAGGUUU
HTT	NM_002111 .6	879	CAAAUGACAAUGAAAUUAAG	UUUUGCAAAUGACAAUGAAAUUAAGGUUUU
HTT	NM_002111 .6	908	AAGGCCUUCAUAGCGAACCU	UGUUAAAGGCCUUCAUAGCGAACCUGAAGU
HTT	NM_002111 .6	1024	ACUAAAUGUGCUCUUAGGCU	UGGCUACUAAAUGUGCUCUUAGGCUUACUC
HTT	NM_002111 .6	1165	CGGAGUGACAAGGAAAGAAA	AGCUUCGGAGUGACAAGGAAAGAAAUGGAA
HTT	NM_002111 .6	1207	GCAGCUUGUCCAGGUUUAUG	GCAGAGCAGCUUGUCCAGGUUUAUGAACUG
HTT	NM_002111 .6	1212	UUGUCCAGGUUUAUGAACUG	GCAGCUUGUCCAGGUUUAUGAACUGACGUU
HTT	NM_002111 .6	1217	CAGGUUUAUGAACUGACGUU	UUGUCCAGGUUUAUGAACUGACGUUACAUC
HTT	NM_002111 .6	1220	GUUUAUGAACUGACGUUACA	UCCAGGUUUAUGAACUGACGUUACAUCAUA
HTT	NM_002111 .6	1223	UAUGAACUGACGUUACAUCA	AGGUUUAUGAACUGACGUUACAUCAUACAC
HTT	NM_002111 .6	1227	AACUGACGUUACAUCAUACA	UUAUGAACUGACGUUACAUCAUACACAGCA
HTT	NM_002111 .6	1229	CUGACGUUACAUCAUACACA	AUGAACUGACGUUACAUCAUACACAGCACC
HTT	NM_002111 .6	1260	ACAAUGUUGUGACCGGAGCC	AGACCACAAUGUUGUGACCGGAGCCCUGGA
HTT	NM_002111 .6	1403	GGGAGUAUUGUGGAACUUAU	GUAGUGGGAGUAUUGUGGAACUUAUAGCUG
HTT	NM_002111 .6	1470	AAGGCAAAGUGCUCUUAGGA	ACAAAAAGGCAAAGUGCUCUUAGGAGAAGA
HTT	NM_002111 .6	1901	GACGGUACCGACAACCAGUA	UGUUAGACGGUACCGACAACCAGUAUUUGG
HTT	NM_002111 .6	1903	CGGUACCGACAACCAGUAUU	UUAGACGGUACCGACAACCAGUAUUUGGGC
HTT	NM_002111 .6	2411	UUGAACUACAUCGAUCAUGG	ACAUCUUGAACUACAUCGAUCAUGGAGACC
HTT	NM_002111 .6	2412	UGAACUACAUCGAUCAUGGA	CAUCUUGAACUACAUCGAUCAUGGAGACCC
HTT	NM_002111 .6	2865	AACGAGUGCUCAAUAAUGUU	GCAAGAACGAGUGCUCAAUAAUGUUGUCAU
HTT	NM_002111 .6	3801	GUCCUGUUACAACAAGUAAA	CUCAGGUCCUGUUACAACAAGUAAAUCCUC
HTT	NM_002111 .6	4040	CUGAAAUCCUGCUUUAGUCG	GAUACCUGAAAUCCUGCUUUAGUCGAGAAC
HTT	NM_002111 .6	4048	CUGCUUUAGUCGAGAACCAA	AAAUCCUGCUUUAGUCGAGAACCAAUGAUG
HTT	NM_002111 .6	4052	UUUAGUCGAGAACCAAUGAU	CCUGCUUUAGUCGAGAACCAAUGAUGGCAA
HTT	NM_002111 .6	4055	AGUCGAGAACCAAUGAUGGC	GCUUUAGUCGAGAACCAAUGAUGGCAACUG
HTT	NM_002111 .6	4083	GUGUUCAACAAUUGUUGAAG	UGUUUGUGUUCAACAAUUGUUGAAGACUCU
HTT	NM_002111 .6	4275	UGAGGAACAUGGUGCAGGCG	CAGCCUGAGGAACAUGGUGCAGGCGGAGCA
HTT	NM_002111 .6	4372	UGUCACAAAGAACCGUGCAG	ACGAGUGUCACAAAGAACCGUGCAGAUAAG
HTT	NM_002111 .6	4374	UCACAAAGAACCGUGCAGAU	GAGUGUCACAAAGAACCGUGCAGAUAAGAA

HTT	NM_002111.6	4376	ACAAAGAACCGUGCAGAUAA	GUGUCACAAAGAACCGUGCAGAUAAGAAUG
HTT	NM_002111.6	4425	UUGAACCUCUUGUUAUAAAA	UUUGUUUGAACCUCUUGUUAUAAAAGCUUU
HTT	NM_002111.6	4562	UUUAUUGGCUUUGUAUUGAA	AGGUGUUUAUUGGCUUUGUAUUGAAACAGU
HTT	NM_002111.6	4692	UCAUUGGAAUUCCUAAAAUC	ACAGAUCAUUGGAAUUCCUAAAAUCAUUCA
HTT	NM_002111.6	4721	UGUGAUGGCAUCAUGGCCAG	AGCUCUGUGAUGGCAUCAUGGCCAGUGGAA
HTT	NM_002111.6	5200	GAUUUCCCAGUCAACUGAAG	GUUCUGAUUUCCCAGUCAACUGAAGAUAUU
HTT	NM_002111.6	5443	GAGUGAGCAGCAACAUACUU	GAAAUGAGUGAGCAGCAACAUACUUUCUAU
HTT	NM_002111.6	5515	GUCUGGAAUGUUCCGGAGAA	UUCAAGUCUGGAAUGUUCCGGAGAAUCACA
HTT	NM_002111.6	8609	UGCGUGAACAUUCACAGCCA	CCCACUGCGUGAACAUUCACAGCCAGCAGC
HTT	NM_002111.6	10130	CUCAGGAUUUAAAAUUUAAU	UUCUUCUCAGGAUUUAAAAUUUAAUUAUAU
HTT	NM_002111.6	10134	GGAUUUAAAAUUUAAUUAUA	UCUCAGGAUUUAAAAUUUAAUUAUAUCAGU
HTT	NM_002111.6	10142	AAUUUAAUUAUAUCAGUAAA	UUUAAAAUUUAAUUAUAUCAGUAAAGAGAU
HTT	NM_002111.6	10169	AUUUUAACGUAACUCUUUCU	GAUUAAUUUUAACGUAACUCUUUCUAUGCC
HTT	NM_002111.6	10182	UCUUUCUAUGCCCGUGUAAA	GUAACUCUUUCUAUGCCCGUGUAAAGUAUG
HTT	NM_002111.6	10186	UCUAUGCCCGUGUAAAGUAU	CUCUUUCUAUGCCCGUGUAAAGUAUGUGAA
HTT	NM_002111.6	10809	CUUUUAGUCAGGAGAGUGCA	GACCCCUUUUAGUCAGGAGAGUGCAGAUCU
HTT	NM_002111.6	11116	UGUUUUGGGUAUUGAAUGUG	GUCGAUGUUUUGGGUAUUGAAUGUGGUAAG
HTT	NM_002111.6	11129	GAAUGUGGUAAGUGGAGGAA	GUAUUGAAUGUGGUAAGUGGAGGAAAUGUU
HTT	NM_002111.6	11134	UGGUAAGUGGAGGAAAUGUU	GAAUGUGGUAAGUGGAGGAAAUGUUGGAAC
HTT	NM_002111.6	11147	AAAUGUUGGAACUCUGUGCA	GGAGGAAAUGUUGGAACUCUGUGCAGGUGC
HTT	NM_002111.6	11412	AUGUUUGAGGAGGCCCUUAA	GUCCGAUGUUUGAGGAGGCCCUUAAGGGAA
HTT	NM_002111.6	11426	CCUUAAGGGAAGCUACUGAA	GAGGCCCUUAAGGGAAGCUACUGAAUUAUA
HTT	NM_002111.6	11443	GAAUUAUAACACGUAAGAAA	CUACUGAAUUAUAACACGUAAGAAAAUCAC
HTT	NM_002111.6	11659	AUGUUUACAUUUGUAAGAAA	GCUAGAUGUUUACAUUUGUAAGAAAUAACA
HTT	NM_002111.6	11666	CAUUUGUAAGAAAUAACACU	GUUUACAUUUGUAAGAAAUAACACUGUGAA
HTT	NM_002111.6	11677	AAUAACACUGUGAAUGUAAA	UAAGAAAUAACACUGUGAAUGUAAAACAGA
HTT	NM_002111.6	11863	AAUAUGAGCUCAUUAGUAAA	AGAUGAAUAUGAGCUCAUUAGUAAAAAUGA
HTT	NM_002111.6	11890	UCACCCACGCAUAUACAUAA	UGACUUCACCCACGCAUAUACAUAAAGUAU
HTT	NM_002111.6	11927	AUAUAGACACAUCUAUAAUU	UGUGCAUAUAGACACAUCUAUAAUUUUACA
HTT	NM_002111.6	11947	UUACACACACACCUCUCAAG	UAAUUUUACACACACACCUCUCAAGACGGA
HTT	NM_002111.6	12163	GACUUUAUCAUGUUCCUAAA	AGGAAGACUUUAUCAUGUUCCUAAAAAUCU
HTT	NM_002111.6	12218	UUGUUGCAAAUGUGAUUAAU	AAAUUUUGUUGCAAAUGUGAUUAAUUUGGU
HTT	NM_002111.6	12223	GCAAAUGUGAUUAAUUUGGU	UUGUUGCAAAUGUGAUUAAUUUGGUUGUCA
HTT	NM_002111.6	12235	AAUUUGGUUGUCAAGUUUUG	UGAUUAAUUUGGUUGUCAAGUUUUGGGGGU
HTT	NM_002111.6	12279	UUUGUUUUCCUGCUGGUAAU	UUGCUUUUGUUUUCCUGCUGGUAAUAUCGG
HTT	NM_002111.6	12282	GUUUUCCUGCUGGUAAUAUC	CUUUUGUUUUCCUGCUGGUAAUAUCGGGAA
HTT	NM_002111.6	12297	AUAUCGGGAAAGAUUUUAAU	UGGUAAUAUCGGGAAAGAUUUUAAUGAAAC
HTT	NM_002111.6	12309	AUUUUAAUGAAACCAGGGUA	GAAAGAUUUUAAUGAAACCAGGGUAGAAUU
HTT	NM_002111.6	12313	UAAUGAAACCAGGGUAGAAU	GAUUUUAAUGAAACCAGGGUAGAAUUGUUU
HTT	NM_002111.6	12331	AUUGUUUGGCAAUGCACUGA	GUAGAAUUGUUUGGCAAUGCACUGAAGCGU
HTT	NM_002111.6	13136	CCCCUCAGUUGUUUCUAAGA	GCCUUCCCCUCAGUUGUUUCUAAGAGCAGA
HTT	NM_002111.6	13398	GGACUGACGAGAGAUGUAUA	GGGAAGGACUGACGAGAGAUGUAUAUUUAA
HTT	NM_002111.6	13403	GACGAGAGAUGUAUAUUUAA	GGACUGACGAGAGAUGUAUAUUUAAUUUU
HTT	NM_002111.6	13423	UUUUUUAACUGCUGCAAACA	UUUAAUUUUUUAACUGCUGCAAACAUUGUA
HTT	NM_002111.6	13428	UAACUGCUGCAAACAUUGUA	UUUUUUAACUGCUGCAAACAUUGUACAUCC
HTT	NM_002111.6	152	ACCCUGGAAAAGCUGAUGAA	UGGCGACCCUGGAAAAGCUGAUGAAGGCCU
HTT	NM_002111.6	170	AAGGCCUUCGAGUCCCUCAA	UGAUGAAGGCCUUCGAGUCCCUCAAGUCCU
HTT	NM_002111.6	402	CGCUGCACCGACCAAAGAAA	GGAGCCGCUGCACCGACCAAAGAAAGAACU
HTT	NM_002111.6	420	AAGAACUUUCAGCUACCAAG	AAAGAAAGAACUUUCAGCUACCAAGAAAGA
HTT	NM_002111.6	430	AGCUACCAAGAAAGACCGUG	CUUUCAGCUACCAAGAAAGACCGUGUGAAU
HTT	NM_002111.6	446	CGUGUGAAUCAUUGUCUGAC	AAGACCGUGUGAAUCAUUGUCUGACAAUAU
HTT	NM_002111.6	454	UCAUUGUCUGACAAUAUGUG	GUGAAUCAUUGUCUGACAAUAUGUGAAAAC
HTT	NM_002111.6	462	UGACAAUAUGUGAAAACAUA	UUGUCUGACAAUAUGUGAAAACAUAGUGGC
HTT	NM_002111.6	467	AUAUGUGAAAACAUAGUGGC	UGACAAUAUGUGAAAACAUAGUGGCACAGU
HTT	NM_002111.6	211	GCAGCAGCAGCAGCAGCAGC	CAGCAGCAGCAGCAGCAGCAGCAGCAG

[0223] Table 1. Additional target sequences according to certain embodiments of the invention.

IV. siRNA Design

[0224] In some embodiments, siRNAs are designed as follows. First, a portion of the target gene (e.g., the *htt* gene), e.g., one or more of the target sequences set forth at Figure 8, is selected, e.g., 10150, 10146 and/or 10125 from the 5' untranslated region of a target gene. Cleavage of mRNA at these sites should eliminate translation of corresponding mutant protein. Sense strands were designed based on the target sequence. (See Figure 8.) Preferably the portion (and corresponding sense strand) includes about 19 to 25 nucleotides, e.g., 19, 20, 21, 22, 23, 24 or 25 nucleotides. More preferably, the portion (and corresponding sense strand) includes 21, 22 or 23 nucleotides. The skilled artisan will appreciate, however, that siRNAs having a length of less than 19 nucleotides or greater than 25 nucleotides can also function to mediate RNAi. Accordingly, siRNAs of such length are also within the scope of the instant invention provided that they retain the ability to mediate RNAi. Longer RNAi agents have been demonstrated to elicit an interferon or PKR response in certain mammalian cells which may be undesirable. Preferably, the RNAi agents of the invention do not elicit a PKR response (i.e., are of a sufficiently short length). However, longer RNAi agents may be useful, for example, in cell types incapable of generating a PRK response or in situations where the PKR response has been down-regulated or dampened by alternative means.

[0225] The sense strand sequence is designed such that the target sequence is essentially in the middle of the strand. Moving the target sequence to an off-center position may, in some instances, reduce efficiency of cleavage by the siRNA. Such compositions, i.e., less efficient compositions, may be desirable for use if off-silencing of the wild-type mRNA is detected.

[0226] The antisense strand is routinely the same length as the sense strand and includes complementary nucleotides. In one embodiment, the strands are fully complementary, i.e., the strands are blunt-ended when aligned or annealed. In another embodiment, the strands comprise align or anneal such that 1-, 2-, 3-, 4-, 5-, 6- or 7-nucleotide overhangs are generated, i.e., the 3' end of the sense strand extends 1, 2, 3, 4, 5, 6 or 7 nucleotides further than the 5' end of the antisense strand and/or the 3' end of the antisense strand extends 1, 2, 3, 4, 5, 6 or 7 nucleotides further than the 5' end of the sense strand. Overhangs can comprise (or consist of) nucleotides corresponding to the target gene sequence (or complement thereof). Alternatively, overhangs can comprise (or consist of) deoxyribonucleotides, for example dTs, or nucleotide analogs, or other suitable non-nucleotide material.

[0227] To facilitate entry of the antisense strand into RISC (and thus increase or improve the efficiency of target cleavage and silencing), the base pair strength between the 5' end of the sense strand and 3' end of the antisense strand can be altered, e.g., lessened or reduced, as described in detail in U.S. Patent Nos. 7,459,547, 7,772,203 and 7,732,593, entitled "Methods and Compositions for Controlling Efficacy of RNA Silencing" (filed Jun. 2, 2003) and U.S. Patent Nos. 8,309,704, 7,750,144, 8,304,530, 8,329,892 and 8,309,705, entitled "Methods and Compositions for Enhancing the Efficacy and Specificity of RNAi" (filed Jun. 2, 2003), the contents of which are incorporated in their entirety by this reference. In one embodiment of these aspects of the invention, the base-pair strength is less due to fewer G:C base pairs between the 5' end of the first or antisense strand and the 3' end of the second or sense strand than between the 3' end of the first or antisense strand and the 5' end of the second or sense strand. In another embodiment, the base pair strength is less due to at least one mismatched base pair between the 5' end of the first or antisense strand and the 3' end of the second or sense strand. In certain exemplary embodiments, the mismatched base pair is selected from the group consisting of G:A, C:A, C:U, G:G, A:A, C:C and U:U. In another embodiment, the base pair strength is less due to at least one wobble base pair, e.g., G:U, between the 5' end of the first or antisense strand and the 3' end of the second or sense strand. In another embodiment, the base pair strength is less due to at least one base pair comprising a rare nucleotide, e.g., inosine (I). In certain exemplary embodiments, the base pair is selected from the group consisting of an I:A, I:U and I:C. In yet another embodiment, the base pair strength is less due to at least one base pair comprising a modified nucleotide. In certain exemplary embodiments, the modified nucleotide is selected from the group consisting of 2-amino-G, 2-amino-A, 2,6-diamino-G, and 2,6-diamino-A.

[0228] The design of siRNAs suitable for targeting the htt target sequences set forth at Figure 8 is described in detail below. siRNAs can be designed according to the above exemplary teachings for any other target sequences found in the *htt* gene. Moreover, the technology is applicable to targeting any other target sequences, e.g., non-disease causing target sequences.

[0229] To validate the effectiveness by which siRNAs destroy mRNAs (e.g., huntingtin mRNA), the siRNA can be incubated with cDNA (e.g., huntingtin cDNA) in a *Drosophila*-based *in vitro* mRNA expression system. Radiolabeled with ^{32}P, newly synthesized mRNAs (e.g., huntingtin mRNA) are detected autoradiographically on an agarose gel. The presence of cleaved mRNA indicates mRNA nuclease activity. Suitable

controls include omission of siRNA. Alternatively, control siRNAs are selected having the same nucleotide composition as the selected siRNA, but without significant sequence complementarity to the appropriate target gene. Such negative controls can be designed by randomly scrambling the nucleotide sequence of the selected siRNA; a homology search can be performed to ensure that the negative control lacks homology to any other gene in the appropriate genome. In addition, negative control siRNAs can be designed by introducing one or more base mismatches into the sequence.

[0230] Sites of siRNA-mRNA complementation are selected which result in optimal mRNA specificity and maximal mRNA cleavage.

[0231] While the instant invention primarily features targeting specific target sequences of a gene (e.g., in *htt*) distinct from the expanded CAG region mutation, the skilled artisan will appreciate that targeting the mutant region may have applicability as a therapeutic strategy in certain situations. Targeting the mutant region can be accomplished using siRNA that complements CAG in series. The $siRNA^{cag}$ would bind to mRNAs with CAG complementation, but might be expected to have greater opportunity to bind to an extended CAG series. Multiple $siRNA^{cag}$ would bind to the mutant huntingtin mRNA (as opposed to fewer for the wild type huntingtin mRNA); thus, the mutant huntingtin mRNA is more likely to be cleaved. Successful mRNA inactivation using this approach would also eliminate normal or wild-type huntingtin mRNA. Also inactivated, at least to some extent, could be other normal genes (approximately 70) which also have CAG repeats, where their mRNAs could interact with the siRNA. This approach would thus rely on an attrition strategy--more of the mutant huntingtin mRNA would be destroyed than wild-type huntingtin mRNA or the other approximately 69 mRNAs that code for polyglutamines.

V. RNAi Agents

[0232] The present invention includes siRNA molecules designed, for example, as described above. The siRNA molecules of the invention can be chemically synthesized, or can be transcribed *in vitro* from a DNA template, or *in vivo* from e.g., shRNA, or by using recombinant human DICER enzyme, to cleave *in vitro* transcribed dsRNA templates into pools of 20-, 21- or 23-bp duplex RNA mediating RNAi. The siRNA molecules can be designed using any method known in the art.

[0233] In one aspect, instead of the RNAi agent being an interfering ribonucleic acid, e.g., an siRNA or shRNA as described above, the RNAi agent can encode an interfering

ribonucleic acid, e.g., an shRNA, as described above. In other words, the RNAi agent can be a transcriptional template of the interfering ribonucleic acid. Thus, RNAi agents of the present invention can also include small hairpin RNAs (shRNAs), and expression constructs engineered to express shRNAs. Transcription of shRNAs is initiated at a polymerase III (pol III) promoter, and is thought to be terminated at position 2 of a 4-5-thymine transcription termination site. Upon expression, shRNAs are thought to fold into a stem-loop structure with 3' UU-overhangs; subsequently, the ends of these shRNAs are processed, converting the shRNAs into siRNA-like molecules of about 21-23 nucleotides (Brummelkamp et al., 2002; Lee et al., 2002, *Supra*; Miyagishi et al., 2002; Paddison et al., 2002, supra; Paul et al., 2002, supra; Sui et al., 2002 supra; Yu et al., 2002, supra. More information about shRNA design and use can be found on the internet at the following addresses: katandin.cshl.org:9331/RNAi/docs/BseRI-BamHI_Strategy.pdf and katandin.cshl.org:9331/RNAi/docs/Web_version_of_PCR_strategy1.pdf).

[0234] Expression constructs of the present invention include any construct suitable for use in the appropriate expression system and include, but are not limited to, retroviral vectors, linear expression cassettes, plasmids and viral or virally-derived vectors, as known in the art. Such expression constructs can include one or more inducible promoters, RNA Pol III promoter systems such as U6 snRNA promoters or H1 RNA polymerase III promoters, or other promoters known in the art. The constructs can include one or both strands of the siRNA. Expression constructs expressing both strands can also include loop structures linking both strands, or each strand can be separately transcribed from separate promoters within the same construct. Each strand can also be transcribed from a separate expression construct. (Tuschl, T., 2002, *Supra*).

[0235] Synthetic siRNAs can be delivered into cells by methods known in the art, including cationic liposome transfection and electroporation. To obtain longer term suppression of the target genes (i.e., *htt* genes) and to facilitate delivery under certain circumstances, one or more siRNA can be expressed within cells from recombinant DNA constructs. Such methods for expressing siRNA duplexes within cells from recombinant DNA constructs to allow longer-term target gene suppression in cells are known in the art, including mammalian Pol III promoter systems (e.g., H1 or U6/snRNA promoter systems (Tuschl, T., 2002, supra) capable of expressing functional double-stranded siRNAs; (Bagella et al., 1998; Lee et al., 2002, supra; Miyagishi et al., 2002, supra; Paul et al., 2002, supra; Yu et al., 2002), supra; Sui et al., 2002, supra). Transcriptional termination by RNA Pol III

occurs at runs of four consecutive T residues in the DNA template, providing a mechanism to end the siRNA transcript at a specific sequence. The siRNA is complementary to the sequence of the target gene in 5'-3' and 3'-5' orientations, and the two strands of the siRNA can be expressed in the same construct or in separate constructs. Hairpin siRNAs, driven by H1 or U6 snRNA promoter and expressed in cells, can inhibit target gene expression (Bagella et al., 1998; Lee et al., 2002, supra; Miyagishi et al., 2002, supra; Paul et al., 2002, supra; Yu et al., 2002), supra; Sui et al., 2002, supra). Constructs containing siRNA sequence under the control of T7 promoter also make functional siRNAs when cotransfected into the cells with a vector expressing T7 RNA polymerase (Jacque et al., 2002, supra). A single construct may contain multiple sequences coding for siRNAs, such as multiple regions of the gene encoding htt, targeting the same gene or multiple genes, and can be driven, for example, by separate PolIII promoter sites.

[0236] Animal cells express a range of noncoding RNAs of approximately 22 nucleotides termed micro RNA (miRNAs) which can regulate gene expression at the post transcriptional or translational level during animal development. One common feature of miRNAs is that they are all excised from an approximately 70 nucleotide precursor RNA stem-loop, probably by Dicer, an RNase III-type enzyme, or a homolog thereof. By substituting the stem sequences of the miRNA precursor with sequence complementary to the target mRNA, a vector construct that expresses the engineered precursor can be used to produce siRNAs to initiate RNAi against specific mRNA targets in mammalian cells (Zeng et al., 2002, supra). When expressed by DNA vectors containing polymerase III promoters, micro-RNA designed hairpins can silence gene expression (McManus et al., 2002, supra). MicroRNAs targeting polymorphisms may also be useful for blocking translation of mutant proteins, in the absence of siRNA-mediated gene-silencing. Such applications may be useful in situations, for example, where a designed siRNA caused off-target silencing of wild type protein.

[0237] Viral-mediated delivery mechanisms can also be used to induce specific silencing of targeted genes through expression of siRNA, for example, by generating recombinant adenoviruses harboring siRNA under RNA Pol II promoter transcription control (Xia et al., 2002, supra). Infection of HeLa cells by these recombinant adenoviruses allows for diminished endogenous target gene expression. Injection of the recombinant adenovirus vectors into transgenic mice expressing the target genes of the siRNA results in *in vivo* reduction of target gene expression. *Id.* In an animal model, whole-embryo electroporation

can efficiently deliver synthetic siRNA into post-implantation mouse embryos (Calegari et al., 2002). In adult mice, efficient delivery of siRNA can be accomplished by "high-pressure" delivery technique, a rapid injection (within 5 seconds) of a large volume of siRNA containing solution into animal via the tail vein (Liu et al., 1999, supra; McCaffrey et al., 2002, supra; Lewis et al., 2002. Nanoparticles and liposomes can also be used to deliver siRNA into animals. In certain exemplary embodiments, recombinant adeno-associated viruses (rAAVs) and their associated vectors can be used to deliver one or more siRNAs into cells, e.g., neural cells (e.g., brain cells) (US Patent Applications 2014/0296486, 2010/0186103, 2008/0269149, 2006/0078542 and 2005/0220766).

[0238] The nucleic acid compositions of the invention include both unmodified siRNAs and modified siRNAs as known in the art, such as crosslinked siRNA derivatives or derivatives having non nucleotide moieties linked, for example to their 3' or 5' ends. Modifying siRNA derivatives in this way may improve cellular uptake or enhance cellular targeting activities of the resulting siRNA derivative as compared to the corresponding siRNA, are useful for tracing the siRNA derivative in the cell, or improve the stability of the siRNA derivative compared to the corresponding siRNA.

[0239] Engineered RNA precursors, introduced into cells or whole organisms as described herein, will lead to the production of a desired siRNA molecule. Such an siRNA molecule will then associate with endogenous protein components of the RNAi pathway to bind to and target a specific mRNA sequence for cleavage and destruction. In this fashion, the mRNA to be targeted by the siRNA generated from the engineered RNA precursor will be depleted from the cell or organism, leading to a decrease in the concentration of the protein encoded by that mRNA in the cell or organism. The RNA precursors are typically nucleic acid molecules that individually encode either one strand of a dsRNA or encode the entire nucleotide sequence of an RNA hairpin loop structure.

[0240] The nucleic acid compositions of the invention can be unconjugated or can be conjugated to another moiety, such as a nanoparticle, to enhance a property of the compositions, e.g., a pharmacokinetic parameter such as absorption, efficacy, bioavailability and/or half-life. The conjugation can be accomplished by methods known in the art, e.g., using the methods of Lambert et al., Drug Deliv. Rev.: 47(1), 99-112 (2001) (describes nucleic acids loaded to polyalkylcyanoacrylate (PACA) nanoparticles); Fattal et al., J. Control Release 53(1-3):137-43 (1998) (describes nucleic acids bound to nanoparticles); Schwab et al., Ann. Oncol. 5 Suppl. 4:55-8 (1994) (describes nucleic acids linked to

intercalating agents, hydrophobic groups, polycations or PACA nanoparticles); and Godard et al., Eur. J. Biochem. 232(2):404-10 (1995) (describes nucleic acids linked to nanoparticles).

[0241] The nucleic acid molecules of the present invention can also be labeled using any method known in the art. For instance, the nucleic acid compositions can be labeled with a fluorophore, e.g., Cy3, fluorescein, or rhodamine. The labeling can be carried out using a kit, e.g., the SILENCER™ siRNA labeling kit (Ambion). Additionally, the siRNA can be radiolabeled, e.g., using ^{3}H, ^{32}P or other appropriate isotope.

[0242] Moreover, because RNAi is believed to progress via at least one single-stranded RNA intermediate, the skilled artisan will appreciate that ss-siRNAs (e.g., the antisense strand of a ds-siRNA) can also be designed (e.g., for chemical synthesis) generated (e.g., enzymatically generated) or expressed (e.g., from a vector or plasmid) as described herein and utilized according to the claimed methodologies. Moreover, in invertebrates, RNAi can be triggered effectively by long dsRNAs (e.g., dsRNAs about 100-1000 nucleotides in length, preferably about 200-500, for example, about 250, 300, 350, 400 or 450 nucleotides in length) acting as effectors of RNAi. (Brondani et al., Proc Natl Acad Sci USA. 2001 Dec. 4; 98(25):14428-33. Epub 2001 Nov. 27.)

VI. Anti-Htt RNA Silencing Agents

[0243] The present invention features anti-huntingtin RNA silencing agents (e.g., siRNA and shRNAs), methods of making said RNA silencing agents, and methods (e.g., research and/or therapeutic methods) for using said improved RNA silencing agents (or portions thereof) for RNA silencing of huntingtin protein (e.g., mutant huntingtin protein). The RNA silencing agents comprise an antisense strand (or portions thereof), wherein the antisense strand has sufficient complementary to a heterozygous single nucleotide polymorphism to mediate an RNA-mediated silencing mechanism (e.g. RNAi).

[0244] In certain embodiments, siRNA compounds are provided having one or any combination of the following properties: (1) fully chemically-stabilized (i.e., no unmodified 2'-OH residues); (2) asymmetry; (3) 11-16 base pair duplexes; (4) alternating pattern of chemically-modified nucleotides (e.g., 2'-fluoro and 2'-methoxy modifications); and (5) single-stranded, fully phosphorothioated tails of 5-8 bases. The number of phosphorothioate modifications is critical. This number is varied from 6 to 17 total in different embodiments.

[0245] In certain embodiments, the siRNA compounds described herein can be conjugated to a variety of targeting agents, including, but not limited to, cholesterol, DHA,

phenyltropanes, cortisol, vitamin A, vitamin D, GalNac, and gangliozides. The cholesterol-modified version showed 5-10 fold improvement in efficacy in vitro versus previously used chemical stabilization patterns (e.g., wherein all purine but not purimidines are modified) in wide range of cell types (e.g., HeLa, neurons, hepatocytes, trophoblasts).

[0246] Certain compounds of the invention having the structural properties described above and herein may be referred to as "hsiRNA-ASP" (hydrophobically-modified, small interfering RNA, featuring an advanced stabilization pattern). In addition, this hsiRNA-ASP pattern showed a dramatically improved distribution through the brain, spinal cord, delivery to liver, placenta, kidney, spleen and several other tissues, making them accessible for therapeutic intervention.

[0247] In liver hsiRNA-ASP delivery specifically to endothelial and kupper cells, but not hepatocytes, making this chemical modification pattern complimentary rather than competitive technology to GalNac conjugates.

[0248] The compounds of the invention can be described in the following aspects and embodiments.

[0249] In a first aspect, provided herein is oligonucleotide of at least 16 contiguous nucleotides, said oligonucleotide having a 5' end, a 3' end and complementarity to a target, wherein: (1) the oligonucleotide comprises alternating 2'-methoxy-ribonucleotides and 2'-fluoro-ribonucleotides; (2) the nucleotides at positions 2 and 14 from the 5' end are not 2'-methoxy-ribonucleotides; (3) the nucleotides are connected via phosphodiester or phosphorothioate linkages; and (4) the nucleotides at positions 1-6 from the 3' end, or positions 1-7 from the 3' end, are connected to adjacent nucleotides via phosphorothioate linkages.

[0250] In a second aspect, provided herein is a double-stranded, chemically-modified nucleic acid, comprising a first oligonucleotide and a second oligonucleotide, wherein: (1) the first oligonucleotide is an oligonucleotide described herein (e.g., comprising SEQ ID Nos:1, 2, 3 or 4); (2) a portion of the first oligonucleotide is complementary to a portion of the second oligonucleotide; (3) the second oligonucleotide comprises alternating 2'-methoxy-ribonucleotides and 2'-fluoro-ribonucleotides; (4) the nucleotides at positions 2 and 14 from the 3' end of the second oligonucleotide are 2'-methoxy-ribonucleotides; and (5) the nucleotides of the second oligonucleotide are connected via phosphodiester or phosphorothioate linkages.

[0251] In a third aspect, provided herein is oligonucleotide having the structure:

X-A(-L-B-L-A)j(-S-B-S-A)r(-S-B)t-OR

wherein: X is a 5' phosphate group; A, for each occurrence, independently is a 2'-methoxy-ribonucleotide; B, for each occurrence, independently is a 2'-fluoro-ribonucleotide; L, for each occurrence independently is a phosphodiester or phosphorothioate linker; S is a phosphorothioate linker; and R is selected from hydrogen and a capping group (e.g., an acyl such as acetyl); j is 4, 5, 6 or 7; r is 2 or 3; and t is 0 or 1.

[0252] In a fourth aspect, provided herein is a double-stranded, chemically-modified nucleic acid comprising a first oligonucleotide and a second oligonucleotide, wherein: (1) the first oligonucleotide is selected from the oligonucleotides of the third aspect; (2) a portion of the first oligonucleotide is complementary to a portion of the second oligonucleotide; and (3) the second oligonucleotide has the structure:

C-L-B(-S-A-S-B)m'(-P-A-P-B)n'(-P-A-S-B)q'(-S-A)r'(-S-B)t'-OR

wherein: C is a hydrophobic molecule; A, for each occurrence, independently is a 2'-methoxy-ribonucleotide; B, for each occurrence, independently is a 2'-fluoro-ribonucleotide; L is a linker comprising one or more moiety selected from the group consisting of: 0-4 repeat units of ethyleneglycol, a phosphodiester, and a phosphorothioate; S is a phosphorothioate linker; P is a phosphodiester linker; R is selected from hydrogen and a capping group (e.g., an acyl such as acetyl); m' is 0 or 1; n' is 4, 5 or 6; q' is 0 or 1; r' is 0 or 1; and t' is 0 or 1.

a) Design of Anti-Htt siRNA Molecules

[0253] An siRNA molecule of the invention is a duplex consisting of a sense strand and complementary antisense strand, the antisense strand having sufficient complementary to an htt mRNA to mediate RNAi. Preferably, the siRNA molecule has a length from about 10-50 or more nucleotides, i.e., each strand comprises 10-50 nucleotides (or nucleotide analogs). More preferably, the siRNA molecule has a length from about 16-30, e.g., 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 nucleotides in each strand, wherein one of the strands is sufficiently complementary to a target region. Preferably, the strands are aligned such that there are at least 1, 2, or 3 bases at the end of the strands which do not align (i.e., for which no complementary bases occur in the opposing strand) such that an overhang of 1, 2 or 3 residues occurs at one or both ends of the duplex when strands are annealed. Preferably, the siRNA molecule has a length from about 10-50 or more nucleotides, i.e., each strand comprises 10-50 nucleotides (or nucleotide analogs). More preferably, the siRNA molecule

has a length from about 16-30, e.g., 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 nucleotides in each strand, wherein one of the strands is substantially complementary to a target sequence, and the other strand is identical or substantially identical to the first strand.

[0254] Generally, siRNAs can be designed by using any method known in the art, for instance, by using the following protocol:

[0255] 1. The siRNA should be specific for a target sequence, e.g., a target sequence set forth in Figure 8. In one embodiment, a target sequence is found in a mutant huntingtin (htt) allele, but not a wild-type huntingtin allele. In another embodiment, a target sequence is found in both a mutant huntingtin (htt) allele, and a wild-type huntingtin allele. In another embodiment, a target sequence is found in a wild-type huntingtin allele. The first strand should be complementary to the target sequence, and the other strand is substantially complementary to the first strand. (See Figure 8 for exemplary sense and antisense strands.) In one embodiment, the target sequence is outside the expanded CAG repeat of the mutant huntingin (htt) allele. In another embodiment, the target sequence is outside a coding region of the target gene. Exemplary target sequences are selected from the 5' untranslated region (5'-UTR) of a target gene. Cleavage of mRNA at these sites should eliminate translation of corresponding mutant protein. Target sequences from other regions of the htt gene are also suitable for targeting. A sense strand is designed based on the target sequence. Further, siRNAs with lower G/C content (35-55%) may be more active than those with G/C content higher than 55%. Thus in one embodiment, the invention includes nucleic acid molecules having 35-55% G/C content.

[0256] 2. The sense strand of the siRNA is designed based on the sequence of the selected target site. Preferably the sense strand includes about 19 to 25 nucleotides, e.g., 19, 20, 21, 22, 23, 24 or 25 nucleotides. More preferably, the sense strand includes 21, 22 or 23 nucleotides. The skilled artisan will appreciate, however, that siRNAs having a length of less than 19 nucleotides or greater than 25 nucleotides can also function to mediate RNAi. Accordingly, siRNAs of such length are also within the scope of the instant invention provided that they retain the ability to mediate RNAi. Longer RNA silencing agents have been demonstrated to elicit an interferon or Protein Kinase R (PKR) response in certain mammalian cells which may be undesirable. Preferably the RNA silencing agents of the invention do not elicit a PKR response (i.e., are of a sufficiently short length). However, longer RNA silencing agents may be useful, for example, in cell types incapable of

generating a PRK response or in situations where the PKR response has been down-regulated or dampened by alternative means.

[0257] The siRNA molecules of the invention have sufficient complementarity with the target sequence such that the siRNA can mediate RNAi. In general, siRNA containing nucleotide sequences sufficiently identical to a target sequence portion of the target gene to effect RISC-mediated cleavage of the target gene are preferred. Accordingly, in a preferred embodiment, the sense strand of the siRNA is designed have to have a sequence sufficiently identical to a portion of the target. For example, the sense strand may have 100% identity to the target site. However, 100% identity is not required. Greater than 80% identity, e.g., 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or even 100% identity, between the sense strand and the target RNA sequence is preferred. The invention has the advantage of being able to tolerate certain sequence variations to enhance efficiency and specificity of RNAi. In one embodiment, the sense strand has 4, 3, 2, 1, or 0 mismatched nucleotide(s) with a target region, such as a target region that differs by at least one base pair between a wild-type and mutant allele, e.g., a target region comprising the gain-of-function mutation, and the other strand is identical or substantially identical to the first strand. Moreover, siRNA sequences with small insertions or deletions of 1 or 2 nucleotides may also be effective for mediating RNAi. Alternatively, siRNA sequences with nucleotide analog substitutions or insertions can be effective for inhibition.

[0258] Sequence identity may be determined by sequence comparison and alignment algorithms known in the art. To determine the percent identity of two nucleic acid sequences (or of two amino acid sequences), the sequences are aligned for optimal comparison purposes (e.g., gaps can be introduced in the first sequence or second sequence for optimal alignment). The nucleotides (or amino acid residues) at corresponding nucleotide (or amino acid) positions are then compared. When a position in the first sequence is occupied by the same residue as the corresponding position in the second sequence, then the molecules are identical at that position. The percent identity between the two sequences is a function of the number of identical positions shared by the sequences (i.e., % homology = number of identical positions / total number of positions x 100), optionally penalizing the score for the number of gaps introduced and/or length of gaps introduced.

[0259] The comparison of sequences and determination of percent identity between two sequences can be accomplished using a mathematical algorithm. In one embodiment, the

alignment generated over a certain portion of the sequence aligned having sufficient identity but not over portions having low degree of identity (i.e., a local alignment). A preferred, non-limiting example of a local alignment algorithm utilized for the comparison of sequences is the algorithm of Karlin and Altschul (1990) Proc. Natl. Acad. Sci. USA 87:2264-68, modified as in Karlin and Altschul (1993) Proc. Natl. Acad. Sci. USA 90:5873-77. Such an algorithm is incorporated into the BLAST programs (version 2.0) of Altschul, et al. (1990) J. Mol. Biol. 215:403-10.

[0260] In another embodiment, the alignment is optimized by introducing appropriate gaps and percent identity is determined over the length of the aligned sequences (i.e., a gapped alignment). To obtain gapped alignments for comparison purposes, Gapped BLAST can be utilized as described in Altschul et al., (1997) Nucleic Acids Res. 25(17):3389-3402. In another embodiment, the alignment is optimized by introducing appropriate gaps and percent identity is determined over the entire length of the sequences aligned (i.e., a global alignment). A preferred, non-limiting example of a mathematical algorithm utilized for the global comparison of sequences is the algorithm of Myers and Miller, CABIOS (1989). Such an algorithm is incorporated into the ALIGN program (version 2.0) which is part of the GCG sequence alignment software package. When utilizing the ALIGN program for comparing amino acid sequences, a PAM120 weight residue table, a gap length penalty of 12, and a gap penalty of 4 can be used.

[0261] 3. The antisense or guide strand of the siRNA is routinely the same length as the sense strand and includes complementary nucleotides. In one embodiment, the guide and sense strands are fully complementary, i.e., the strands are blunt-ended when aligned or annealed. In another embodiment, the strands of the siRNA can be paired in such a way as to have a 3' overhang of 1 to 7 (e.g., 2, 3, 4, 5, 6 or 7), or 1 to 4, e.g., 2, 3 or 4 nucleotides. Overhangs can comprise (or consist of) nucleotides corresponding to the target gene sequence (or complement thereof). Alternatively, overhangs can comprise (or consist of) deoxyribonucleotides, for example dTs, or nucleotide analogs, or other suitable non-nucleotide material. Thus in another embodiment, the nucleic acid molecules may have a 3' overhang of 2 nucleotides, such as TT. The overhanging nucleotides may be either RNA or DNA. As noted above, it is desirable to choose a target region wherein the mutant:wild type mismatch is a purine:purine mismatch.

[0262] 4. Using any method known in the art, compare the potential targets to the appropriate genome database (human, mouse, rat, etc.) and eliminate from consideration any

target sequences with significant homology to other coding sequences. One such method for such sequence homology searches is known as BLAST, which is available at National Center for Biotechnology Information website.

[0263] 5. Select one or more sequences that meet your criteria for evaluation.

[0264] Further general information about the design and use of siRNA may be found in "The siRNA User Guide," available at The Max-Plank-Institut fur Biophysikalishe Chemie website.

[0265] Alternatively, the siRNA may be defined functionally as a nucleotide sequence (or oligonucleotide sequence) that is capable of hybridizing with the target sequence (e.g., 400 mM NaCl, 40 mM PIPES pH 6.4, 1 mM EDTA, 50 °C or 70 °C hybridization for 12-16 hours; followed by washing). Additional preferred hybridization conditions include hybridization at 70 °C in 1xSSC or 50 °C in 1xSSC, 50% formamide followed by washing at 70 °C in 0.3xSSC or hybridization at 70 °C in 4xSSC or 50 °C in 4xSSC, 50% formamide followed by washing at 67 °C in 1xSSC. The hybridization temperature for hybrids anticipated to be less than 50 base pairs in length should be 5-10 °C less than the melting temperature (Tm) of the hybrid, where Tm is determined according to the following equations. For hybrids less than 18 base pairs in length, Tm(°C)=2(# of A+T bases)+4(# of G+C bases). For hybrids between 18 and 49 base pairs in length, Tm(°C)=81.5+16.6(log 10[Na+])+0.41(% G+C)-(600/N), where N is the number of bases in the hybrid, and [Na+] is the concentration of sodium ions in the hybridization buffer ([Na+] for 1xSSC=0.165 M). Additional examples of stringency conditions for polynucleotide hybridization are provided in Sambrook, J., E. F. Fritsch, and T. Maniatis, 1989, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., chapters 9 and 11, and Current Protocols in Molecular Biology, 1995, F. M. Ausubel et al., eds., John Wiley & Sons, Inc., sections 2.10 and 6.3-6.4, incorporated herein by reference.

[0266] Negative control siRNAs should have the same nucleotide composition as the selected siRNA, but without significant sequence complementarity to the appropriate genome. Such negative controls may be designed by randomly scrambling the nucleotide sequence of the selected siRNA. A homology search can be performed to ensure that the negative control lacks homology to any other gene in the appropriate genome. In addition,

negative control siRNAs can be designed by introducing one or more base mismatches into the sequence.

[0267] 6. To validate the effectiveness by which siRNAs destroy target mRNAs (e.g., wild-type or mutant huntingtin mRNA), the siRNA may be incubated with target cDNA (e.g., huntingtin cDNA) in a *Drosophila*-based *in vitro* mRNA expression system. Radiolabeled with ^{32}P, newly synthesized target mRNAs (e.g., huntingtin mRNA) are detected autoradiographically on an agarose gel. The presence of cleaved target mRNA indicates mRNA nuclease activity. Suitable controls include omission of siRNA and use of non-target cDNA. Alternatively, control siRNAs are selected having the same nucleotide composition as the selected siRNA, but without significant sequence complementarity to the appropriate target gene. Such negative controls can be designed by randomly scrambling the nucleotide sequence of the selected siRNA. A homology search can be performed to ensure that the negative control lacks homology to any other gene in the appropriate genome. In addition, negative control siRNAs can be designed by introducing one or more base mismatches into the sequence.

[0268] Anti-htt siRNAs may be designed to target any of the target sequences described supra. Said siRNAs comprise an antisense strand which is sufficiently complementary with the target sequence to mediate silencing of the target sequence. In certain embodiments, the RNA silencing agent is a siRNA.

[0269] In certain embodiments, the siRNA comprises a sense strand comprising a sequence set forth at Figure 8, and an antisense strand comprising a sequence set forth at Figure 8.

[0270] Sites of siRNA-mRNA complementation are selected which result in optimal mRNA specificity and maximal mRNA cleavage.

b) siRNA-Like Molecules

[0271] siRNA-like molecules of the invention have a sequence (i.e., have a strand having a sequence) that is "sufficiently complementary" to a target sequence of a htt mRNA to direct gene silencing either by RNAi or translational repression. siRNA-like molecules are designed in the same way as siRNA molecules, but the degree of sequence identity between the sense strand and target RNA approximates that observed between an miRNA and its target. In general, as the degree of sequence identity between a miRNA sequence and the corresponding target gene sequence is decreased, the tendency to mediate post-transcriptional

gene silencing by translational repression rather than RNAi is increased. Therefore, in an alternative embodiment, where post-transcriptional gene silencing by translational repression of the target gene is desired, the miRNA sequence has partial complementarity with the target gene sequence. In certain embodiments, the miRNA sequence has partial complementarity with one or more short sequences (complementarity sites) dispersed within the target mRNA (e.g. within the 3'-UTR of the target mRNA) (Hutvagner and Zamore, Science, 2002; Zeng et al., Mol. Cell, 2002; Zeng et al., RNA, 2003; Doench et al., Genes & Dev., 2003). Since the mechanism of translational repression is cooperative, multiple complementarity sites (e.g., 2, 3, 4, 5, or 6) may be targeted in certain embodiments.

[0272] The capacity of a siRNA-like duplex to mediate RNAi or translational repression may be predicted by the distribution of non-identical nucleotides between the target gene sequence and the nucleotide sequence of the silencing agent at the site of complementarity. In one embodiment, where gene silencing by translational repression is desired, at least one non-identical nucleotide is present in the central portion of the complementarity site so that duplex formed by the miRNA guide strand and the target mRNA contains a central "bulge" (Doench J G et al., Genes & Dev., 2003). In another embodiment 2, 3, 4, 5, or 6 contiguous or non-contiguous non-identical nucleotides are introduced. The non-identical nucleotide may be selected such that it forms a wobble base pair (e.g., G:U) or a mismatched base pair (G:A, C:A, C:U, G:G, A:A, C:C, U:U). In a further preferred embodiment, the "bulge" is centered at nucleotide positions 12 and 13 from the 5' end of the miRNA molecule.

c) Short Hairpin RNA (shRNA) Molecules

[0273] In certain featured embodiments, the instant invention provides shRNAs capable of mediating RNA silencing of an htt target sequence with enhanced selectivity. In contrast to siRNAs, shRNAs mimic the natural precursors of micro RNAs (miRNAs) and enter at the top of the gene silencing pathway. For this reason, shRNAs are believed to mediate gene silencing more efficiently by being fed through the entire natural gene silencing pathway.

[0274] miRNAs are noncoding RNAs of approximately 22 nucleotides which can regulate gene expression at the post transcriptional or translational level during plant and animal development. One common feature of miRNAs is that they are all excised from an approximately 70 nucleotide precursor RNA stem-loop termed pre-miRNA, probably by

Dicer, an RNase III-type enzyme, or a homolog thereof. Naturally-occurring miRNA precursors (pre-miRNA) have a single strand that forms a duplex stem including two portions that are generally complementary, and a loop, that connects the two portions of the stem. In typical pre-miRNAs, the stem includes one or more bulges, e.g., extra nucleotides that create a single nucleotide "loop" in one portion of the stem, and/or one or more unpaired nucleotides that create a gap in the hybridization of the two portions of the stem to each other. Short hairpin RNAs, or engineered RNA precursors, of the invention are artificial constructs based on these naturally occurring pre-miRNAs, but which are engineered to deliver desired RNA silencing agents (e.g., siRNAs of the invention). By substituting the stem sequences of the pre-miRNA with sequence complementary to the target mRNA, a shRNA is formed. The shRNA is processed by the entire gene silencing pathway of the cell, thereby efficiently mediating RNAi.

[0275] The requisite elements of a shRNA molecule include a first portion and a second portion, having sufficient complementarity to anneal or hybridize to form a duplex or double-stranded stem portion. The two portions need not be fully or perfectly complementary. The first and second "stem" portions are connected by a portion having a sequence that has insufficient sequence complementarity to anneal or hybridize to other portions of the shRNA. This latter portion is referred to as a "loop" portion in the shRNA molecule. The shRNA molecules are processed to generate siRNAs. shRNAs can also include one or more bulges, i.e., extra nucleotides that create a small nucleotide "loop" in a portion of the stem, for example a one-, two- or three-nucleotide loop. The stem portions can be the same length, or one portion can include an overhang of, for example, 1-5 nucleotides. The overhanging nucleotides can include, for example, uracils (Us), e.g., all Us. Such Us are notably encoded by thymidines (Ts) in the shRNA-encoding DNA which signal the termination of transcription.

[0276] In shRNAs (or engineered precursor RNAs) of the instant invention, one portion of the duplex stem is a nucleic acid sequence that is complementary (or anti-sense) to the htt target sequence. Preferably, one strand of the stem portion of the shRNA is sufficiently complementary (e.g., antisense) to a target RNA (e.g., mRNA) sequence to mediate degradation or cleavage of said target RNA via RNA interference (RNAi). Thus, engineered RNA precursors include a duplex stem with two portions and a loop connecting the two stem portions. The antisense portion can be on the 5' or 3' end of the stem. The stem portions of a shRNA are preferably about 15 to about 50 nucleotides in length. Preferably the

two stem portions are about 18 or 19 to about 21, 22, 23, 24, 25, 30, 35, 37, 38, 39, or 40 or more nucleotides in length. In preferred embodiments, the length of the stem portions should be 21 nucleotides or greater. When used in mammalian cells, the length of the stem portions should be less than about 30 nucleotides to avoid provoking non-specific responses like the interferon pathway. In non-mammalian cells, the stem can be longer than 30 nucleotides. In fact, the stem can include much larger sections complementary to the target mRNA (up to, and including the entire mRNA). In fact, a stem portion can include much larger sections complementary to the target mRNA (up to, and including the entire mRNA).

[0277] The two portions of the duplex stem must be sufficiently complementary to hybridize to form the duplex stem. Thus, the two portions can be, but need not be, fully or perfectly complementary. In addition, the two stem portions can be the same length, or one portion can include an overhang of 1, 2, 3, or 4 nucleotides. The overhanging nucleotides can include, for example, uracils (Us), e.g., all Us. The loop in the shRNAs or engineered RNA precursors may differ from natural pre-miRNA sequences by modifying the loop sequence to increase or decrease the number of paired nucleotides, or replacing all or part of the loop sequence with a tetraloop or other loop sequences. Thus, the loop in the shRNAs or engineered RNA precursors can be 2, 3, 4, 5, 6, 7, 8, 9, or more, e.g., 15 or 20, or more nucleotides in length.

[0278] The loop in the shRNAs or engineered RNA precursors may differ from natural pre-miRNA sequences by modifying the loop sequence to increase or decrease the number of paired nucleotides, or replacing all or part of the loop sequence with a tetraloop or other loop sequences. Thus, the loop portion in the shRNA can be about 2 to about 20 nucleotides in length, i.e., about 2, 3, 4, 5, 6, 7, 8, 9, or more, e.g., 15 or 20, or more nucleotides in length. A preferred loop consists of or comprises a "tetraloop" sequences. Exemplary tetraloop sequences include, but are not limited to, the sequences GNRA, where N is any nucleotide and R is a purine nucleotide, GGGG, and UUUU.

[0279] In certain embodiments, shRNAs of the invention include the sequences of a desired siRNA molecule described supra. In other embodiments, the sequence of the antisense portion of a shRNA can be designed essentially as described above or generally by selecting an 18, 19, 20, 21 nucleotide, or longer, sequence from within the target RNA (e.g., htt mRNA), for example, from a region 100 to 200 or 300 nucleotides upstream or downstream of the start of translation. In general, the sequence can be selected from any portion of the target RNA (e.g., mRNA) including the 5' UTR (untranslated region), coding

sequence, or 3' UTR, provided said portion is distant from the site of the gain-of-function mutation. This sequence can optionally follow immediately after a region of the target gene containing two adjacent AA nucleotides. The last two nucleotides of the nucleotide sequence can be selected to be UU. This 21 or so nucleotide sequence is used to create one portion of a duplex stem in the shRNA. This sequence can replace a stem portion of a wild-type pre-miRNA sequence, e.g., enzymatically, or is included in a complete sequence that is synthesized. For example, one can synthesize DNA oligonucleotides that encode the entire stem-loop engineered RNA precursor, or that encode just the portion to be inserted into the duplex stem of the precursor, and using restriction enzymes to build the engineered RNA precursor construct, e.g., from a wild-type pre-miRNA.

[0280] Engineered RNA precursors include in the duplex stem the 21-22 or so nucleotide sequences of the siRNA or siRNA-like duplex desired to be produced *in vivo*. Thus, the stem portion of the engineered RNA precursor includes at least 18 or 19 nucleotide pairs corresponding to the sequence of an exonic portion of the gene whose expression is to be reduced or inhibited. The two 3' nucleotides flanking this region of the stem are chosen so as to maximize the production of the siRNA from the engineered RNA precursor and to maximize the efficacy of the resulting siRNA in targeting the corresponding mRNA for translational repression or destruction by RNAi *in vivo* and *in vitro*.

[0281] In certain embodiments, shRNAs of the invention include miRNA sequences, optionally end-modified miRNA sequences, to enhance entry into RISC. The miRNA sequence can be similar or identical to that of any naturally occurring miRNA (see e.g. The miRNA Registry; Griffiths-Jones S, Nuc. Acids Res., 2004). Over one thousand natural miRNAs have been identified to date and together they are thought to comprise about 1% of all predicted genes in the genome. Many natural miRNAs are clustered together in the introns of pre-mRNAs and can be identified *in silico* using homology-based searches (Pasquinelli et al., 2000; Lagos-Quintana et al., 2001; Lau et al., 2001; Lee and Ambros, 2001) or computer algorithms (e.g. MiRScan, MiRSeeker) that predict the capability of a candidate miRNA gene to form the stem loop structure of a pri-mRNA (Grad et al., Mol. Cell., 2003; Lim et al., Genes Dev., 2003; Lim et al., Science, 2003; Lai E C et al., Genome Bio., 2003). An online registry provides a searchable database of all published miRNA sequences (The miRNA Registry at the Sanger Institute website; Griffiths-Jones S, Nuc. Acids Res., 2004). Exemplary, natural miRNAs include lin-4, let-7, miR-10, mirR-15, miR-16, miR-168, miR-175, miR-196 and their homologs, as well as other natural miRNAs from

humans and certain model organisms including *Drosophila melanogaster*, *Caenorhabditis elegans*, zebrafish, *Arabidopsis thalania*, Mus musculus, and *Rattus norvegicus* as described in International PCT Publication No. WO 03/029459.

[0282] Naturally-occurring miRNAs are expressed by endogenous genes *in vivo* and are processed from a hairpin or stem-loop precursor (pre-miRNA or pri-miRNAs) by Dicer or other RNAses (Lagos-Quintana et al., Science, 2001; Lau et al., Science, 2001; Lee and Ambros, Science, 2001; Lagos-Quintana et al., Curr. Biol., 2002; Mourelatos et al., Genes Dev., 2002; Reinhart et al., Science, 2002; Ambros et al., Curr. Biol., 2003; Brennecke et al., 2003; Lagos-Quintana et al., RNA, 2003; Lim et al., Genes Dev., 2003; Lim et al., Science, 2003). miRNAs can exist transiently *in vivo* as a double-stranded duplex, but only one strand is taken up by the RISC complex to direct gene silencing. Certain miRNAs, e.g., plant miRNAs, have perfect or near-perfect complementarity to their target mRNAs and, hence, direct cleavage of the target mRNAs. Other miRNAs have less than perfect complementarity to their target mRNAs and, hence, direct translational repression of the target mRNAs. The degree of complementarity between an miRNA and its target mRNA is believed to determine its mechanism of action. For example, perfect or near-perfect complementarity between a miRNA and its target mRNA is predictive of a cleavage mechanism (Yekta et al., Science, 2004), whereas less than perfect complementarity is predictive of a translational repression mechanism. In particular embodiments, the miRNA sequence is that of a naturally-occurring miRNA sequence, the aberrant expression or activity of which is correlated with an miRNA disorder.

d) Dual Functional Oligonucleotide Tethers

[0283] In other embodiments, the RNA silencing agents of the present invention include dual functional oligonucleotide tethers useful for the intercellular recruitment of a miRNA. Animal cells express a range of miRNAs, noncoding RNAs of approximately 22 nucleotides which can regulate gene expression at the post transcriptional or translational level. By binding a miRNA bound to RISC and recruiting it to a target mRNA, a dual functional oligonucleotide tether can repress the expression of genes involved e.g., in the arteriosclerotic process. The use of oligonucleotide tethers offer several advantages over existing techniques to repress the expression of a particular gene. First, the methods described herein allow an endogenous molecule (often present in abundance), an miRNA, to mediate RNA silencing. Accordingly, the methods described herein obviate the need to introduce foreign molecules (e.g., siRNAs) to mediate RNA silencing. Second, the RNA-

silencing agents and, in particular, the linking moiety (e.g., oligonucleotides such as the 2'-O-methyl oligonucleotide), can be made stable and resistant to nuclease activity. As a result, the tethers of the present invention can be designed for direct delivery, obviating the need for indirect delivery (e.g. viral) of a precursor molecule or plasmid designed to make the desired agent within the cell. Third, tethers and their respective moieties, can be designed to conform to specific mRNA sites and specific miRNAs. The designs can be cell and gene product specific. Fourth, the methods disclosed herein leave the mRNA intact, allowing one skilled in the art to block protein synthesis in short pulses using the cell's own machinery. As a result, these methods of RNA silencing are highly regulatable.

[0284] The dual functional oligonucleotide tethers ("tethers") of the invention are designed such that they recruit miRNAs (e.g., endogenous cellular miRNAs) to a target mRNA so as to induce the modulation of a gene of interest. In preferred embodiments, the tethers have the formula T-L-μ, wherein T is an mRNA targeting moiety, L is a linking moiety, and μ is an miRNA recruiting moiety. Any one or more moiety may be double stranded. Preferably, however, each moiety is single stranded.

[0285] Moieties within the tethers can be arranged or linked (in the 5' to 3' direction) as depicted in the formula T-L-μ (i.e., the 3' end of the targeting moiety linked to the 5' end of the linking moiety and the 3' end of the linking moiety linked to the 5' end of the miRNA recruiting moiety). Alternatively, the moieties can be arranged or linked in the tether as follows: μ-T-L (i.e., the 3' end of the miRNA recruiting moiety linked to the 5' end of the linking moiety and the 3' end of the linking moiety linked to the 5' end of the targeting moiety).

[0286] The mRNA targeting moiety, as described above, is capable of capturing a specific target mRNA. According to the invention, expression of the target mRNA is undesirable, and, thus, translational repression of the mRNA is desired. The mRNA targeting moiety should be of sufficient size to effectively bind the target mRNA. The length of the targeting moiety will vary greatly depending, in part, on the length of the target mRNA and the degree of complementarity between the target mRNA and the targeting moiety. In various embodiments, the targeting moiety is less than about 200, 100, 50, 30, 25, 20, 19, 18, 17, 16, 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, or 5 nucleotides in length. In a particular embodiment, the targeting moiety is about 15 to about 25 nucleotides in length.

[0287] The miRNA recruiting moiety, as described above, is capable of associating with a miRNA. According to the invention, the miRNA may be any miRNA capable of repressing the target mRNA. Mammals are reported to have over 250 endogenous miRNAs (Lagos-Quintana et al. (2002) Current Biol. 12:735-739; Lagos-Quintana et al. (2001) Science 294:858-862; and Lim et al. (2003) Science 299:1540). In various embodiments, the miRNA may be any art-recognized miRNA.

[0288] The linking moiety is any agent capable of linking the targeting moieties such that the activity of the targeting moieties is maintained. Linking moieties are preferably oligonucleotide moieties comprising a sufficient number of nucleotides such that the targeting agents can sufficiently interact with their respective targets. Linking moieties have little or no sequence homology with cellular mRNA or miRNA sequences. Exemplary linking moieties include one or more 2'-O-methylnucleotides, e.g., 2'-β-methyladenosine, 2'-O-methylthymidine, 2'-O-methylguanosine or 2'-O-methyluridine.

e) Gene Silencing Oligonucleotides

[0289] In certain exemplary embodiments, gene expression (i.e., *htt* gene expression) can be modulated using oligonucleotide-based compounds comprising two or more single stranded antisense oligonucleotides that are linked through their 5'-ends that allow the presence of two or more accessible 3'-ends to effectively inhibit or decrease *htt* gene expression. Such linked oligonucleotides are also known as Gene Silencing Oligonucleotides (GSOs). (*See*, e.g., US 8,431,544 assigned to Idera Pharmaceuticals, Inc., incorporated herein by reference in its entirety for all purposes.)

[0290] The linkage at the 5' ends of the GSOs is independent of the other oligonucleotide linkages and may be directly via 5', 3' or 2' hydroxyl groups, or indirectly, via a non-nucleotide linker or a nucleoside, utilizing either the 2' or 3' hydroxyl positions of the nucleoside. Linkages may also utilize a functionalized sugar or nucleobase of a 5' terminal nucleotide.

[0291] GSOs can comprise two identical or different sequences conjugated at their 5'-5' ends via a phosphodiester, phosphorothioate or non-nucleoside linker. Such compounds may comprise 15 to 27 nucleotides that are complementary to specific portions of mRNA targets of interest for antisense down regulation of gene product. GSOs that comprise identical sequences can bind to a specific mRNA via Watson-Crick hydrogen bonding interactions and inhibit protein expression. GSOs that comprise different sequences are able

to bind to two or more different regions of one or more mRNA target and inhibit protein expression. Such compounds are comprised of heteronucleotide sequences complementary to target mRNA and form stable duplex structures through Watson-Crick hydrogen bonding. Under certain conditions, GSOs containing two free 3'-ends (5'-5'-attached antisense) can be more potent inhibitors of gene expression than those containing a single free 3'-end or no free 3'-end.

[0292] In some embodiments, the non-nucleotide linker is glycerol or a glycerol homolog of the formula HO--$(CH_2)_o$--CH(OH)--$(CH_2)_p$--OH, wherein o and p independently are integers from 1 to about 6, from 1 to about 4 or from 1 to about 3. In some other embodiments, the non-nucleotide linker is a derivative of 1,3-diamino-2-hydroxypropane. Some such derivatives have the formula HO--$(CH_2)_m$--C(O)NH--CH_2--CH(OH)--CH_2--NHC(O)--$(CH_2)_m$--OH, wherein m is an integer from 0 to about 10, from 0 to about 6, from 2 to about 6 or from 2 to about 4.

[0293] Some non-nucleotide linkers permit attachment of more than two GSO components. For example, the non-nucleotide linker glycerol has three hydroxyl groups to which GSO components may be covalently attached. Some oligonucleotide-based compounds of the invention, therefore, comprise two or more oligonucleotides linked to a nucleotide or a non-nucleotide linker. Such oligonucleotides according to the invention are referred to as being "branched."

[0294] In certain embodiments, GSOs are at least 14 nucleotides in length. In certain exemplary embodiments, GSOs are 15 to 40 nucleotides long or 20 to 30 nucleotides in length. Thus, the component oligonucleotides of GSOs can independently be 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 or 40 nucleotides in length.

[0295] These oligonucleotides can be prepared by the art recognized methods such as phosphoramidate or H-phosphonate chemistry which can be carried out manually or by an automated synthesizer. These oligonucleotides may also be modified in a number of ways without compromising their ability to hybridize to mRNA. Such modifications may include at least one internucleotide linkage of the oligonucleotide being an alkylphosphonate, phosphorothioate, phosphorodithioate, methylphosphonate, phosphate ester, alkylphosphonothioate, phosphoramidate, carbamate, carbonate, phosphate hydroxyl, acetamidate or carboxymethyl ester or a combination of these and other internucleotide

linkages between the 5' end of one nucleotide and the 3' end of another nucleotide in which the 5' nucleotide phosphodiester linkage has been replaced with any number of chemical groups.

VII. Modified Anti-Htt RNA Silencing Agents

[0296] In certain aspects of the invention, an RNA silencing agent (or any portion thereof) of the invention as described supra may be modified such that the activity of the agent is further improved. For example, the RNA silencing agents described in Section II supra may be modified with any of the modifications described infra. The modifications can, in part, serve to further enhance target discrimination, to enhance stability of the agent (e.g., to prevent degradation), to promote cellular uptake, to enhance the target efficiency, to improve efficacy in binding (e.g., to the targets), to improve patient tolerance to the agent, and/or to reduce toxicity.

1) Modifications to Enhance Target Discrimination

[0297] In certain embodiments, the RNA silencing agents of the invention may be substituted with a destabilizing nucleotide to enhance single nucleotide target discrimination (see U.S. application Ser. No. 11/698,689, filed Jan. 25, 2007 and U.S. Provisional Application No. 60/762,225 filed Jan. 25, 2006, both of which are incorporated herein by reference). Such a modification may be sufficient to abolish the specificity of the RNA silencing agent for a non-target mRNA (e.g. wild-type mRNA), without appreciably affecting the specificity of the RNA silencing agent for a target mRNA (e.g. gain-of-function mutant mRNA).

[0298] In preferred embodiments, the RNA silencing agents of the invention are modified by the introduction of at least one universal nucleotide in the antisense strand thereof. Universal nucleotides comprise base portions that are capable of base pairing indiscriminately with any of the four conventional nucleotide bases (e.g. A, G, C, U). A universal nucleotide is preferred because it has relatively minor effect on the stability of the RNA duplex or the duplex formed by the guide strand of the RNA silencing agent and the target mRNA. Exemplary universal nucleotide include those having an inosine base portion or an inosine analog base portion selected from the group consisting of deoxyinosine (e.g. 2'-deoxyinosine), 7-deaza-2'-deoxyinosine, 2'-aza-2'-deoxyinosine, PNA-inosine, morpholino-inosine, LNA-inosine, phosphoramidate-inosine, 2'-O-methoxyethyl-inosine, and 2'-OMe-

inosine. In particularly preferred embodiments, the universal nucleotide is an inosine residue or a naturally occurring analog thereof.

[0299] In certain embodiments, the RNA silencing agents of the invention are modified by the introduction of at least one destabilizing nucleotide within 5 nucleotides from a specificity-determining nucleotide (i.e., the nucleotide which recognizes the disease-related polymorphism). For example, the destabilizing nucleotide may be introduced at a position that is within 5, 4, 3, 2, or 1 nucleotide(s) from a specificity-determining nucleotide. In exemplary embodiments, the destabilizing nucleotide is introduced at a position which is 3 nucleotides from the specificity-determining nucleotide (i.e., such that there are 2 stabilizing nucleotides between the destablilizing nucleotide and the specificity-determining nucleotide). In RNA silencing agents having two strands or strand portions (e.g. siRNAs and shRNAs), the destabilizing nucleotide may be introduced in the strand or strand portion that does not contain the specificity-determining nucleotide. In preferred embodiments, the destabilizing nucleotide is introduced in the same strand or strand portion that contains the specificity-determining nucleotide.

2) Modifications to Enhance Efficacy and Specificity

[0300] In certain embodiments, the RNA silencing agents of the invention may be altered to facilitate enhanced efficacy and specificity in mediating RNAi according to asymmetry design rules (see U.S. Patent Nos. 8,309,704, 7,750,144, 8,304,530, 8,329,892 and 8,309,705). Such alterations facilitate entry of the antisense strand of the siRNA (e.g., a siRNA designed using the methods of the invention or an siRNA produced from a shRNA) into RISC in favor of the sense strand, such that the antisense strand preferentially guides cleavage or translational repression of a target mRNA, and thus increasing or improving the efficiency of target cleavage and silencing. Preferably the asymmetry of an RNA silencing agent is enhanced by lessening the base pair strength between the antisense strand 5' end (AS 5') and the sense strand 3' end (S 3') of the RNA silencing agent relative to the bond strength or base pair strength between the antisense strand 3' end (AS 3') and the sense strand 5' end (S '5) of said RNA silencing agent.

[0301] In one embodiment, the asymmetry of an RNA silencing agent of the invention may be enhanced such that there are fewer G:C base pairs between the 5' end of the first or antisense strand and the 3' end of the sense strand portion than between the 3' end of the first or antisense strand and the 5' end of the sense strand portion. In another

embodiment, the asymmetry of an RNA silencing agent of the invention may be enhanced such that there is at least one mismatched base pair between the 5' end of the first or antisense strand and the 3' end of the sense strand portion. Preferably, the mismatched base pair is selected from the group consisting of G:A, C:A, C:U, G:G, A:A, C:C and U:U. In another embodiment, the asymmetry of an RNA silencing agent of the invention may be enhanced such that there is at least one wobble base pair, e.g., G:U, between the 5' end of the first or antisense strand and the 3' end of the sense strand portion. In another embodiment, the asymmetry of an RNA silencing agent of the invention may be enhanced such that there is at least one base pair comprising a rare nucleotide, e.g., inosine (I). Preferably, the base pair is selected from the group consisting of an I:A, I:U and I:C. In yet another embodiment, the asymmetry of an RNA silencing agent of the invention may be enhanced such that there is at least one base pair comprising a modified nucleotide. In preferred embodiments, the modified nucleotide is selected from the group consisting of 2-amino-G, 2-amino-A, 2,6-diamino-G, and 2,6-diamino-A.

3) RNA Silencing Agents with Enhanced Stability

[0302] The RNA silencing agents of the present invention can be modified to improve stability in serum or in growth medium for cell cultures. In order to enhance the stability, the 3'-residues may be stabilized against degradation, e.g., they may be selected such that they consist of purine nucleotides, particularly adenosine or guanosine nucleotides. Alternatively, substitution of pyrimidine nucleotides by modified analogues, e.g., substitution of uridine by 2'-deoxythymidine is tolerated and does not affect the efficiency of RNA interference.

[0303] In a preferred aspect, the invention features RNA silencing agents that include first and second strands wherein the second strand and/or first strand is modified by the substitution of internal nucleotides with modified nucleotides, such that *in vivo* stability is enhanced as compared to a corresponding unmodified RNA silencing agent. As defined herein, an "internal" nucleotide is one occurring at any position other than the 5' end or 3' end of nucleic acid molecule, polynucleotide or oligonucleotide. An internal nucleotide can be within a single-stranded molecule or within a strand of a duplex or double-stranded molecule. In one embodiment, the sense strand and/or antisense strand is modified by the substitution of at least one internal nucleotide. In another embodiment, the sense strand and/or antisense strand is modified by the substitution of at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25 or more internal nucleotides. In another embodiment, the sense strand and/or antisense strand is modified by the substitution of at least 5%, 10%, 15%,

20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95% or more of the internal nucleotides. In yet another embodiment, the sense strand and/or antisense strand is modified by the substitution of all of the internal nucleotides.

[0304] In a preferred embodiment of the present invention, the RNA silencing agents may contain at least one modified nucleotide analogue. The nucleotide analogues may be located at positions where the target-specific silencing activity, e.g., the RNAi mediating activity or translational repression activity is not substantially effected, e.g., in a region at the 5'-end and/or the 3'-end of the siRNA molecule. Particularly, the ends may be stabilized by incorporating modified nucleotide analogues.

[0305] Exemplary nucleotide analogues include sugar- and/or backbone-modified ribonucleotides (i.e., include modifications to the phosphate-sugar backbone). For example, the phosphodiester linkages of natural RNA may be modified to include at least one of a nitrogen or sulfur heteroatom. In exemplary backbone-modified ribonucleotides, the phosphoester group connecting to adjacent ribonucleotides is replaced by a modified group, e.g., of phosphothioate group. In exemplary sugar-modified ribonucleotides, the 2' OH-group is replaced by a group selected from H, OR, R, halo, SH, SR, NH_2, NHR, NR_2 or ON, wherein R is C_1-C_6 alkyl, alkenyl or alkynyl and halo is F, Cl, Br or I.

[0306] In particular embodiments, the modifications are 2'-fluoro, 2'-amino and/or 2'-thio modifications. Particularly preferred modifications include 2'-fluoro-cytidine, 2'-fluoro-uridine, 2'-fluoro-adenosine, 2'-fluoro-guanosine, 2'-amino-cytidine, 2'-amino-uridine, 2'-amino-adenosine, 2'-amino-guanosine, 2,6-diaminopurine, 4-thio-uridine, and/or 5-amino-allyl-uridine. In a particular embodiment, the 2'-fluoro ribonucleotides are every uridine and cytidine. Additional exemplary modifications include 5-bromo-uridine, 5-iodo-uridine, 5-methyl-cytidine, ribo-thymidine, 2-aminopurine, 2'-amino-butyryl-pyrene-uridine, 5-fluoro-cytidine, and 5-fluoro-uridine. 2'-deoxy-nucleotides and 2'-Ome nucleotides can also be used within modified RNA-silencing agents moities of the instant invention. Additional modified residues include, deoxy-abasic, inosine, N3-methyl-uridine, N6,N6-dimethyl-adenosine, pseudouridine, purine ribonucleoside and ribavirin. In a particularly preferred embodiment, the 2' moiety is a methyl group such that the linking moiety is a 2'-O-methyl oligonucleotide.

[0307] In an exemplary embodiment, the RNA silencing agent of the invention comprises Locked Nucleic Acids (LNAs). LNAs comprise sugar-modified nucleotides that resist nuclease activities (are highly stable) and possess single nucleotide discrimination for

mRNA (Elmen et al., Nucleic Acids Res., (2005), 33(1): 439-447; Braasch et al. (2003) Biochemistry 42:7967-7975, Petersen et al. (2003) Trends Biotechnol 21:74-81). These molecules have 2'-O,4'-C-ethylene-bridged nucleic acids, with possible modifications such as 2'-deoxy-2''-fluorouridine. Moreover, LNAs increase the specificity of oligonucleotides by constraining the sugar moiety into the 3'-endo conformation, thereby pre-organizing the nucleotide for base pairing and increasing the melting temperature of the oligonucleotide by as much as 10 ^{o}C per base.

[0308] In another exemplary embodiment, the RNA silencing agent of the invention comprises Peptide Nucleic Acids (PNAs). PNAs comprise modified nucleotides in which the sugar-phosphate portion of the nucleotide is replaced with a neutral 2-amino ethylglycine moiety capable of forming a polyamide backbone which is highly resistant to nuclease digestion and imparts improved binding specificity to the molecule (Nielsen, et al., Science, (2001), 254: 1497-1500).

[0309] Also preferred are nucleobase-modified ribonucleotides, i.e., ribonucleotides, containing at least one non-naturally occurring nucleobase instead of a naturally occurring nucleobase. Bases may be modified to block the activity of adenosine deaminase. Exemplary modified nucleobases include, but are not limited to, uridine and/or cytidine modified at the 5-position, e.g., 5-(2-amino)propyl uridine, 5-bromo uridine; adenosine and/or guanosines modified at the 8 position, e.g., 8-bromo guanosine; deaza nucleotides, e.g., 7-deaza-adenosine; O- and N-alkylated nucleotides, e.g., N6-methyl adenosine are suitable. It should be noted that the above modifications may be combined.

[0310] In other embodiments, cross-linking can be employed to alter the pharmacokinetics of the RNA silencing agent, for example, to increase half-life in the body. Thus, the invention includes RNA silencing agents having two complementary strands of nucleic acid, wherein the two strands are crosslinked. The invention also includes RNA silencing agents which are conjugated or unconjugated (e.g., at its 3' terminus) to another moiety (e.g. a non-nucleic acid moiety such as a peptide), an organic compound (e.g., a dye), or the like). Modifying siRNA derivatives in this way may improve cellular uptake or enhance cellular targeting activities of the resulting siRNA derivative as compared to the corresponding siRNA, are useful for tracing the siRNA derivative in the cell, or improve the stability of the siRNA derivative compared to the corresponding siRNA.

[0311] Other exemplary modifications include: (a) 2' modification, e.g., provision of a 2' OMe moiety on a U in a sense or antisense strand, but especially on a sense strand, or provision of a 2' OMe moiety in a 3' overhang, e.g., at the 3' terminus (3' terminus means at the 3' atom of the molecule or at the most 3' moiety, e.g., the most 3' P or 2' position, as indicated by the context); (b) modification of the backbone, e.g., with the replacement of an 0 with an S, in the phosphate backbone, e.g., the provision of a phosphorothioate modification, on the U or the A or both, especially on an antisense strand; e.g., with the replacement of a O with an S; (c) replacement of the U with a C5 amino linker; (d) replacement of an A with a G (sequence changes are preferred to be located on the sense strand and not the antisense strand); and (d) modification at the 2', 6', 7', or 8' position. Exemplary embodiments are those in which one or more of these modifications are present on the sense but not the antisense strand, or embodiments where the antisense strand has fewer of such modifications. Yet other exemplary modifications include the use of a methylated P in a 3' overhang, e.g., at the 3' terminus; combination of a 2' modification, e.g., provision of a 2' O Me moiety and modification of the backbone, e.g., with the replacement of a O with an S, e.g., the provision of a phosphorothioate modification, or the use of a methylated P, in a 3' overhang, e.g., at the 3' terminus; modification with a 3' alkyl; modification with an abasic pyrrolidone in a 3' overhang, e.g., at the 3' terminus; modification with naproxen, ibuprofen, or other moieties which inhibit degradation at the 3' terminus.

4) Modifications to Enhance Cellular Uptake

[0312] In other embodiments, RNA silencing agents may be modified with chemical moieties, for example, to enhance cellular uptake by target cells (e.g., neuronal cells). Thus, the invention includes RNA silencing agents which are conjugated or unconjugated (e.g., at its 3' terminus) to another moiety (e.g. a non-nucleic acid moiety such as a peptide), an organic compound (e.g., a dye), or the like. The conjugation can be accomplished by methods known in the art, e.g., using the methods of Lambert et al., Drug Deliv. Rev.: 47(1), 99-112 (2001) (describes nucleic acids loaded to polyalkylcyanoacrylate (PACA) nanoparticles); Fattal et al., J. Control Release 53(1-3):137-43 (1998) (describes nucleic acids bound to nanoparticles); Schwab et al., Ann. Oncol. 5 Suppl. 4:55-8 (1994) (describes nucleic acids linked to intercalating agents, hydrophobic groups, polycations or PACA nanoparticles); and Godard et al., Eur. J. Biochem. 232(2):404-10 (1995) (describes nucleic acids linked to nanoparticles).

[0313] In a particular embodiment, an RNA silencing agent of invention is conjugated to a lipophilic moiety. In one embodiment, the lipophilic moiety is a ligand that includes a cationic group. In another embodiment, the lipophilic moiety is attached to one or both strands of an siRNA. In an exemplary embodiment, the lipophilic moiety is attached to one end of the sense strand of the siRNA. In another exemplary embodiment, the lipophilic moiety is attached to the 3' end of the sense strand. In certain embodiments, the lipophilic moiety is selected from the group consisting of cholesterol, vitamin E, vitamin K, vitamin A, folic acid, or a cationic dye (e.g., Cy3). In an exemplary embodiment, the lipophilic moiety is a cholesterol. Other lipophilic moieties include cholic acid, adamantane acetic acid, 1-pyrene butyric acid, dihydrotestosterone, 1,3-Bis-O(hexadecyl)glycerol, geranyloxyhexyl group, hexadecylglycerol, borneol, menthol, 1,3-propanediol, heptadecyl group, palmitic acid, myristic acid, O3-(oleoyl)lithocholic acid, O3-(oleoyl)cholenic acid, dimethoxytrityl, or phenoxazine.

5) Tethered Ligands

[0314] Other entities can be tethered to an RNA silencing agent of the invention. For example, a ligand tethered to an RNA silencing agent to improve stability, hybridization thermodynamics with a target nucleic acid, targeting to a particular tissue or cell-type, or cell permeability, e.g., by an endocytosis-dependent or -independent mechanism. Ligands and associated modifications can also increase sequence specificity and consequently decrease off-site targeting. A tethered ligand can include one or more modified bases or sugars that can function as intercalators. These are preferably located in an internal region, such as in a bulge of RNA silencing agent/target duplex. The intercalator can be an aromatic, e.g., a polycyclic aromatic or heterocyclic aromatic compound. A polycyclic intercalator can have stacking capabilities, and can include systems with 2, 3, or 4 fused rings. The universal bases described herein can be included on a ligand. In one embodiment, the ligand can include a cleaving group that contributes to target gene inhibition by cleavage of the target nucleic acid. The cleaving group can be, for example, a bleomycin (e.g., bleomycin-A5, bleomycin-A2, or bleomycin-B2), pyrene, phenanthroline (e.g., O-phenanthroline), a polyamine, a tripeptide (e.g., lys-tyr-lys tripeptide), or metal ion chelating group. The metal ion chelating group can include, e.g., an Lu(III) or EU(III) macrocyclic complex, a Zn(II) 2,9-dimethylphenanthroline derivative, a Cu(II) terpyridine, or acridine, which can promote the selective cleavage of target RNA at the site of the bulge by free metal ions, such as Lu(III). In some embodiments, a peptide ligand can be tethered to a RNA silencing agent to promote

cleavage of the target RNA, e.g., at the bulge region. For example, 1,8-dimethyl-1,3,6,8,10,13-hexaazacyclotetradecane (cyclam) can be conjugated to a peptide (e.g., by an amino acid derivative) to promote target RNA cleavage. A tethered ligand can be an aminoglycoside ligand, which can cause an RNA silencing agent to have improved hybridization properties or improved sequence specificity. Exemplary aminoglycosides include glycosylated polylysine, galactosylated polylysine, neomycin B, tobramycin, kanamycin A, and acridine conjugates of aminoglycosides, such as Neo-N-acridine, Neo-S-acridine, Neo-C-acridine, Tobra-N-acridine, and KanaA-N-acridine. Use of an acridine analog can increase sequence specificity. For example, neomycin B has a high affinity for RNA as compared to DNA, but low sequence-specificity. An acridine analog, neo-5-acridine has an increased affinity for the HIV Rev-response element (RRE). In some embodiments the guanidine analog (the guanidinoglycoside) of an aminoglycoside ligand is tethered to an RNA silencing agent. In a guanidinoglycoside, the amine group on the amino acid is exchanged for a guanidine group. Attachment of a guanidine analog can enhance cell permeability of an RNA silencing agent. A tethered ligand can be a poly-arginine peptide, peptoid or peptidomimetic, which can enhance the cellular uptake of an oligonucleotide agent.

[0315] Exemplary ligands are coupled, preferably covalently, either directly or indirectly via an intervening tether, to a ligand-conjugated carrier. In exemplary embodiments, the ligand is attached to the carrier via an intervening tether. In exemplary embodiments, a ligand alters the distribution, targeting or lifetime of an RNA silencing agent into which it is incorporated. In exemplary embodiments, a ligand provides an enhanced affinity for a selected target, e.g., molecule, cell or cell type, compartment, e.g., a cellular or organ compartment, tissue, organ or region of the body, as, e.g., compared to a species absent such a ligand.

[0316] Exemplary ligands can improve transport, hybridization, and specificity properties and may also improve nuclease resistance of the resultant natural or modified RNA silencing agent, or a polymeric molecule comprising any combination of monomers described herein and/or natural or modified ribonucleotides. Ligands in general can include therapeutic modifiers, e.g., for enhancing uptake; diagnostic compounds or reporter groups e.g., for monitoring distribution; cross-linking agents; nuclease-resistance conferring moieties; and natural or unusual nucleobases. General examples include lipophiles, lipids, steroids (e.g., uvaol, hecigenin, diosgenin), terpenes (e.g., triterpenes, e.g., sarsasapogenin, Friedelin,

epifriedelanol derivatized lithocholic acid), vitamins (e.g., folic acid, vitamin A, biotin, pyridoxal), carbohydrates, proteins, protein binding agents, integrin targeting molecules, polycationics, peptides, polyamines, and peptide mimics. Ligands can include a naturally occurring substance, (e.g., human serum albumin (HSA), low-density lipoprotein (LDL), or globulin); carbohydrate (e.g., a dextran, pullulan, chitin, chitosan, inulin, cyclodextrin or hyaluronic acid); amino acid, or a lipid. The ligand may also be a recombinant or synthetic molecule, such as a synthetic polymer, e.g., a synthetic polyamino acid. Examples of polyamino acids include polyamino acid is a polylysine (PLL), poly L-aspartic acid, poly L-glutamic acid, styrene-maleic acid anhydride copolymer, poly(L-lactide-co-glycolied) copolymer, divinyl ether-maleic anhydride copolymer, N-(2-hydroxypropyl)methacrylamide copolymer (HMPA), polyethylene glycol (PEG), polyvinyl alcohol (PVA), polyurethane, poly(2-ethylacryllic acid), N-isopropylacrylamide polymers, or polyphosphazine. Example of polyamines include: polyethylenimine, polylysine (PLL), spermine, spermidine, polyamine, pseudopeptide-polyamine, peptidomimetic polyamine, dendrimer polyamine, arginine, amidine, protamine, cationic lipid, cationic porphyrin, quaternary salt of a polyamine, or an alpha helical peptide.

[0317] Ligands can also include targeting groups, e.g., a cell or tissue targeting agent, e.g., a lectin, glycoprotein, lipid or protein, e.g., an antibody, that binds to a specified cell type such as a kidney cell. A targeting group can be a thyrotropin, melanotropin, lectin, glycoprotein, surfactant protein A, mucin carbohydrate, multivalent lactose, multivalent galactose, N-acetyl-galactosamine, N-acetyl-glucosamine, multivalent mannose, multivalent fucose, glycosylated polyaminoacids, multivalent galactose, transferrin, bisphosphonate, polyglutamate, polyaspartate, a lipid, cholesterol, a steroid, bile acid, folate, vitamin B12, biotin, or an RGD peptide or RGD peptide mimetic. Other examples of ligands include dyes, intercalating agents (e.g. acridines and substituted acridines), cross-linkers (e.g. psoralene, mitomycin C), porphyrins (TPPC4, texaphyrin, Sapphyrin), polycyclic aromatic hydrocarbons (e.g., phenazine, dihydrophenazine, phenanthroline, pyrenes), lys-tyr-lys tripeptide, aminoglycosides, guanidium aminoglycodies, artificial endonucleases (e.g. EDTA), lipophilic molecules, e.g, cholesterol (and thio analogs thereof), cholic acid, cholanic acid, lithocholic acid, adamantane acetic acid, 1-pyrene butyric acid, dihydrotestosterone, glycerol (e.g., esters (e.g., mono, bis, or tris fatty acid esters, e.g., C_{10}, C_{11}, C_{12}, C_{13}, C_{14}, C_{15}, C_{16}, C_{17}, C_{18}, C_{19}, or C_{20} fatty acids) and ethers thereof, e.g., C_{10}, C_{11}, C_{12}, C_{13}, C_{14}, C_{15}, C_{16}, C_{17}, C_{18}, C_{19}, or C_{20} alkyl; e.g., 1,3-bis-O(hexadecyl)glycerol, 1,3-bis-O(octaadecyl)glycerol),

geranyloxyhexyl group, hexadecylglycerol, borneol, menthol, 1,3-propanediol, heptadecyl group, palmitic acid, stearic acid (e.g., glyceryl distearate), oleic acid, myristic acid, O3-(oleoyl)lithocholic acid, O3-(oleoyl)cholenic acid, dimethoxytrityl, or phenoxazine) and peptide conjugates (e.g., antennapedia peptide, Tat peptide), alkylating agents, phosphate, amino, mercapto, PEG (e.g., PEG-40K), MPEG, $[MPEG]_2$, polyamino, alkyl, substituted alkyl, radiolabeled markers, enzymes, haptens (e.g. biotin), transport/absorption facilitators (e.g., aspirin, naproxen, vitamin E, folic acid), synthetic ribonucleases (e.g., imidazole, bisimidazole, histamine, imidazole clusters, acridine-imidazole conjugates, Eu3+ complexes of tetraazamacrocycles), dinitrophenyl, HRP or AP.

[0318] Ligands can be proteins, e.g., glycoproteins, or peptides, e.g., molecules having a specific affinity for a co-ligand, or antibodies e.g., an antibody, that binds to a specified cell type such as a cancer cell, endothelial cell, or bone cell. Ligands may also include hormones and hormone receptors. They can also include non-peptidic species, such as lipids, lectins, carbohydrates, vitamins, cofactors, multivalent lactose, multivalent galactose, N-acetyl-galactosamine, N-acetyl-glucosamine multivalent mannose, or multivalent fucose. The ligand can be, for example, a lipopolysaccharide, an activator of p38 MAP kinase, or an activator of NF-κB.

[0319] The ligand can be a substance, e.g., a drug, which can increase the uptake of the RNA silencing agent into the cell, for example, by disrupting the cell's cytoskeleton, e.g., by disrupting the cell's microtubules, microfilaments, and/or intermediate filaments. The drug can be, for example, taxon, vincristine, vinblastine, cytochalasin, nocodazole, japlakinolide, latrunculin A, phalloidin, swinholide A, indanocine, or myoservin. The ligand can increase the uptake of the RNA silencing agent into the cell by activating an inflammatory response, for example. Exemplary ligands that would have such an effect include tumor necrosis factor alpha (TNFα), interleukin-1 beta, or gamma interferon. In one aspect, the ligand is a lipid or lipid-based molecule. Such a lipid or lipid-based molecule preferably binds a serum protein, e.g., human serum albumin (HSA). An HSA binding ligand allows for distribution of the conjugate to a target tissue, e.g., a non-kidney target tissue of the body. For example, the target tissue can be the liver, including parenchymal cells of the liver. Other molecules that can bind HSA can also be used as ligands. For example, neproxin or aspirin can be used. A lipid or lipid-based ligand can (a) increase resistance to degradation of the conjugate, (b) increase targeting or transport into a target cell or cell membrane, and/or (c) can be used to adjust binding to a serum protein, e.g., HSA. A lipid

based ligand can be used to modulate, e.g., control the binding of the conjugate to a target tissue. For example, a lipid or lipid-based ligand that binds to HSA more strongly will be less likely to be targeted to the kidney and therefore less likely to be cleared from the body. A lipid or lipid-based ligand that binds to HSA less strongly can be used to target the conjugate to the kidney. In a preferred embodiment, the lipid based ligand binds HSA. A lipid-based ligand can bind HSA with a sufficient affinity such that the conjugate will be preferably distributed to a non-kidney tissue. However, it is preferred that the affinity not be so strong that the HSA-ligand binding cannot be reversed. In another preferred embodiment, the lipid based ligand binds HSA weakly or not at all, such that the conjugate will be preferably distributed to the kidney. Other moieties that target to kidney cells can also be used in place of or in addition to the lipid based ligand.

[0320] In another aspect, the ligand is a moiety, e.g., a vitamin, which is taken up by a target cell, e.g., a proliferating cell. These are particularly useful for treating disorders characterized by unwanted cell proliferation, e.g., of the malignant or non-malignant type, e.g., cancer cells. Exemplary vitamins include vitamin A, E, and K. Other exemplary vitamins include are B vitamin, e.g., folic acid, B12, riboflavin, biotin, pyridoxal or other vitamins or nutrients taken up by cancer cells. Also included are HSA and low density lipoprotein (LDL).

[0321] In another aspect, the ligand is a cell-permeation agent, preferably a helical cell-permeation agent. Preferably, the agent is amphipathic. An exemplary agent is a peptide such as tat or antennopedia. If the agent is a peptide, it can be modified, including a peptidylmimetic, invertomers, non-peptide or pseudo-peptide linkages, and use of D-amino acids. The helical agent is preferably an alpha-helical agent, which preferably has a lipophilic and a lipophobic phase.

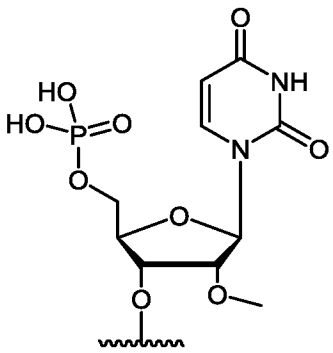
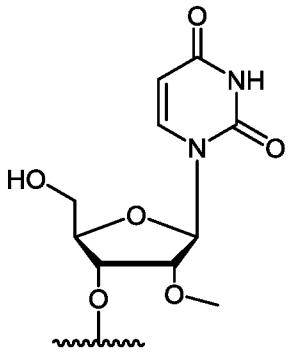
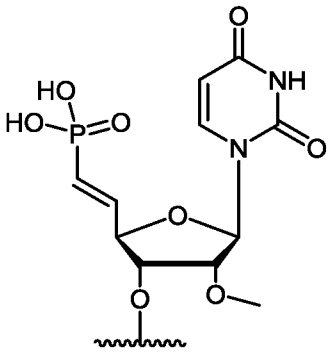
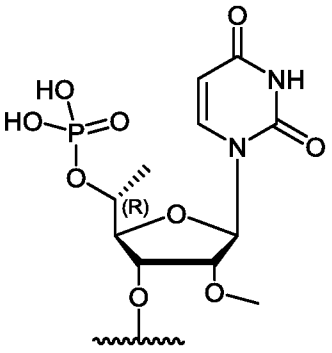
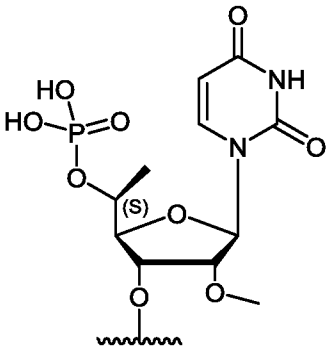
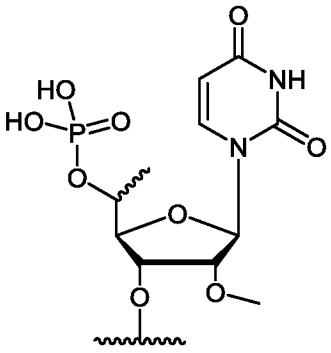
[0322] The ligand can be a peptide or peptidomimetic. A peptidomimetic (also referred to herein as an oligopeptidomimetic) is a molecule capable of folding into a defined three-dimensional structure similar to a natural peptide. The attachment of peptide and peptidomimetics to oligonucleotide agents can affect pharmacokinetic distribution of the RNA silencing agent, such as by enhancing cellular recognition and absorption. The peptide or peptidomimetic moiety can be about 5-50 amino acids long, e.g., about 5, 10, 15, 20, 25, 30, 35, 40, 45, or 50 amino acids long. A peptide or peptidomimetic can be, for example, a cell permeation peptide, cationic peptide, amphipathic peptide, or hydrophobic peptide (e.g., consisting primarily of Tyr, Trp or Phe). The peptide moiety can be a dendrimer peptide,

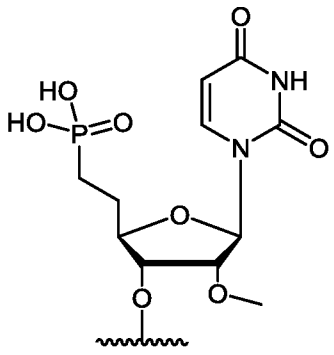
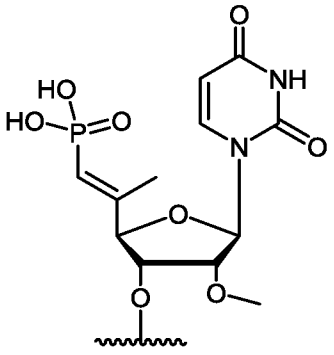
constrained peptide or crosslinked peptide. The peptide moiety can be an L-peptide or D-peptide. In another alternative, the peptide moiety can include a hydrophobic membrane translocation sequence (MTS). A peptide or peptidomimetic can be encoded by a random sequence of DNA, such as a peptide identified from a phage-display library, or one-bead-one-compound (OBOC) combinatorial library (Lam et al., Nature 354:82-84, 1991). In exemplary embodiments, the peptide or peptidomimetic tethered to an RNA silencing agent via an incorporated monomer unit is a cell targeting peptide such as an arginine-glycine-aspartic acid (RGD)-peptide, or RGD mimic. A peptide moiety can range in length from about 5 amino acids to about 40 amino acids. The peptide moieties can have a structural modification, such as to increase stability or direct conformational properties. Any of the structural modifications described below can be utilized.

6) Compounds

[0323] In one aspect, provided herein is a compound of the Formula shown in Figure 81, or a pharmaceutically acceptable salt thereof, wherein

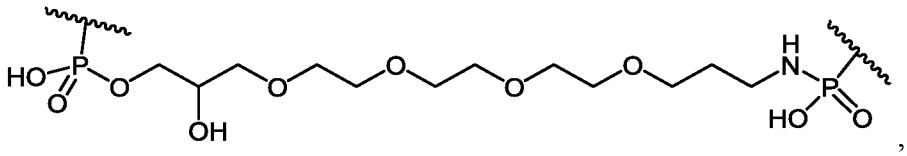
R^1 is selected from the group consisting of:



, and ;

R^3 is independently selected at each occurrence from the group consisting of an internucleotide linker as shown in Figure 82; and

L is a linker connecting two moieties, wherein the linker is selected from the group consisting of an ethylene glycol chain, an alkyl chain, a peptide, an RNA, a DNA,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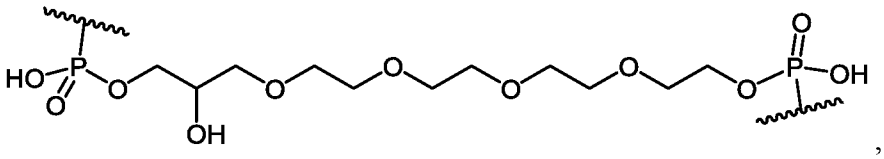
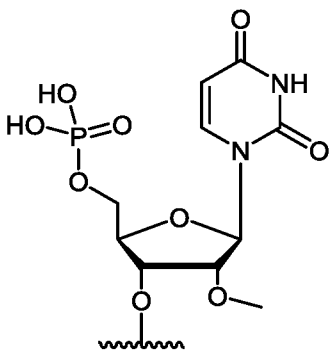

,

or a combination thereof.

[0324] In one embodiment, R^1 is selected from the group consisting of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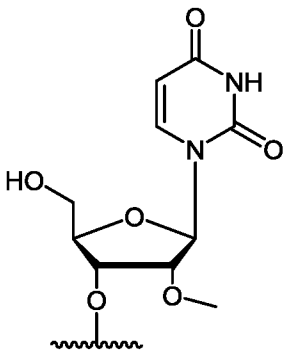
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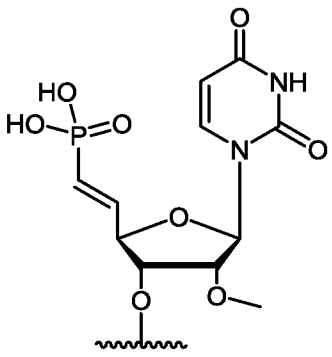
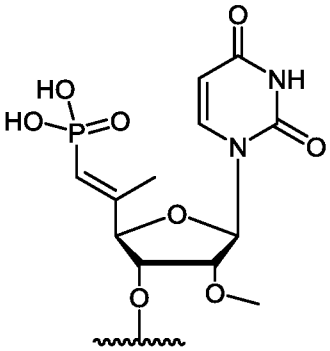
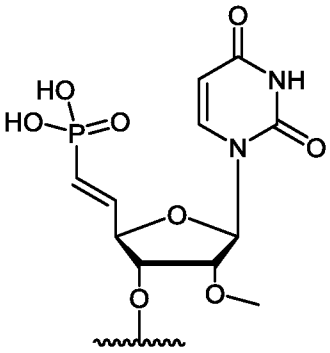

, and



[0325] In another embodiment, R^1 is



[0326] In another embodiment, R^3 is an internucleotide linker independently selected at each occurrence from the group consisting of a phosphorothioate, a phosphorodithioate, a methylphosphonate, a methylenephosphonate, a phosphotriester, and a boranophosphate.

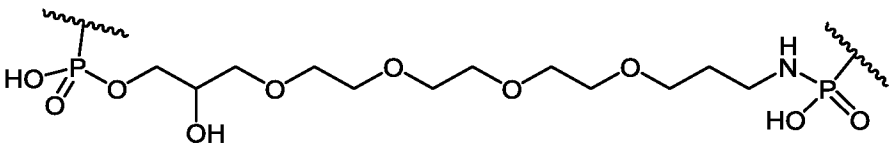
[0327] In another embodiment, R^3 is an internucleotide linker independently selected at each occurrence from the group consisting of a phosphorothioate, a phosphorodithioate, and a boranophosphate.

[0328] In another embodiment, R^3 is a phosphorothioate.

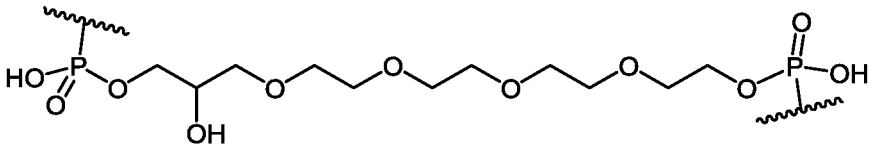
[0329] In another embodiment, L is selected from the group consisting of an ethylene glycol chain, an alkyl chain, and a peptide.

[0330] In another embodiment, L is selected from an ethylene glycol chain or a peptide.

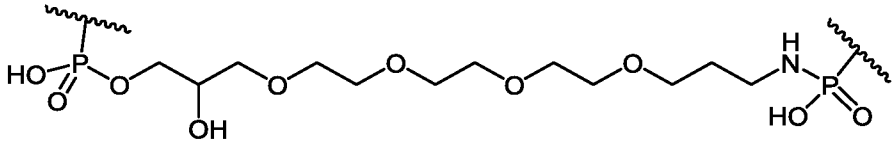
[0331] In yet another embodiment, L is



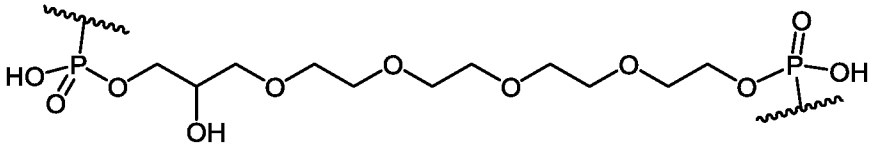
or



[0332] In still another embodiment, L is



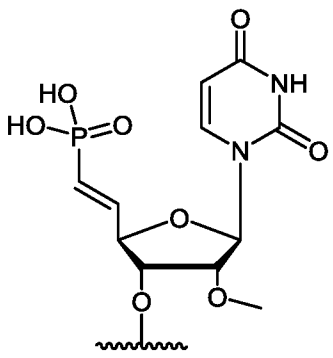
[0333] In another embodiment, L is



[0334] In one embodiment, the compound of the Formula shown in Figure 81 is a compound the Formula shown in Figure 83.

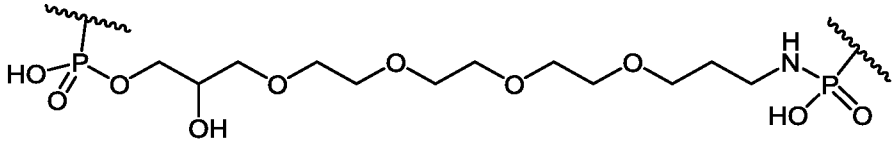
[0335] In another embodiment, the compound of the Formula shown in Figure 81 is a compound of the Formula shown in Figure 83, or a pharmaceutically acceptable salt thereof, wherein

R^1 is



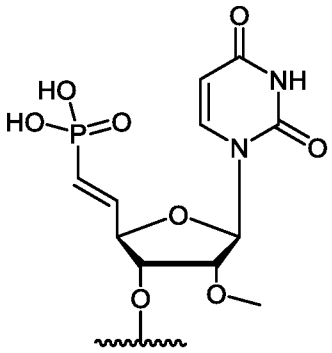
; and

L is

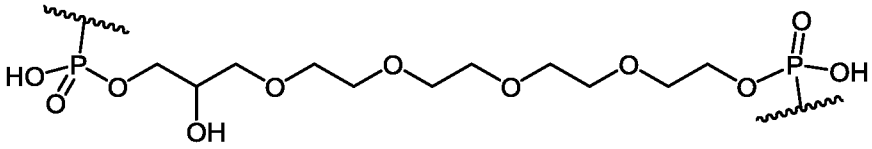


[0336] In another embodiment, the compound of the Formula shown in Figure 81 is a compound of the Formula shown in Figure 83, or a pharmaceutically acceptable salt thereof, wherein

R^1 is

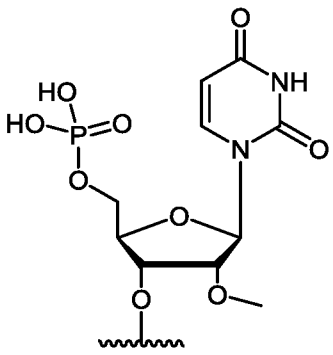
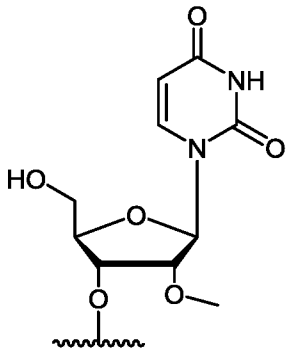
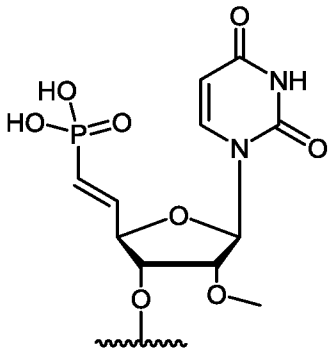

; and

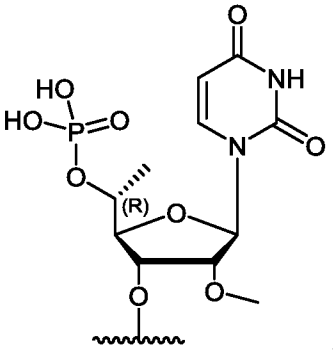
L is


.

[0337] In another aspect, provided herein is a compound of the Formula shown in Figure 84, or a pharmaceutically acceptable salt thereof, wherein

R^1 is selected from the group consisting of


,

,

,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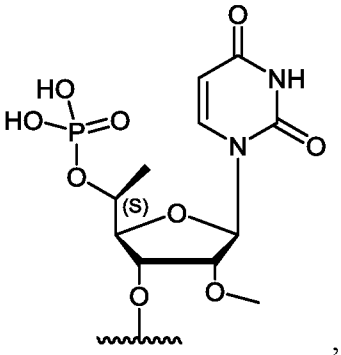
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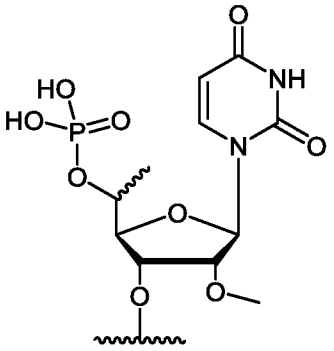
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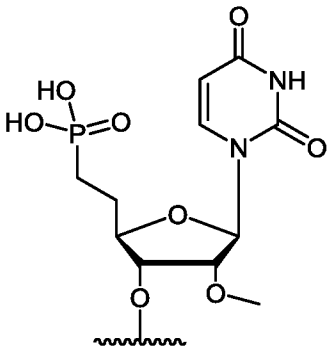
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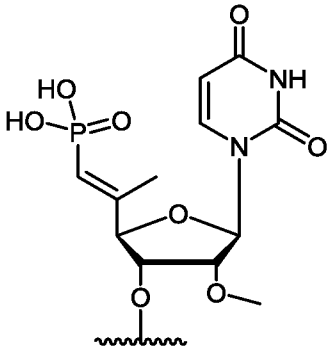
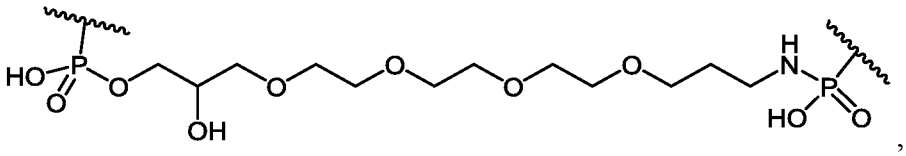

;

R^3 is independently selected at each occurrence from the group consisting of an internucleotide linker as shown in Figure 82;

L is a linker connecting two moieties, wherein the linker is selected from the group consisting of an ethylene glycol chain, an alkyl chain, a peptide, an RNA, a DNA,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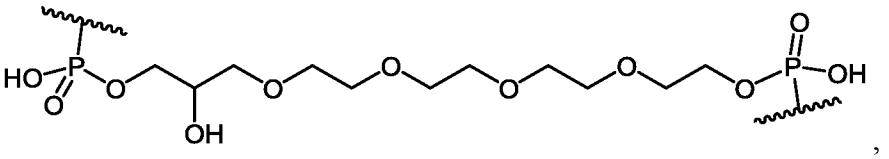
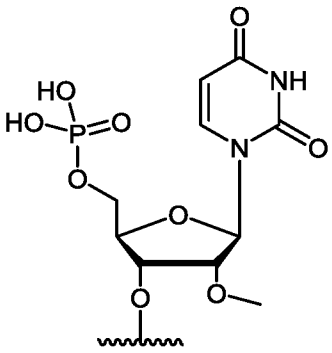

,

or a combination thereof; and

B is a branch point between two or more linkers, wherein the branch point is selected from the group consisting of a glycol, an amino acid, or any poly-valent organic species.

[0338] In one embodiment, R^1 is selected from the group consisting of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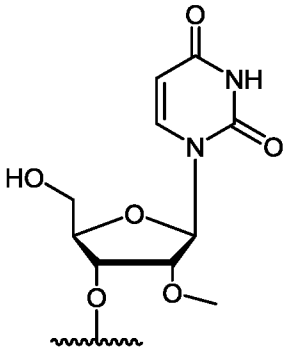
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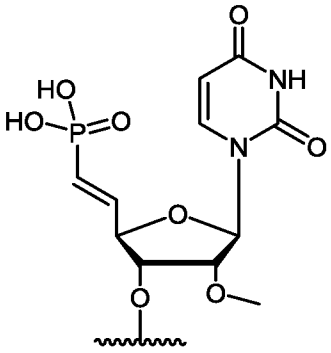
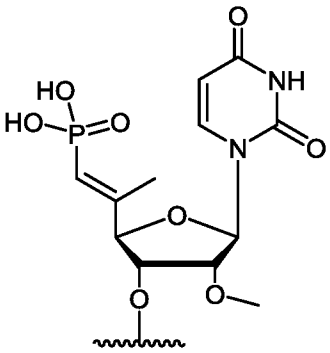
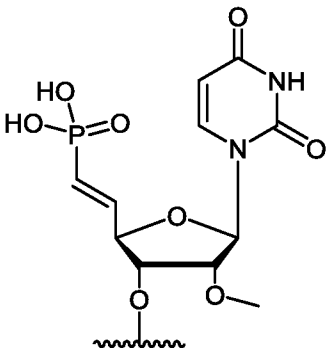

, and

.

[0339] In another embodiment, R^1 is


.

[0340] In another embodiment, R^3 is an internucleotide linker independently selected at each occurrence from the group consisting of a phosphorothioate, a phosphorodithioate, a methylphosphonate, a methylenephosphonate, a phosphotriester, and a boranophosphate.

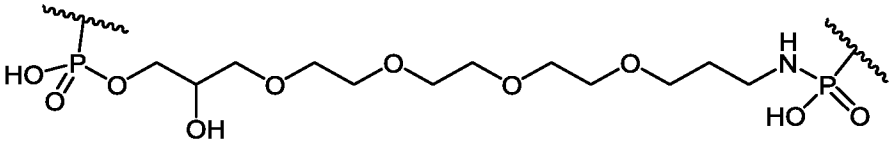
[0341] In another embodiment, R^3 is an internucleotide linker independently selected at each occurrence from the group consisting of a phosphorothioate, a phosphorodithioate, and a boranophosphate.

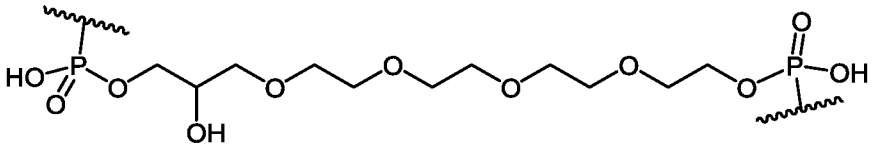
[0342] In another embodiment, R^3 is a phosphorothioate.

[0343] In another embodiment, L is selected from the group consisting of an ethylene glycol chain, an alkyl chain, and a peptide.

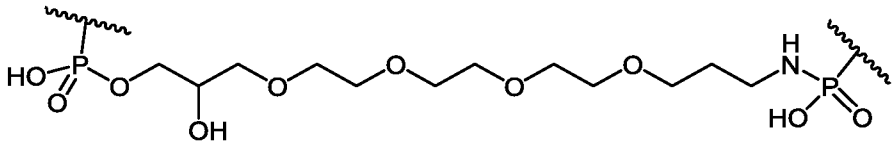
[0344] In another embodiment, L is selected from an ethylene glycol chain or a peptide.

[0345] In yet another embodiment, L is

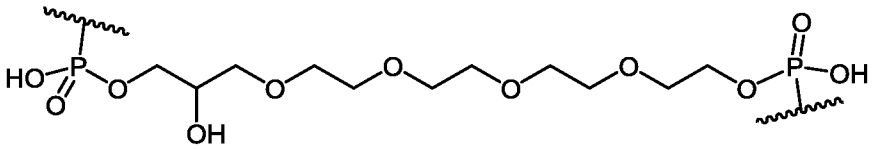

or


.

[0346] In still another embodiment, L is


.

[0347] In another embodiment, L is

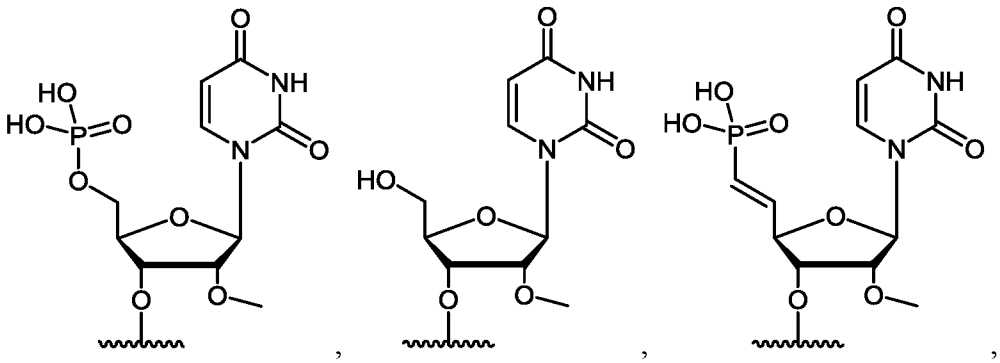

.

[0348] In one embodiment, B is a branch point between two or more linkers, wherein the branch point is selected a glycol or an amino acid. In another embodiment, the branch point is a glycol. In another embodiment, the branch point is an amino acid.

[0349] In another embodiment of the compound of the Formula shown in Figure 84, Y is defined as shown in Figure 86.

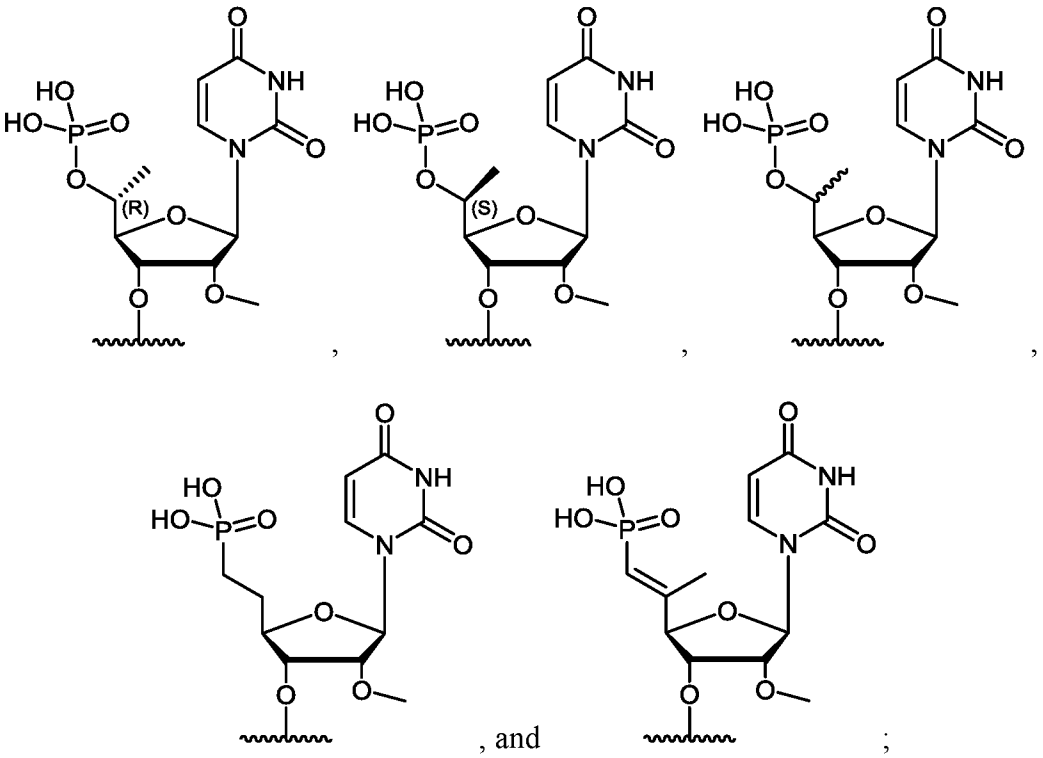
[0350] In another aspect, provided herein is a compound of the Formula shown in Figure 85, or a pharmaceutically acceptable salt thereof, wherein

[0351] R^1 is selected from the group consisting of


,

,

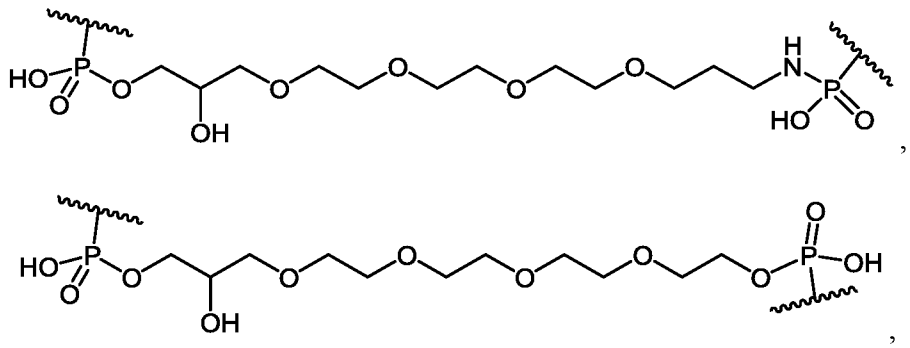
,



, and ;

R^3 is independently selected at each occurrence from the group consisting of an internucleotide linker as shown in Figure 82;

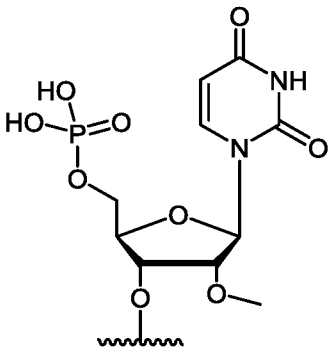
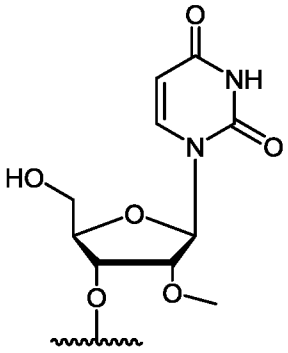
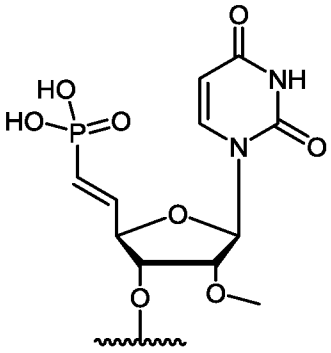
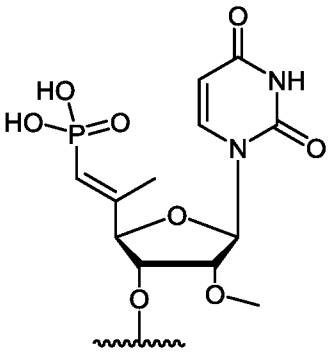
L is a linker connecting two moieties, wherein the linker is selected from the group consisting of an ethylene glycol chain, an alkyl chain, a peptide, an RNA, a DNA,



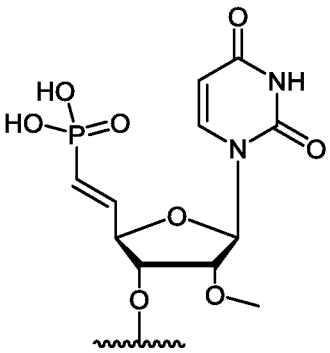
or a combination thereof; and

B is a branch point between two or more linkers, wherein the branch point is selected from the group consisting of a glycol, an amino acid, or any polyvalent organic species.

[0352] In one embodiment, R^1 is selected from the group consisting of


,

,

, and

.

[0353] In another embodiment, R^1 is


.

[0354] In another embodiment, R^3 is an internucleotide linker independently selected at each occurrence from the group consisting of a phosphorothioate, a phosphorodithioate, a methylphosphonate, a methylenephosphonate, a phosphotriester, and a boranophosphate.

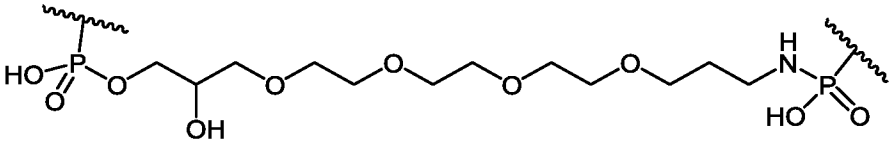
[0355] In another embodiment, R^3 is an internucleotide linker independently selected at each occurrence from the group consisting of a phosphorothioate, a phosphorodithioate, and a boranophosphate.

[0356] In another embodiment, R^3 is a phosphorothioate.

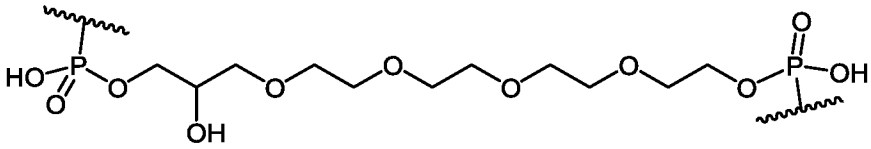
[0357] In another embodiment, L is selected from the group consisting of an ethylene glycol chain, an alkyl chain, and a peptide.

[0358] In another embodiment, L is selected from an ethylene glycol chain or a peptide.

[0359] In yet another embodiment, L is

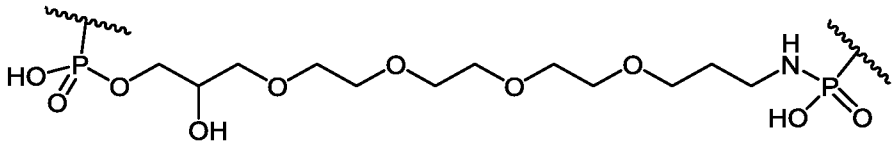


or



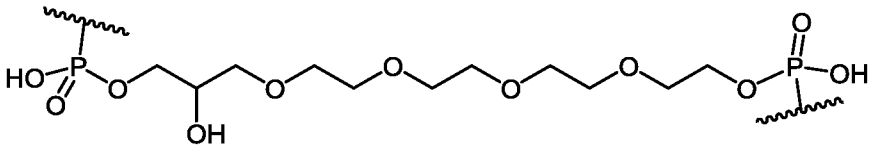
.

[0360] In still another embodiment, L is



.

[0361] In another embodiment, L is



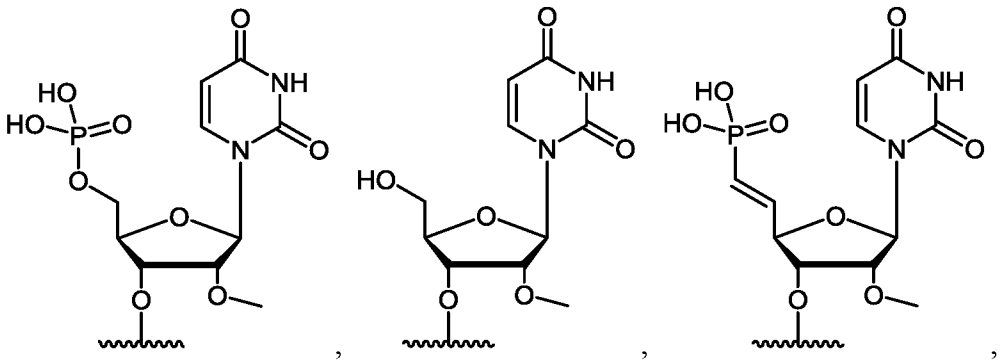
.

[0362] In one embodiment, B is a branch point between two or more linkers, wherein the branch point is selected a glycol or an amino acid. In another embodiment, the branch point is a glycol. In another embodiment, the branch point is an amino acid.

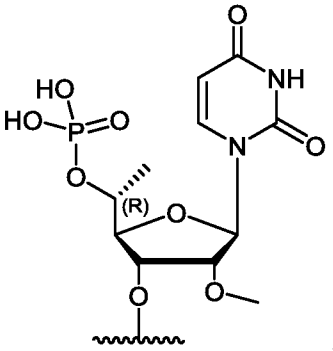
[0363] In one embodiment of the compound of the Formula shown in Figure 85, Y is defined as shown in Figure 86.

[0364] In another aspect, provided herein is a compound of the Formula shown in Figure 87, or a pharmaceutically acceptable salt thereof, wherein

R^1 is selected from the group consisting of



, , ,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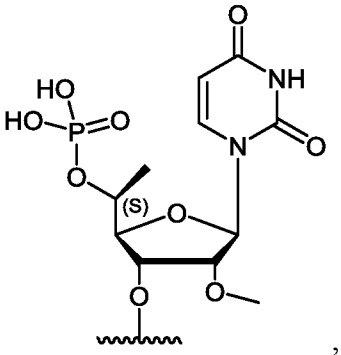
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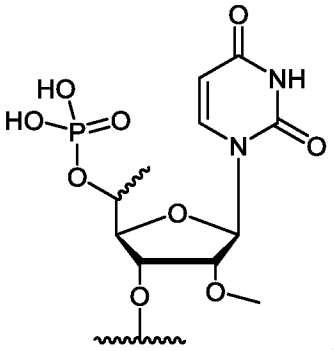
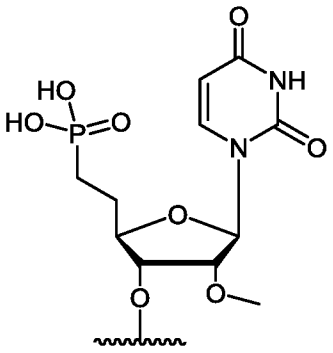
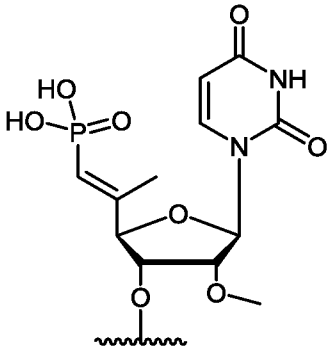
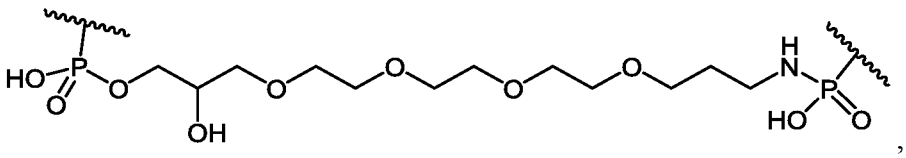

,

, and

;

R^3 is independently selected at each occurrence from the group consisting of an internucleotide linker as shown in Figure 82;

L is a linker connecting two moieties, wherein the linker is selected from the group consisting of an ethylene glycol chain, an alkyl chain, a peptide, an RNA, a DNA,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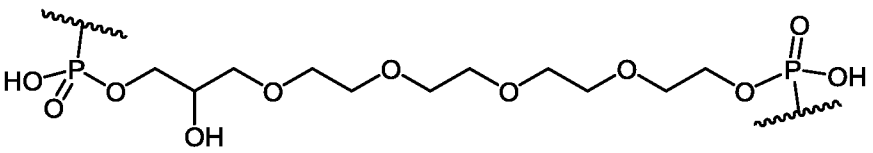
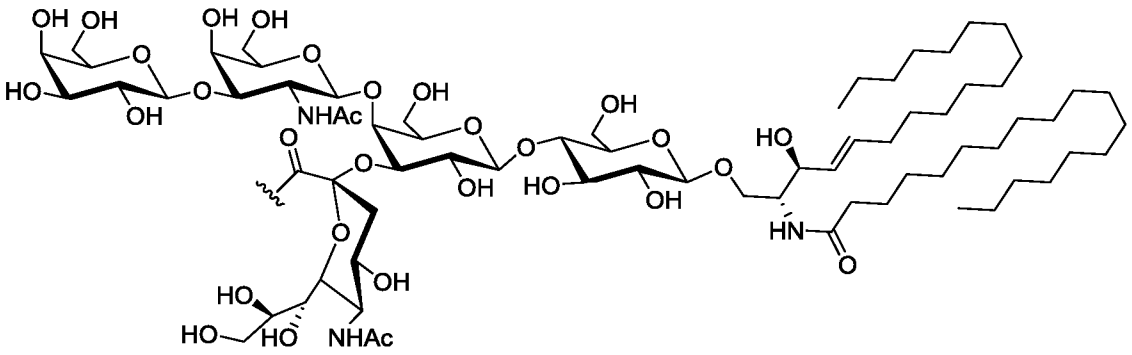

,

or a combination thereof; and

R^2 is selected from the group consisting of an alkyl chain (e.g., C_{1-6}, C_{1-10}, C_{1-20}, C_{1-30}, or C_{1-40}), a vitamin, a ligand, a peptide, a bioactive conjugate (including, but not limited to glycosphingolipids, polyunsaturated fatty acids, secosteroids, steroid hormones, or sterol lipids),



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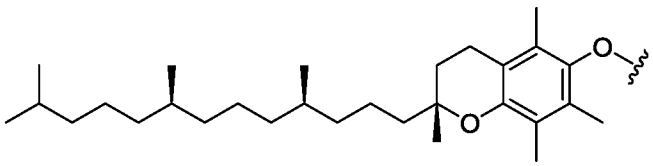

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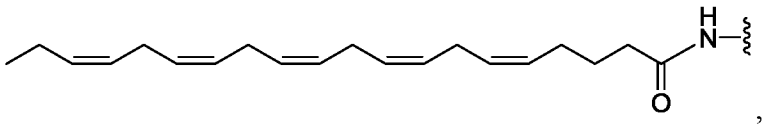

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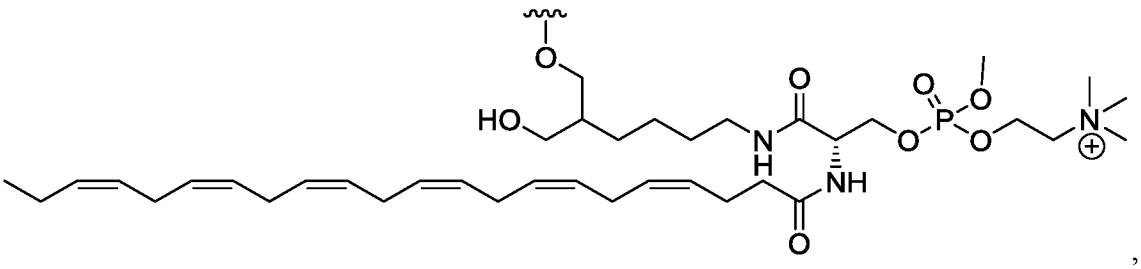

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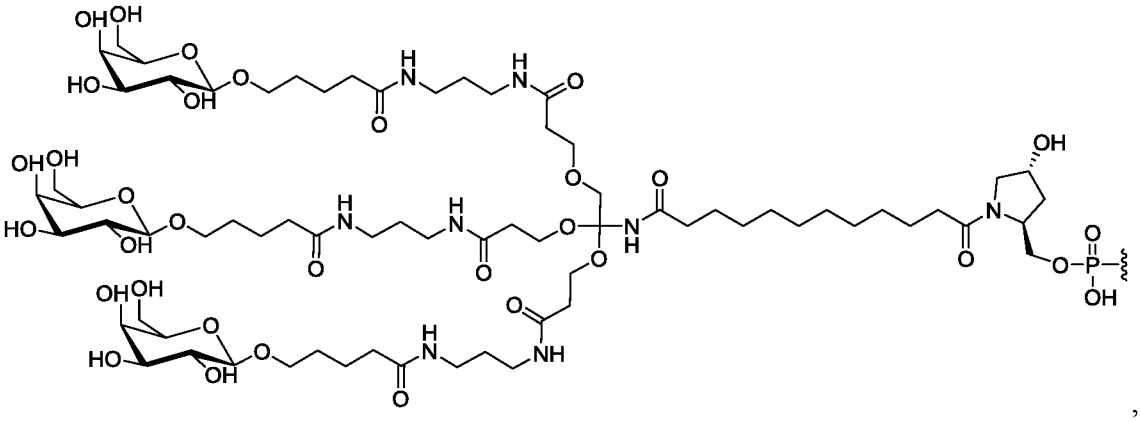

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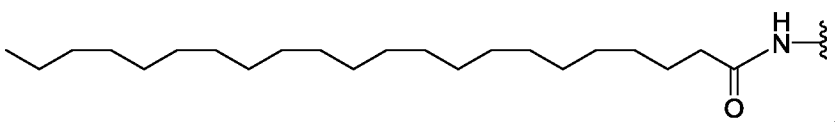

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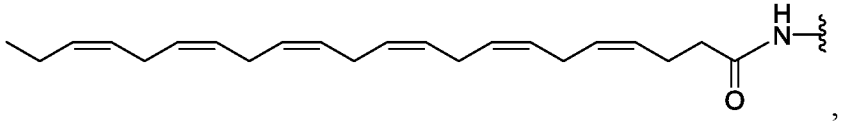

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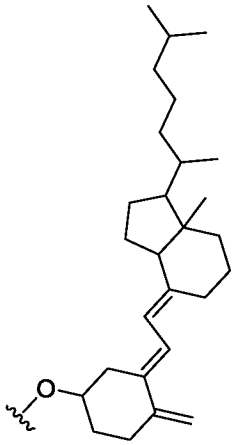
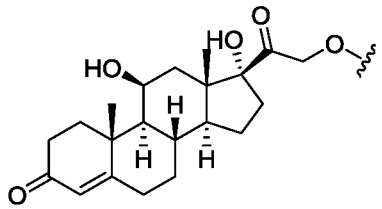
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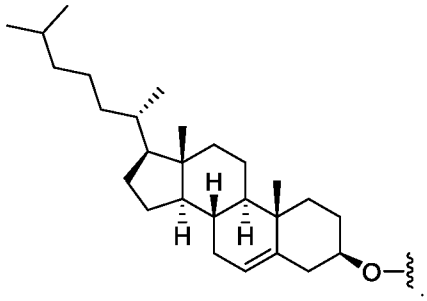
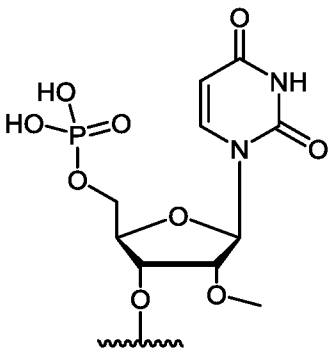

.

[0365] In one embodiment, R^1 is selected from the group consisting of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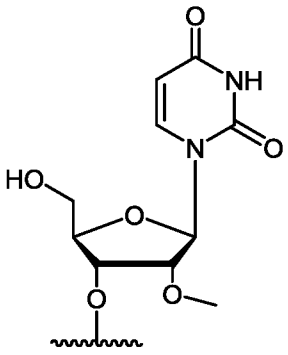
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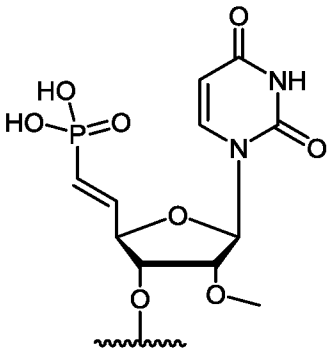
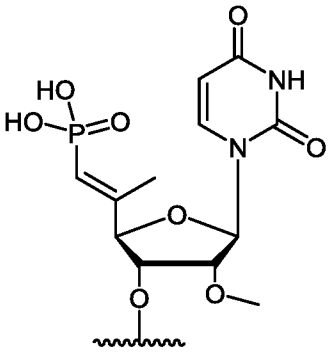
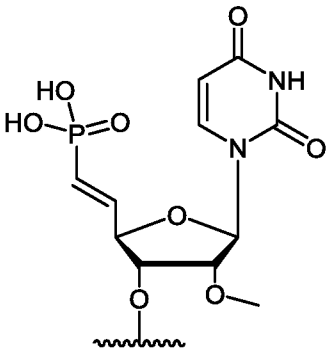
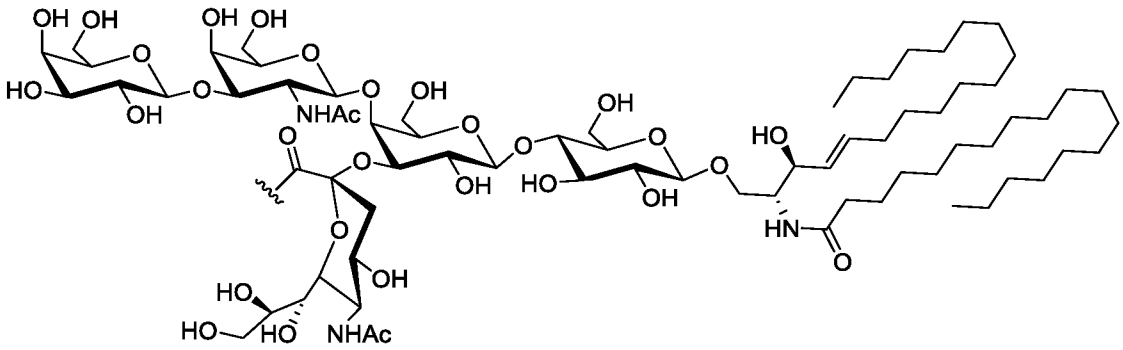

, and

.

[0366] In another embodiment, R^1 is


.

[0367] In another embodiment, R^2 is selected from the group consisting of



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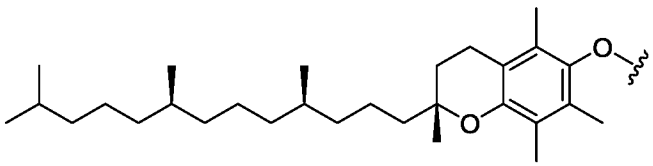

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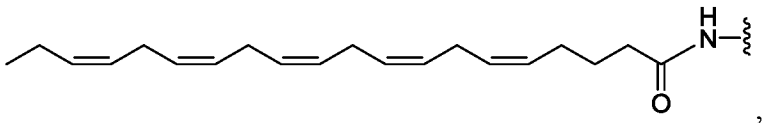

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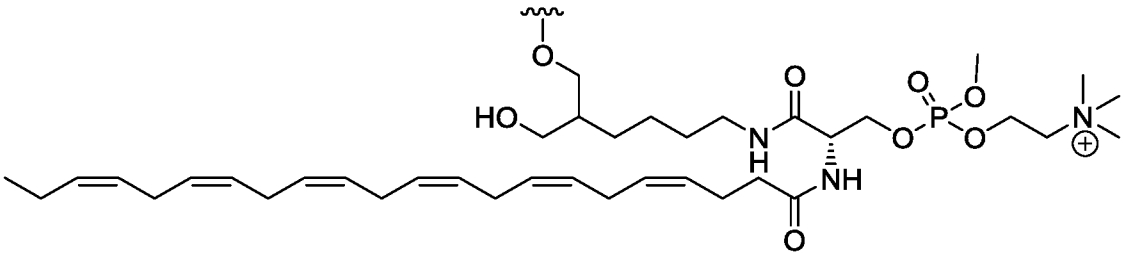

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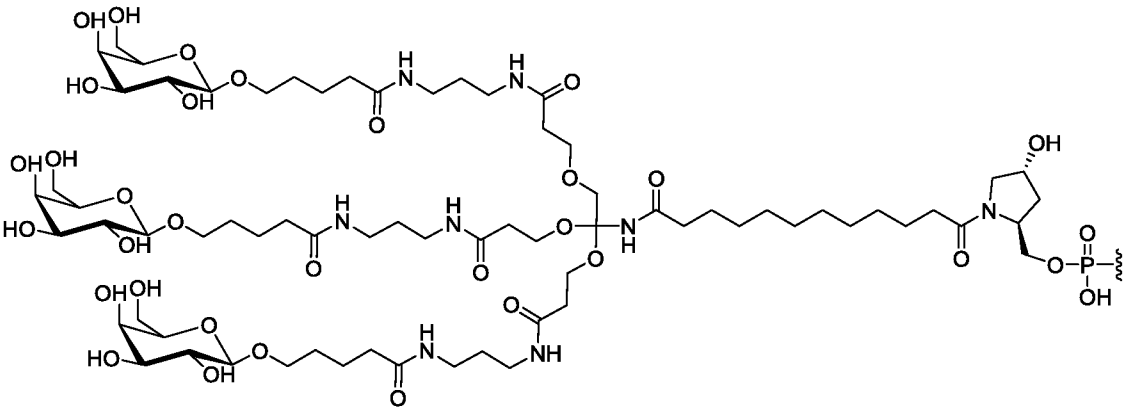

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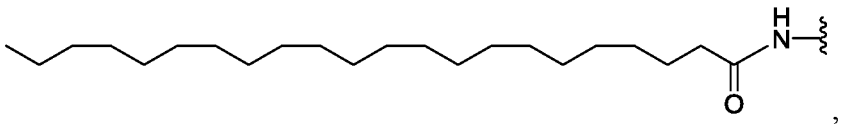

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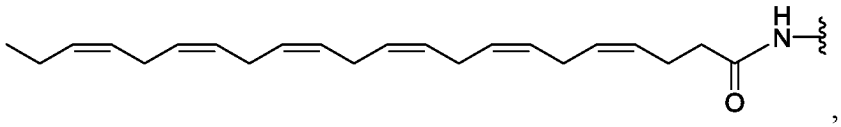

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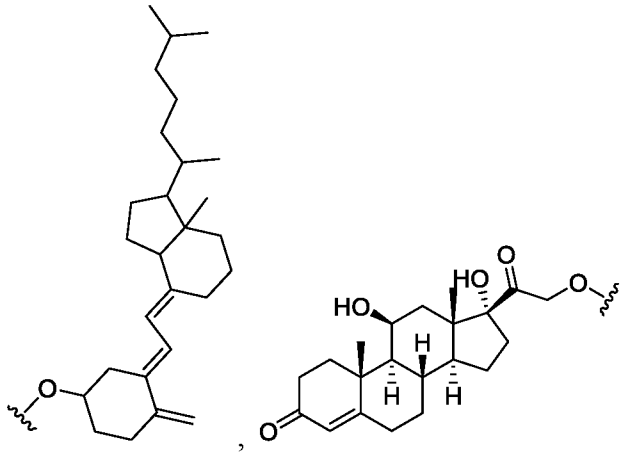
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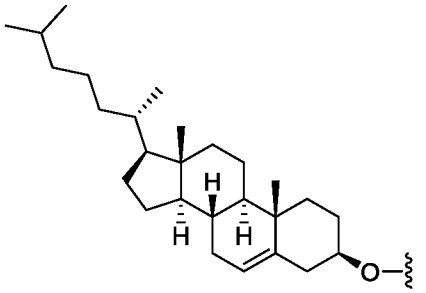

.

[0368] In another embodiment, R^3 is an internucleotide linker independently selected at each occurrence from the group consisting of a phosphorothioate, a phosphorodithioate, a methylphosphonate, a methylenephosphonate, a phosphotriester, and a boranophosphate.

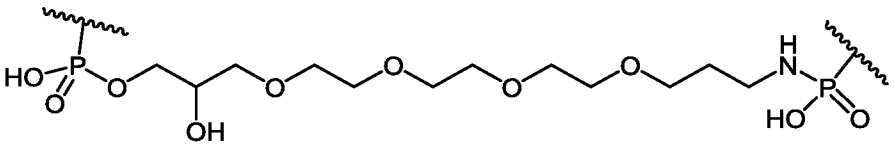
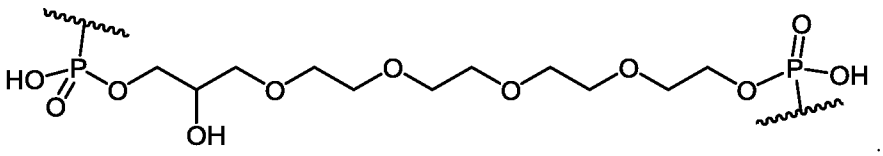
[0369] In another embodiment, R^3 is an internucleotide linker independently selected at each occurrence from the group consisting of a phosphorothioate, a phosphorodithioate, and a boranophosphate.

[0370] In another embodiment, R^3 is a phosphorothioate.

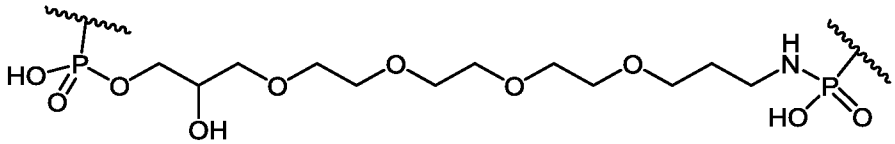
[0371] In another embodiment, L is selected from the group consisting of an ethylene glycol chain, an alkyl chain, and a peptide.

[0372] In another embodiment, L is selected from an ethylene glycol chain or a peptide.

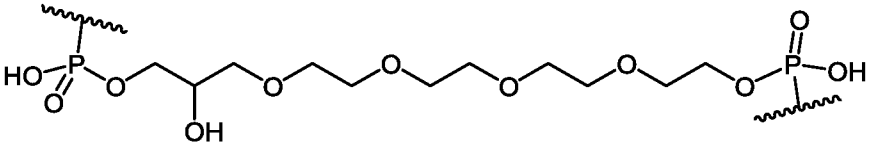
[0373] In yet another embodiment, L is


or

.

[0374] In still another embodiment, L is


.

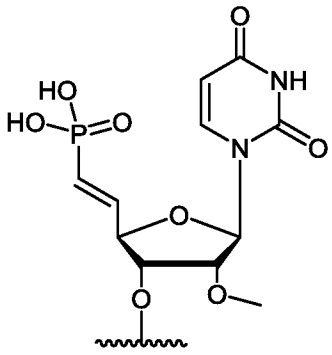
[0375] In another embodiment, L is



[0376] In one embodiment, the compound of the Formula shown in Figure 87 is a compound the Formula shown in Figure 88.

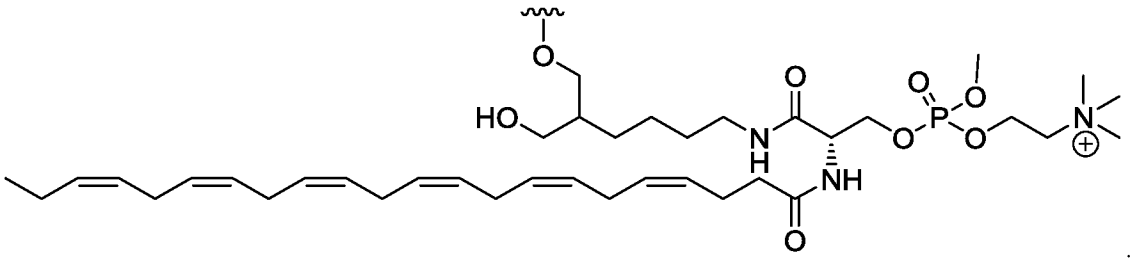
[0377] In another embodiment, the compound of the Formula shown in Figure 87, is a compound of the Formula shown in Figure 88, or a pharmaceutically acceptable salt thereof, wherein

R^1 is



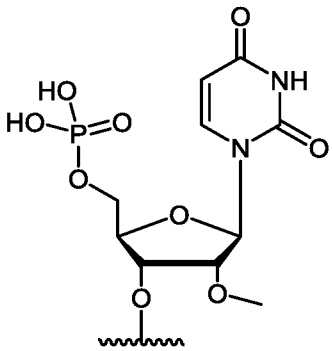
, and

R^2 is



[0378] In another aspect, provided herein is a compound of the Formula shown in Figure 89, or a pharmaceutically acceptable salt thereof, wherein

R^1 is selected from the group consisting of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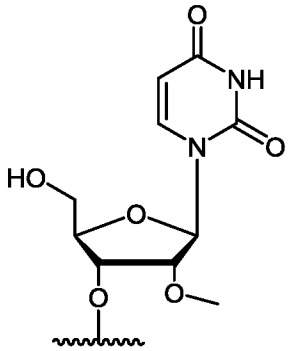
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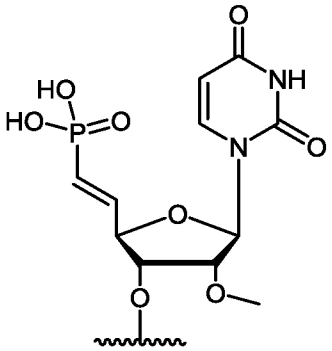
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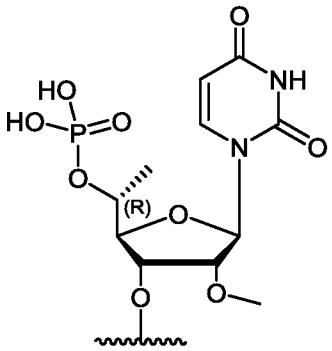
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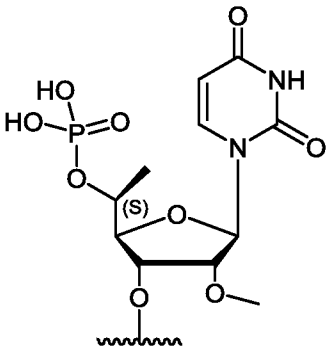
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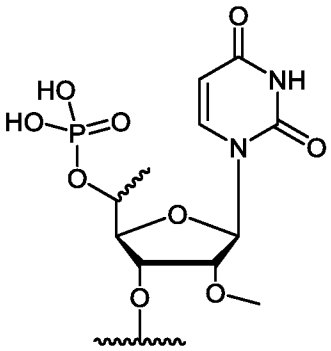
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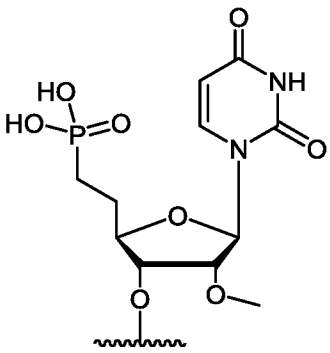
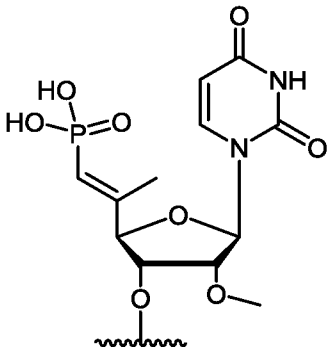
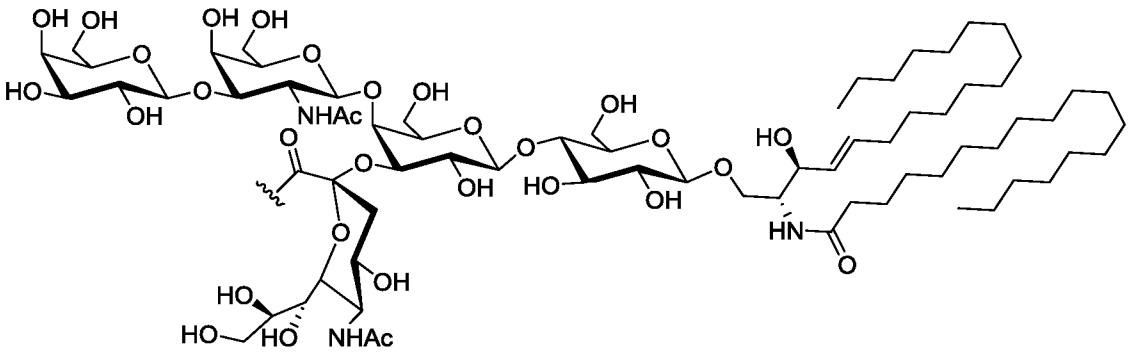
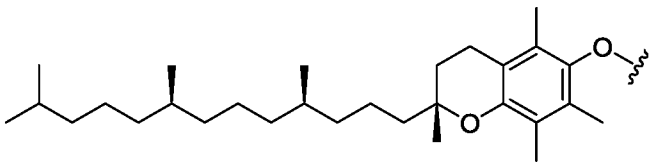

, and

;

R^3 is independently selected at each occurrence from the group consisting of an internucleotide linker as shown in Figure 82; and

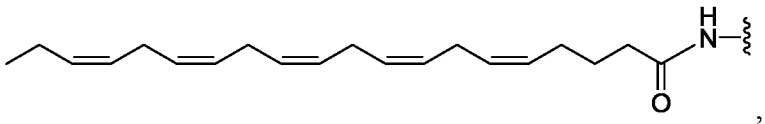
R^2 is selected from the group consisting of an alkyl chain (e.g., C_{1-6}, C_{1-10}, C_{1-20}, C_{1-30}, or C_{1-40}), a vitamin, a ligand, a peptide, a bioactive conjugate (including, but not limited to glycosphingolipids, polyunsaturated fatty acids, secosteroids, steroid hormones, or sterol lipids),



,



,



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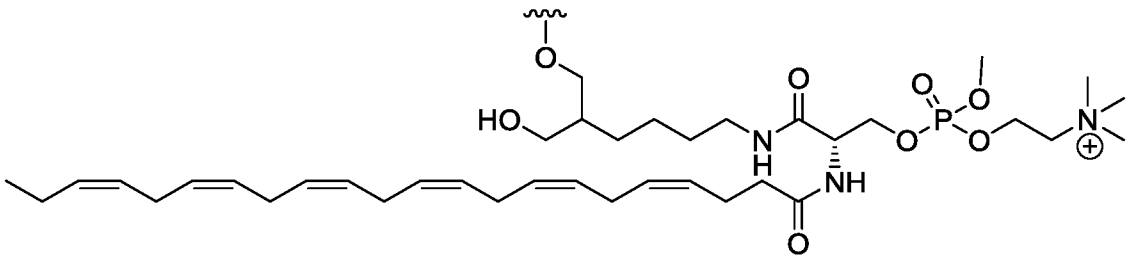

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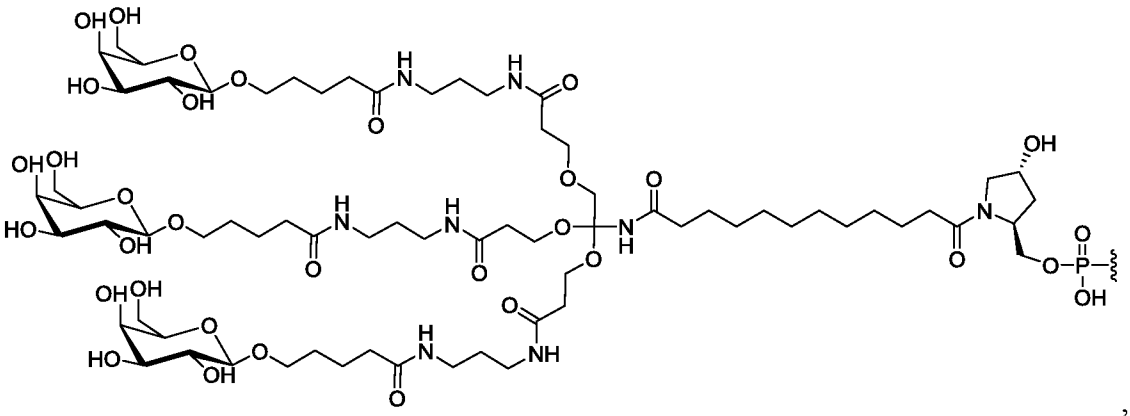

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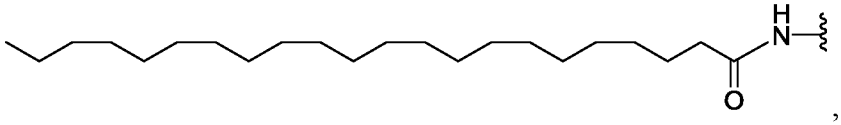

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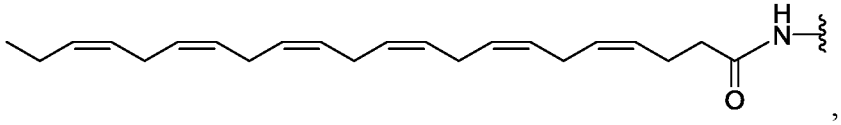

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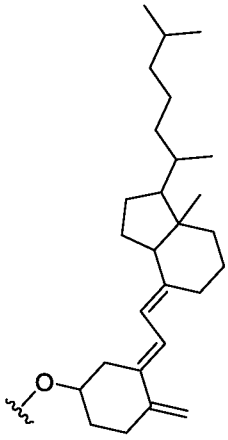
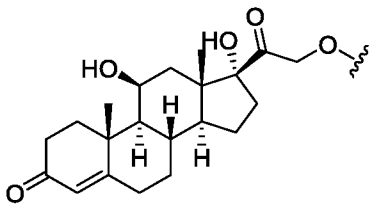
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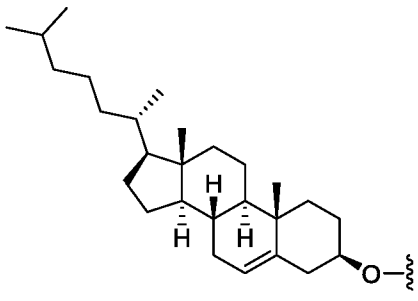
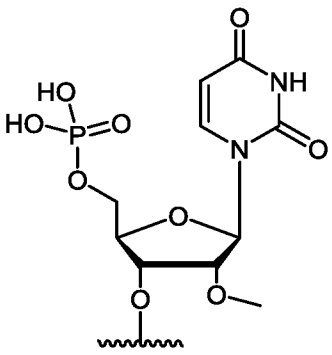


[0379] In one embodiment, R^1 is selected from the group consisting of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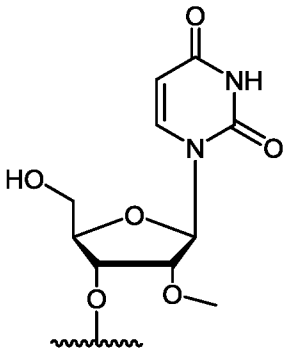
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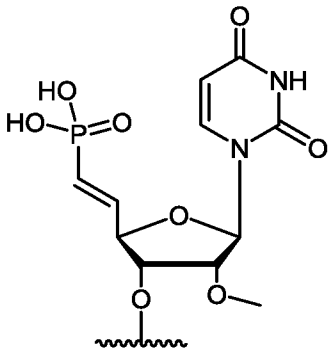
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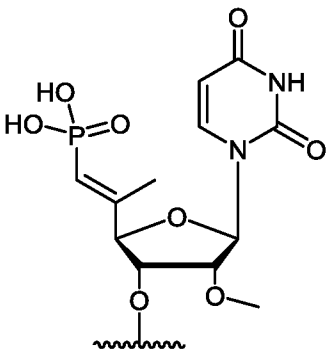
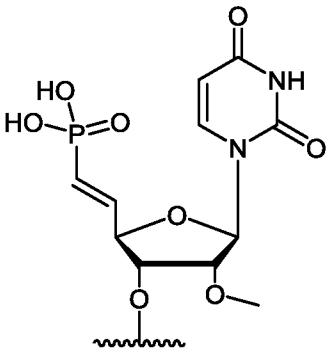
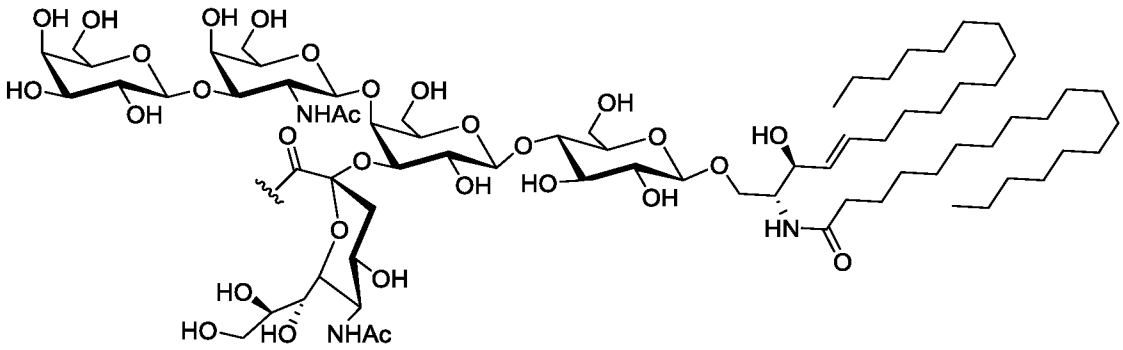


[0380] In another embodiment, R^1 is



[0381] In another embodiment, R^2 is selected from the group consisting of



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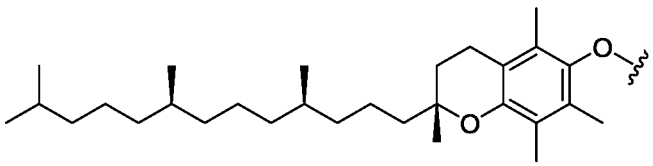

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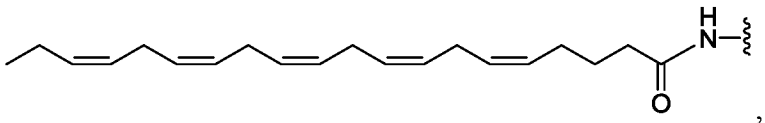

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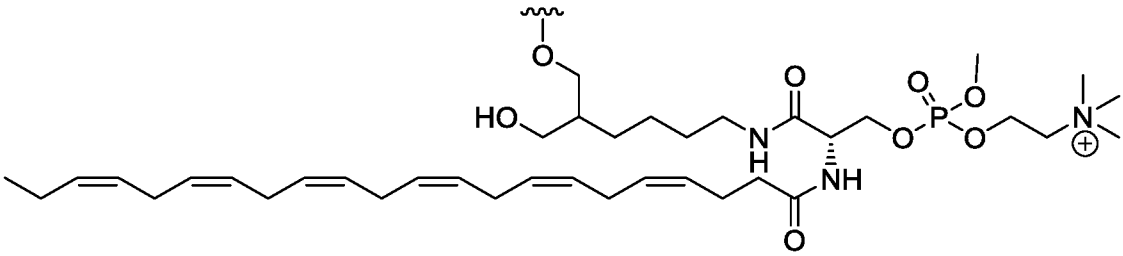

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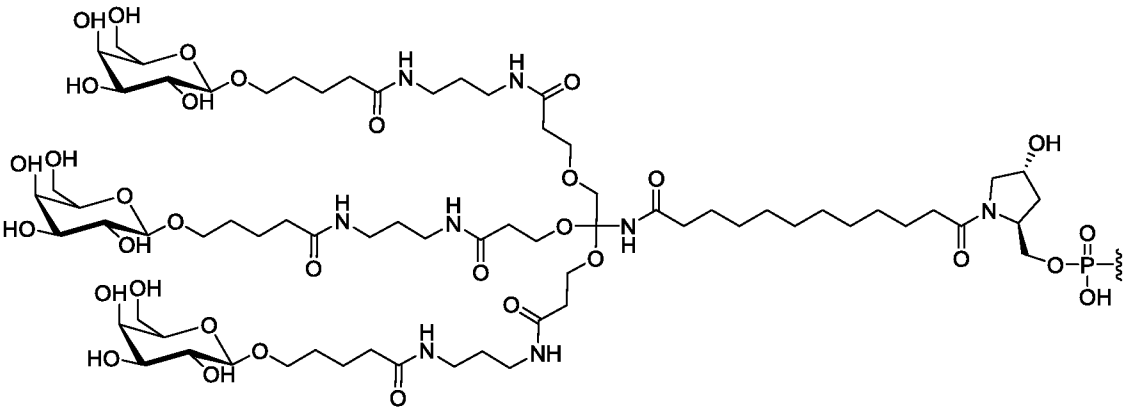

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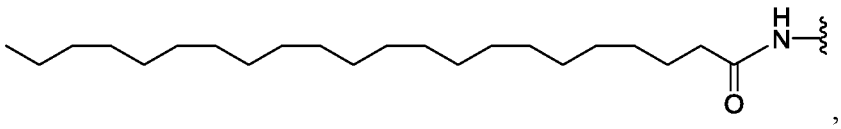

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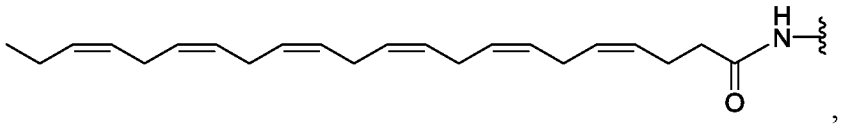

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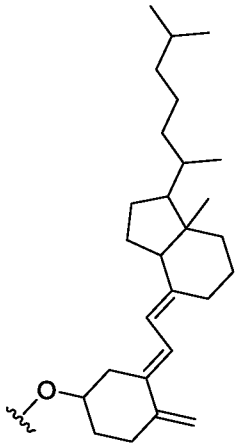
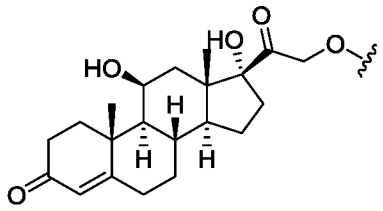
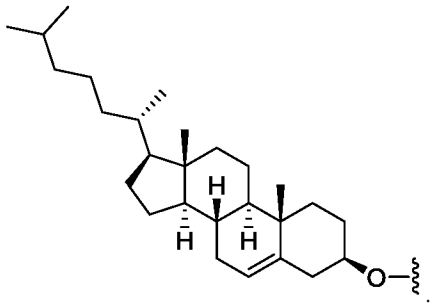




[0382] In another embodiment, R^3 is an internucleotide linker independently selected at each occurrence from the group consisting of a phosphorothioate, a phosphorodithioate, a methylphosphonate, a methylenephosphonate, a phosphotriester, and a boranophosphate.

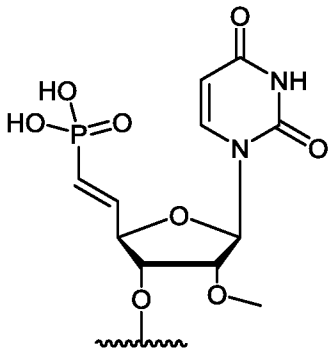
[0383] In another embodiment, R^3 is an internucleotide linker independently selected at each occurrence from the group consisting of a phosphorothioate, a phosphorodithioate, and a boranophosphate.

[0384] In another embodiment, R^3 is a phosphorothioate.

[0385] In one embodiment, the compound of the Formula shown in Figure 89 is a compound of the Formula shown in Figure 90.

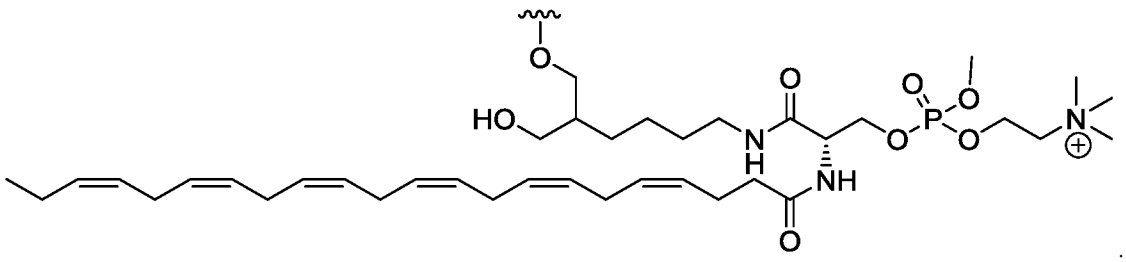
[0386] In one embodiment, the compound of the Formula shown in Figure 89, is a compound of the Formula shown in Figure 90, or a pharmaceutically acceptable salt thereof, wherein

R^1 is



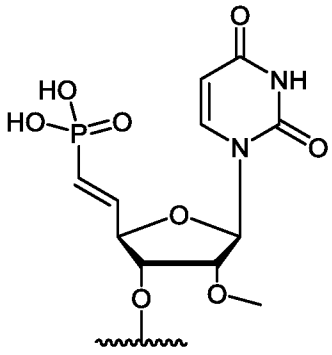
, and

R^2 is



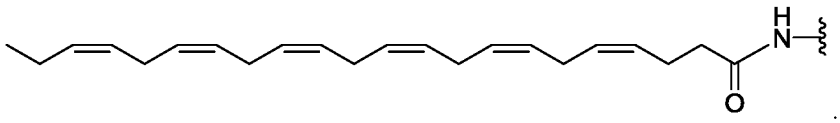
[0387] In one embodiment, the compound of the Formula shown in Figure 89, is a compound of the Formula shown in Figure 90, or a pharmaceutically acceptable salt thereof, wherein

R^1 is



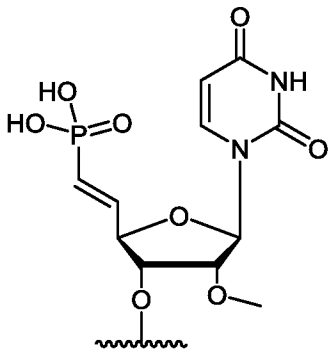
, and

R^2 is



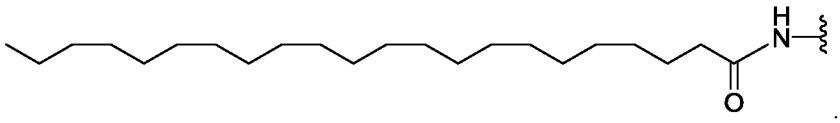
[0388] In one embodiment, the compound of the Formula shown in Figure 89, is a compound of the Formula shown in Figure 90, or a pharmaceutically acceptable salt thereof, wherein

R^1 is



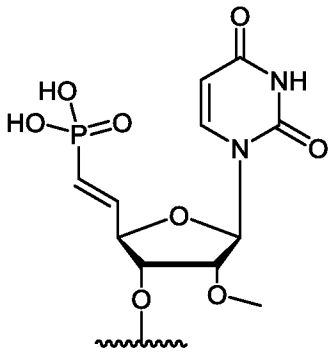
, and

R^2 is



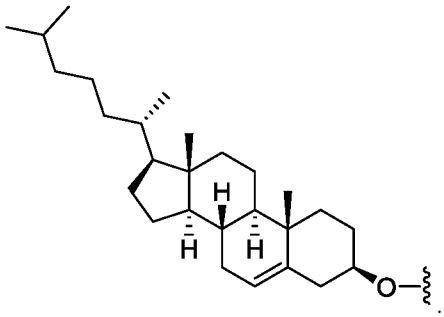
[0389] In one embodiment, the compound of the Formula shown in Figure 89, is a compound of the Formula shown in Figure 90, or a pharmaceutically acceptable salt thereof, wherein

R^1 is



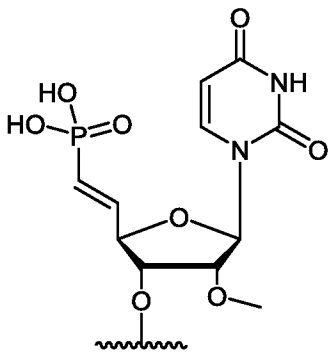
, and

R^2 is



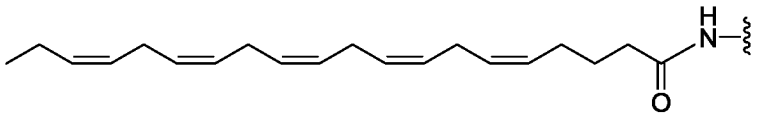
[0390] In one embodiment, the compound of the Formula shown in Figure 89, is a compound of the Formula shown in Figure 90, or a pharmaceutically acceptable salt thereof, wherein

R^1 is



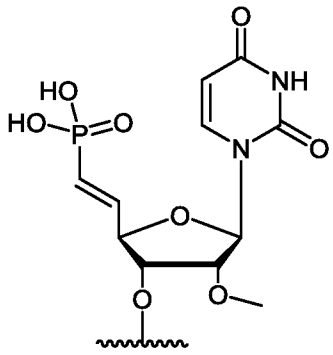
, and

R^2 is



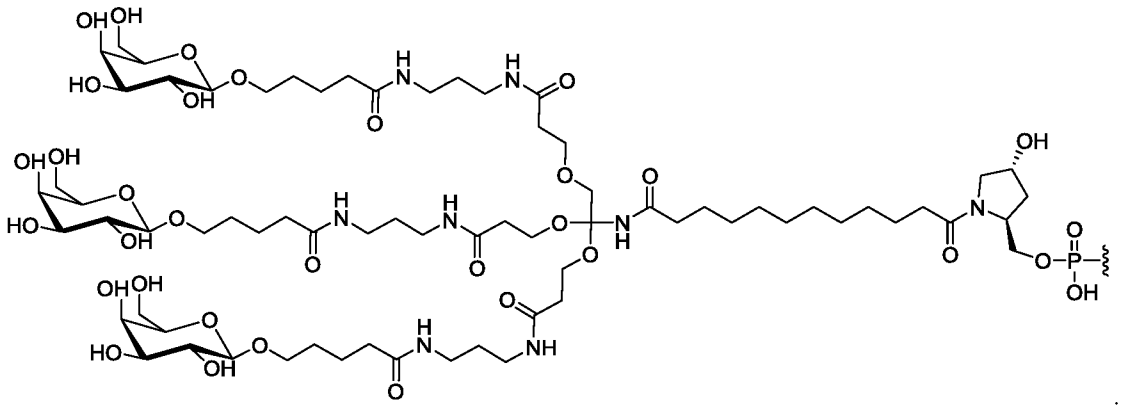
[0391] In one embodiment, the compound of the Formula shown in Figure 89, is a compound of the Formula shown in Figure 90, or a pharmaceutically acceptable salt thereof, wherein

R^1 is



, and

R^2 is



VIII. Methods of Introducing Nucleic Acids, Vectors and Host Cells

[0392] RNA silencing agents of the invention may be directly introduced into the cell (e.g., a neural cell) (i.e., intracellularly); or introduced extracellularly into a cavity, interstitial space, into the circulation of an organism, introduced orally, or may be introduced by bathing a cell or organism in a solution containing the nucleic acid. Vascular or extravascular circulation, the blood or lymph system, and the cerebrospinal fluid are sites where the nucleic acid may be introduced.

[0393] The RNA silencing agents of the invention can be introduced using nucleic acid delivery methods known in art including injection of a solution containing the nucleic acid, bombardment by particles covered by the nucleic acid, soaking the cell or organism in a

solution of the nucleic acid, or electroporation of cell membranes in the presence of the nucleic acid. Other methods known in the art for introducing nucleic acids to cells may be used, such as lipid-mediated carrier transport, chemical-mediated transport, and cationic liposome transfection such as calcium phosphate, and the like. The nucleic acid may be introduced along with other components that perform one or more of the following activities: enhance nucleic acid uptake by the cell or other-wise increase inhibition of the target gene.

[0394] Physical methods of introducing nucleic acids include injection of a solution containing the RNA, bombardment by particles covered by the RNA, soaking the cell or organism in a solution of the RNA, or electroporation of cell membranes in the presence of the RNA. A viral construct packaged into a viral particle would accomplish both efficient introduction of an expression construct into the cell and transcription of RNA encoded by the expression construct. Other methods known in the art for introducing nucleic acids to cells may be used, such as lipid-mediated carrier transport, chemical-mediated transport, such as calcium phosphate, and the like. Thus the RNA may be introduced along with components that perform one or more of the following activities: enhance RNA uptake by the cell, inhibit annealing of single strands, stabilize the single strands, or other-wise increase inhibition of the target gene.

[0395] RNA may be directly introduced into the cell (i.e., intracellularly); or introduced extracellularly into a cavity, interstitial space, into the circulation of an organism, introduced orally, or may be introduced by bathing a cell or organism in a solution containing the RNA. Vascular or extravascular circulation, the blood or lymph system, and the cerebrospinal fluid are sites where the RNA may be introduced.

[0396] The cell having the target gene may be from the germ line or somatic, totipotent or pluripotent, dividing or non-dividing, parenchyma or epithelium, immortalized or transformed, or the like. The cell may be a stem cell or a differentiated cell. Cell types that are differentiated include adipocytes, fibroblasts, myocytes, cardiomyocytes, endothelium, neurons, glia, blood cells, megakaryocytes, lymphocytes, macrophages, neutrophils, eosinophils, basophils, mast cells, leukocytes, granulocytes, keratinocytes, chondrocytes, osteoblasts, osteoclasts, hepatocytes, and cells of the endocrine or exocrine glands.

[0397] Depending on the particular target gene and the dose of double stranded RNA material delivered, this process may provide partial or complete loss of function for the target

gene. A reduction or loss of gene expression in at least 50%, 60%, 70%, 80%, 90%, 95% or 99% or more of targeted cells is exemplary. Inhibition of gene expression refers to the absence (or observable decrease) in the level of protein and/or mRNA product from a target gene. Specificity refers to the ability to inhibit the target gene without manifest effects on other genes of the cell. The consequences of inhibition can be confirmed by examination of the outward properties of the cell or organism (as presented below in the examples) or by biochemical techniques such as RNA solution hybridization, nuclease protection, Northern hybridization, reverse transcription, gene expression monitoring with a microarray, antibody binding, Enzyme Linked ImmunoSorbent Assay (ELISA), Western blotting, RadioImmunoAssay (RIA), other immunoassays, and Fluorescence Activated Cell Sorting (FACS).

[0398] For RNA-mediated inhibition in a cell line or whole organism, gene expression is conveniently assayed by use of a reporter or drug resistance gene whose protein product is easily assayed. Such reporter genes include acetohydroxyacid synthase (AHAS), alkaline phosphatase (AP), beta galactosidase (LacZ), beta glucoronidase (GUS), chloramphenicol acetyltransferase (CAT), green fluorescent protein (GFP), horseradish peroxidase (HRP), luciferase (Luc), nopaline synthase (NOS), octopine synthase (OCS), and derivatives thereof. Multiple selectable markers are available that confer resistance to ampicillin, bleomycin, chloramphenicol, gentarnycin, hygromycin, kanamycin, lincomycin, methotrexate, phosphinothricin, puromycin, and tetracyclin. Depending on the assay, quantitation of the amount of gene expression allows one to determine a degree of inhibition which is greater than 10%, 33%, 50%, 90%, 95% or 99% as compared to a cell not treated according to the present invention. Lower doses of injected material and longer times after administration of RNAi agent may result in inhibition in a smaller fraction of cells (e.g., at least 10%, 20%, 50%, 75%, 90%, or 95% of targeted cells). Quantization of gene expression in a cell may show similar amounts of inhibition at the level of accumulation of target mRNA or translation of target protein. As an example, the efficiency of inhibition may be determined by assessing the amount of gene product in the cell; mRNA may be detected with a hybridization probe having a nucleotide sequence outside the region used for the inhibitory double-stranded RNA, or translated polypeptide may be detected with an antibody raised against the polypeptide sequence of that region.

[0399] The RNA may be introduced in an amount which allows delivery of at least one copy per cell. Higher doses (e.g., at least 5, 10, 100, 500 or 1000 copies per cell) of

material may yield more effective inhibition; lower doses may also be useful for specific applications.

[0400] In an exemplary aspect, the efficacy of an RNAi agent of the invention (e.g., an siRNA targeting an htt target sequence) is tested for its ability to specifically degrade mutant mRNA (e.g., htt mRNA and/or the production of huntingtin protein) in cells, in particular, in neurons (e.g., striatal or cortical neuronal clonal lines and/or primary neurons). Also suitable for cell-based validation assays are other readily transfectable cells, for example, HeLa cells or COS cells. Cells are transfected with human wild type or mutant cDNAs (e.g., human wild type or mutant huntingtin cDNA). Standard siRNA, modified siRNA or vectors able to produce siRNA from U-looped mRNA are co-transfected. Selective reduction in target mRNA (e.g., huntingtin mRNA) and/or target protein (e.g., huntingtin protein) is measured. Reduction of target mRNA or protein can be compared to levels of target mRNA or protein in the absence of an RNAi agent or in the presence of an RNAi agent that does not target htt mRNA. Exogenously-introduced mRNA or protein (or endogenous mRNA or protein) can be assayed for comparison purposes. When utilizing neuronal cells, which are known to be somewhat resistant to standard transfection techniques, it may be desirable to introduce RNAi agents (e.g., siRNAs) by passive uptake.

Recombinant Adeno-Associated Viruses and Vectors

[0401] In certain exemplary embodiments, recombinant adeno-associated viruses (rAAVs) and their associated vectors can be used to deliver one or more siRNAs into cells, e.g., neural cells (e.g., brain cells). AAV is able to infect many different cell types, although the infection efficiency varies based upon serotype, which is determined by the sequence of the capsid protein. Several native AAV serotypes have been identified, with serotypes 1-9 being the most commonly used for recombinant AAV. AAV-2 is the most well-studied and published serotype. The AAV-DJ system includes serotypes AAV-DJ and AAV-DJ/8. These serotypes were created through DNA shuffling of multiple AAV serotypes to produce AAV with hybrid capsids that have improved transduction efficiencies *in vitro* (AAV-DJ) and *in vivo* (AAV-DJ/8) in a variety of cells and tissues.

[0402] In particular embodiments, widespread central nervous system (CNS) delivery can be achieved by intravascular delivery of recombinant adeno-associated virus 7 (rAAV7), RAAV9 and rAAV10, or other suitable rAAVs (Zhang et al. (2011) *Mol. Ther.* 19(8):1440-8. doi: 10.1038/mt.2011.98. Epub 2011 May 24). rAAVs and their associated vectors are well-

known in the art and are described in US Patent Applications 2014/0296486, 2010/0186103, 2008/0269149, 2006/0078542 and 2005/0220766, each of which is incorporated herein by reference in its entirety for all purposes.

[0403] rAAVs may be delivered to a subject in compositions according to any appropriate methods known in the art. An rAAV can be suspended in a physiologically compatible carrier (i.e., in a composition), and may be administered to a subject, i.e., a host animal, such as a human, mouse, rat, cat, dog, sheep, rabbit, horse, cow, goat, pig, guinea pig, hamster, chicken, turkey, a non-human primate (e.g., Macaque) or the like. In certain embodiments, a host animal is a non-human host animal.

[0404] Delivery of one or more rAAVs to a mammalian subject may be performed, for example, by intramuscular injection or by administration into the bloodstream of the mammalian subject. Administration into the bloodstream may be by injection into a vein, an artery, or any other vascular conduit. In certain embodiments, one or more rAAVs are administered into the bloodstream by way of isolated limb perfusion, a technique well known in the surgical arts, the method essentially enabling the artisan to isolate a limb from the systemic circulation prior to administration of the rAAV virions. A variant of the isolated limb perfusion technique, described in U.S. Pat. No. 6,177,403, can also be employed by the skilled artisan to administer virions into the vasculature of an isolated limb to potentially enhance transduction into muscle cells or tissue. Moreover, in certain instances, it may be desirable to deliver virions to the central nervous system (CNS) of a subject. By "CNS" is meant all cells and tissue of the brain and spinal cord of a vertebrate. Thus, the term includes, but is not limited to, neuronal cells, glial cells, astrocytes, cerebrospinal fluid (CSF), interstitial spaces, bone, cartilage and the like. Recombinant AAVs may be delivered directly to the CNS or brain by injection into, e.g., the ventricular region, as well as to the striatum (e.g., the caudate nucleus or putamen of the striatum), spinal cord and neuromuscular junction, or cerebellar lobule, with a needle, catheter or related device, using neurosurgical techniques known in the art, such as by stereotactic injection (see, e.g., Stein et al., J Virol 73:3424-3429, 1999; Davidson et al., PNAS 97:3428-3432, 2000; Davidson et al., Nat. Genet. 3:219-223, 1993; and Alisky and Davidson, Hum. Gene Ther. 11:2315-2329, 2000).

[0405] The compositions of the invention may comprise an rAAV alone, or in combination with one or more other viruses (e.g., a second rAAV encoding having one or more different transgenes). In certain embodiments, a composition comprises 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more different rAAVs each having one or more different transgenes.

[0406] An effective amount of an rAAV is an amount sufficient to target infect an animal, target a desired tissue. In some embodiments, an effective amount of an rAAV is an amount sufficient to produce a stable somatic transgenic animal model. The effective amount will depend primarily on factors such as the species, age, weight, health of the subject, and the tissue to be targeted, and may thus vary among animal and tissue. For example, an effective amount of one or more rAAVs is generally in the range of from about 1 ml to about 100 ml of solution containing from about 10^9 to 10^{16} genome copies. In some cases, a dosage between about 10^{11} to 10^{12} rAAV genome copies is appropriate. In certain embodiments, 10^{12} rAAV genome copies is effective to target heart, liver, and pancreas tissues. In some cases, stable transgenic animals are produced by multiple doses of an rAAV.

[0407] In some embodiments, rAAV compositions are formulated to reduce aggregation of AAV particles in the composition, particularly where high rAAV concentrations are present (e.g., about 10^{13} genome copies/mL or more). Methods for reducing aggregation of rAAVs are well known in the art and, include, for example, addition of surfactants, pH adjustment, salt concentration adjustment, etc. (See, e.g., Wright et al. (2005) Molecular Therapy 12:171-178, the contents of which are incorporated herein by reference.)

[0408] "Recombinant AAV (rAAV) vectors" comprise, at a minimum, a transgene and its regulatory sequences, and 5' and 3' AAV inverted terminal repeats (ITRs). It is this recombinant AAV vector which is packaged into a capsid protein and delivered to a selected target cell. In some embodiments, the transgene is a nucleic acid sequence, heterologous to the vector sequences, which encodes a polypeptide, protein, functional RNA molecule (e.g., siRNA) or other gene product, of interest. The nucleic acid coding sequence is operatively linked to regulatory components in a manner which permits transgene transcription, translation, and/or expression in a cell of a target tissue.

[0409] The AAV sequences of the vector typically comprise the cis-acting 5' and 3' inverted terminal repeat (ITR) sequences (See, e.g., B. J. Carter, in "Handbook of Parvoviruses", ed., P. Tijsser, CRC Press, pp. 155 168 (1990)). The ITR sequences are usually about 145 basepairs in length. In certain embodiments, substantially the entire sequences encoding the ITRs are used in the molecule, although some degree of minor modification of these sequences is permissible. The ability to modify these ITR sequences is within the skill of the art. (See, e.g., texts such as Sambrook et al, "Molecular Cloning. A Laboratory Manual", 2d ed., Cold Spring Harbor Laboratory, New York (1989); and K.

Fisher et al., J Virol., 70:520 532 (1996)). An example of such a molecule employed in the present invention is a "cis-acting" plasmid containing the transgene, in which the selected transgene sequence and associated regulatory elements are flanked by the 5' and 3' AAV ITR sequences. The AAV ITR sequences may be obtained from any known AAV, including mammalian AAV types described further herein.

IX. Methods of Treatment

[0410] The present invention provides for both prophylactic and therapeutic methods of treating a subject at risk of (or susceptible to) a disease or disorder caused, in whole or in part, by a gain of function mutant protein. In one embodiment, the disease or disorder is a trinucleotide repeat disease or disorder. In another embodiment, the disease or disorder is a polyglutamine disorder. In a preferred embodiment, the disease or disorder is a disorder associated with the expression of huntingtin and in which alteration of huntingtin, especially the amplification of CAG repeat copy number, leads to a defect in huntingtin gene (structure or function) or huntingtin protein (structure or function or expression), such that clinical manifestations include those seen in Huntington's disease patients.

[0411] "Treatment," or "treating," as used herein, is defined as the application or administration of a therapeutic agent (e.g., a RNA agent or vector or transgene encoding same) to a patient, or application or administration of a therapeutic agent to an isolated tissue or cell line from a patient, who has the disease or disorder, a symptom of disease or disorder or a predisposition toward a disease or disorder, with the purpose to cure, heal, alleviate, relieve, alter, remedy, ameliorate, improve or affect the disease or disorder, the symptoms of the disease or disorder, or the predisposition toward disease.

[0412] In one aspect, the invention provides a method for preventing in a subject, a disease or disorder as described above, by administering to the subject a therapeutic agent (e.g., an RNAi agent or vector or transgene encoding same). Subjects at risk for the disease can be identified by, for example, any or a combination of diagnostic or prognostic assays as described herein. Administration of a prophylactic agent can occur prior to the manifestation of symptoms characteristic of the disease or disorder, such that the disease or disorder is prevented or, alternatively, delayed in its progression.

[0413] Another aspect of the invention pertains to methods treating subjects therapeutically, i.e., alter onset of symptoms of the disease or disorder. In an exemplary embodiment, the modulatory method of the invention involves contacting a cell expressing a

gain-of-function mutant with a therapeutic agent (e.g., a RNAi agent or vector or transgene encoding same) that is specific for a target sequence within the gene (e.g., SEQ ID NOs:1, 2 or 3), such that sequence specific interference with the gene is achieved. These methods can be performed *in vitro* (e.g., by culturing the cell with the agent) or, alternatively, *in vivo* (e.g., by administering the agent to a subject).

[0414] With regards to both prophylactic and therapeutic methods of treatment, such treatments may be specifically tailored or modified, based on knowledge obtained from the field of pharmacogenomics. "Pharmacogenomics," as used herein, refers to the application of genomics technologies such as gene sequencing, statistical genetics, and gene expression analysis to drugs in clinical development and on the market. More specifically, the term refers the study of how a patient's genes determine his or her response to a drug (e.g., a patient's "drug response phenotype," or "drug response genotype"). Thus, another aspect of the invention provides methods for tailoring an individual's prophylactic or therapeutic treatment with either the target gene molecules of the present invention or target gene modulators according to that individual's drug response genotype. Pharmacogenomics allows a clinician or physician to target prophylactic or therapeutic treatments to patients who will most benefit from the treatment and to avoid treatment of patients who will experience toxic drug-related side effects.

[0415] Therapeutic agents can be tested in an appropriate animal model. For example, an RNAi agent (or expression vector or transgene encoding same) as described herein can be used in an animal model to determine the efficacy, toxicity, or side effects of treatment with said agent. Alternatively, a therapeutic agent can be used in an animal model to determine the mechanism of action of such an agent. For example, an agent can be used in an animal model to determine the efficacy, toxicity, or side effects of treatment with such an agent. Alternatively, an agent can be used in an animal model to determine the mechanism of action of such an agent.

[0416] A pharmaceutical composition containing an RNA silencing agent of the invention can be administered to any patient diagnosed as having or at risk for developing a neurological disorder, such as Huntington's disease. In one embodiment, the patient is diagnosed as having a neurological disorder, and the patient is otherwise in general good health. For example, the patient is not terminally ill, and the patient is likely to live at least 2, 3, 5 or more years following diagnosis. The patient can be treated immediately following diagnosis, or treatment can be delayed until the patient is experiencing more debilitating

symptoms, such as motor fluctuations and dyskinesis in Parkinson's disease patients. In another embodiment, the patient has not reached an advanced stage of the disease.

[0417] An RNA silencing agent modified for enhance uptake into neural cells can be administered at a unit dose less than about 1.4 mg per kg of bodyweight, or less than 10, 5, 2, 1, 0.5, 0.1, 0.05, 0.01, 0.005, 0.001, 0.0005, 0.0001, 0.00005 or 0.00001 mg per kg of bodyweight, and less than 200 nmole of RNA agent (e.g., about 4.4 x 10^{16} copies) per kg of bodyweight, or less than 1500, 750, 300, 150, 75, 15, 7.5, 1.5, 0.75, 0.15, 0.075, 0.015, 0.0075, 0.0015, 0.00075, 0.00015 nmole of RNA silencing agent per kg of bodyweight. The unit dose, for example, can be administered by injection (e.g., intravenous or intramuscular, intrathecally, or directly into the brain), an inhaled dose, or a topical application. Particularly preferred dosages are less than 2, 1, or 0.1 mg/kg of body weight.

[0418] Delivery of an RNA silencing agent directly to an organ (e.g., directly to the brain) can be at a dosage on the order of about 0.00001 mg to about 3 mg per organ, or preferably about 0.0001-0.001 mg per organ, about 0.03-3.0 mg per organ, about 0.1-3.0 mg per eye or about 0.3-3.0 mg per organ. The dosage can be an amount effective to treat or prevent a neurological disease or disorder, e.g., Huntington's disease. In one embodiment, the unit dose is administered less frequently than once a day, e.g., less than every 2, 4, 8 or 30 days. In another embodiment, the unit dose is not administered with a frequency (e.g., not a regular frequency). For example, the unit dose may be administered a single time. In one embodiment, the effective dose is administered with other traditional therapeutic modalities.

[0419] In one embodiment, a subject is administered an initial dose, and one or more maintenance doses of an RNA silencing agent. The maintenance dose or doses are generally lower than the initial dose, e.g., one-half less of the initial dose. A maintenance regimen can include treating the subject with a dose or doses ranging from 0.01 μg to 1.4 mg/kg of body weight per day, e.g., 10, 1, 0.1, 0.01, 0.001, or 0.00001 mg per kg of bodyweight per day. The maintenance doses are preferably administered no more than once every 5, 10, or 30 days. Further, the treatment regimen may last for a period of time which will vary depending upon the nature of the particular disease, its severity and the overall condition of the patient. In preferred embodiments the dosage may be delivered no more than once per day, e.g., no more than once per 24, 36, 48, or more hours, e.g., no more than once every 5 or 8 days. Following treatment, the patient can be monitored for changes in his condition and for alleviation of the symptoms of the disease state. The dosage of the compound may either be increased in the event the patient does not respond significantly to current dosage levels, or

the dose may be decreased if an alleviation of the symptoms of the disease state is observed, if the disease state has been ablated, or if undesired side-effects are observed.

[0420] The effective dose can be administered in a single dose or in two or more doses, as desired or considered appropriate under the specific circumstances. If desired to facilitate repeated or frequent infusions, implantation of a delivery device, e.g., a pump, semi-permanent stent (e.g., intravenous, intraperitoneal, intracisternal or intracapsular), or reservoir may be advisable. In one embodiment, a pharmaceutical composition includes a plurality of RNA silencing agent species. In another embodiment, the RNA silencing agent species has sequences that are non-overlapping and non-adjacent to another species with respect to a naturally occurring target sequence. In another embodiment, the plurality of RNA silencing agent species is specific for different naturally occurring target genes. In another embodiment, the RNA silencing agent is allele specific. In another embodiment, the plurality of RNA silencing agent species target two or more target sequences (e.g., two, three, four, five, six, or more target sequences).

[0421] Following successful treatment, it may be desirable to have the patient undergo maintenance therapy to prevent the recurrence of the disease state, wherein the compound of the invention is administered in maintenance doses, ranging from 0.01 μg to 100 g per kg of body weight (see U.S. Pat. No. 6,107,094).

[0422] The concentration of the RNA silencing agent composition is an amount sufficient to be effective in treating or preventing a disorder or to regulate a physiological condition in humans. The concentration or amount of RNA silencing agent administered will depend on the parameters determined for the agent and the method of administration, e.g. nasal, buccal, or pulmonary. For example, nasal formulations tend to require much lower concentrations of some ingredients in order to avoid irritation or burning of the nasal passages. It is sometimes desirable to dilute an oral formulation up to 10-100 times in order to provide a suitable nasal formulation.

[0423] Certain factors may influence the dosage required to effectively treat a subject, including but not limited to the severity of the disease or disorder, previous treatments, the general health and/or age of the subject, and other diseases present. Moreover, treatment of a subject with a therapeutically effective amount of an RNA silencing agent can include a single treatment or, preferably, can include a series of treatments. It will also be appreciated that the effective dosage of an RNA silencing agent for treatment may increase or decrease

over the course of a particular treatment. Changes in dosage may result and become apparent from the results of diagnostic assays as described herein. For example, the subject can be monitored after administering an RNA silencing agent composition. Based on information from the monitoring, an additional amount of the RNA silencing agent composition can be administered.

[0424] Dosing is dependent on severity and responsiveness of the disease condition to be treated, with the course of treatment lasting from several days to several months, or until a cure is effected or a diminution of disease state is achieved. Optimal dosing schedules can be calculated from measurements of drug accumulation in the body of the patient. Persons of ordinary skill can easily determine optimum dosages, dosing methodologies and repetition rates. Optimum dosages may vary depending on the relative potency of individual compounds, and can generally be estimated based on EC50s found to be effective in in vitro and in vivo animal models. In some embodiments, the animal models include transgenic animals that express a human gene, e.g., a gene that produces a target RNA, e.g., an RNA expressed in a neural cell. The transgenic animal can be deficient for the corresponding endogenous RNA. In another embodiment, the composition for testing includes an RNA silencing agent that is complementary, at least in an internal region, to a sequence that is conserved between the target RNA in the animal model and the target RNA in a human.

X. Pharmaceutical Compositions and Methods of Administration

[0425] The invention pertains to uses of the above-described agents for prophylactic and/or therapeutic treatments as described *Infra*. Accordingly, the modulators (e.g., RNAi agents) of the present invention can be incorporated into pharmaceutical compositions suitable for administration. Such compositions typically comprise the nucleic acid molecule, protein, antibody, or modulatory compound and a pharmaceutically acceptable carrier. As used herein the language "pharmaceutically acceptable carrier" is intended to include any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like, compatible with pharmaceutical administration. The use of such media and agents for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active compound, use thereof in the compositions is contemplated. Supplementary active compounds can also be incorporated into the compositions.

[0426] A pharmaceutical composition of the invention is formulated to be compatible with its intended route of administration. Examples of routes of administration include parenteral, e.g., intravenous, intradermal, subcutaneous, intraperitoneal, intramuscular, oral (e.g., inhalation), transdermal (topical), and transmucosal administration. In certain exemplary embodiments, a pharmaceutical composition of the invention is delivered to the cerebrospinal fluid (CSF) by a route of administration that includes, but is not limited to, intrastriatal (IS) administration, intracerebroventricular (ICV) administration and intrathecal (IT) administration (e.g., via a pump, an infusion or the like). Solutions or suspensions used for parenteral, intradermal, or subcutaneous application can include the following components: a sterile diluent such as water for injection, saline solution, fixed oils, polyethylene glycols, glycerine, propylene glycol or other synthetic solvents; antibacterial agents such as benzyl alcohol or methyl parabens; antioxidants such as ascorbic acid or sodium bisulfite; chelating agents such as ethylenediaminetetraacetic acid; buffers such as acetates, citrates or phosphates and agents for the adjustment of tonicity such as sodium chloride or dextrose. pH can be adjusted with acids or bases, such as hydrochloric acid or sodium hydroxide. The parenteral preparation can be enclosed in ampoules, disposable syringes or multiple dose vials made of glass or plastic.

[0427] Pharmaceutical compositions suitable for injectable use include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. For intravenous, IS, ICV and/or IT administration, suitable carriers include physiological saline, bacteriostatic water, Cremophor EL™ (BASF, Parsippany, N.J.) or phosphate buffered saline (PBS). In all cases, the composition must be sterile and should be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. Prevention of the action of microorganisms can be achieved by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, ascorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars, polyalcohols such as mannitol,

sorbitol, sodium chloride in the composition. Prolonged absorption of the injectable compositions can be brought about by including in the composition an agent which delays absorption, for example, aluminum monostearate and gelatin.

[0428] Sterile injectable solutions can be prepared by incorporating the active compound in the required amount in an appropriate solvent with one or a combination of ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the active compound into a sterile vehicle which contains a basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and freeze-drying which yields a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

[0429] Oral compositions generally include an inert diluent or an edible carrier. They can be enclosed in gelatin capsules or compressed into tablets. For the purpose of oral therapeutic administration, the active compound can be incorporated with excipients and used in the form of tablets, troches, or capsules. Oral compositions can also be prepared using a fluid carrier for use as a mouthwash, wherein the compound in the fluid carrier is applied orally and swished and expectorated or swallowed. Pharmaceutically compatible binding agents, and/or adjuvant materials can be included as part of the composition. The tablets, pills, capsules, troches and the like can contain any of the following ingredients, or compounds of a similar nature: a binder such as microcrystalline cellulose, gum tragacanth or gelatin; an excipient such as starch or lactose, a disintegrating agent such as alginic acid, Primogel, or corn starch; a lubricant such as magnesium stearate or Sterotes; a glidant such as colloidal silicon dioxide; a sweetening agent such as sucrose or saccharin; or a flavoring agent such as peppermint, methyl salicylate, or orange flavoring.

[0430] For administration by inhalation, the compounds are delivered in the form of an aerosol spray from pressured container or dispenser which contains a suitable propellant, e.g., a gas such as carbon dioxide, or a nebulizer.

[0431] Systemic administration can also be by transmucosal or transdermal means. For transmucosal or transdermal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art, and include, for example, for transmucosal administration, detergents, bile salts, and fusidic acid

derivatives. Transmucosal administration can be accomplished through the use of nasal sprays or suppositories. For transdermal administration, the active compounds are formulated into ointments, salves, gels, or creams as generally known in the art.

[0432] The compounds can also be prepared in the form of suppositories (e.g., with conventional suppository bases such as cocoa butter and other glycerides) or retention enemas for rectal delivery.

[0433] The RNA silencing agents can also be administered by transfection or infection using methods known in the art, including but not limited to the methods described in McCaffrey et al. (2002), Nature, 418(6893), 38-9 (hydrodynamic transfection); Xia et al. (2002), Nature Biotechnol., 20(10), 1006-10 (viral-mediated delivery); or Putnam (1996), Am. J. Health Syst. Pharm. 53(2), 151-160, erratum at Am. J. Health Syst. Pharm. 53(3), 325 (1996).

[0434] The RNA silencing agents can also be administered by any method suitable for administration of nucleic acid agents, such as a DNA vaccine. These methods include gene guns, bio injectors, and skin patches as well as needle-free methods such as the micro-particle DNA vaccine technology disclosed in U.S. Pat. No. 6,194,389, and the mammalian transdermal needle-free vaccination with powder-form vaccine as disclosed in U.S. Pat. No. 6,168,587. Additionally, intranasal delivery is possible, as described in, inter alia, Hamajima et al. (1998), Clin. Immunol. Immunopathol., 88(2), 205-10. Liposomes (e.g., as described in U.S. Pat. No. 6,472,375) and microencapsulation can also be used. Biodegradable targetable microparticle delivery systems can also be used (e.g., as described in U.S. Pat. No. 6,471,996).

[0435] In one embodiment, the active compounds are prepared with carriers that will protect the compound against rapid elimination from the body, such as a controlled release formulation, including implants and microencapsulated delivery systems. Biodegradable, biocompatible polymers can be used, such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters, and polylactic acid. Methods for preparation of such formulations will be apparent to those skilled in the art. The materials can also be obtained commercially from Alza Corporation and Nova Pharmaceuticals, Inc. Liposomal suspensions (including liposomes targeted to infected cells with monoclonal antibodies to viral antigens) can also be used as pharmaceutically acceptable carriers. These can be

prepared according to methods known to those skilled in the art, for example, as described in U.S. Pat. No. 4,522,811.

[0436] It is especially advantageous to formulate oral or parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the subject to be treated; each unit containing a predetermined quantity of active compound calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. The specification for the dosage unit forms of the invention are dictated by and directly dependent on the unique characteristics of the active compound and the particular therapeutic effect to be achieved, and the limitations inherent in the art of compounding such an active compound for the treatment of individuals.

[0437] Toxicity and therapeutic efficacy of such compounds can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, e.g., for determining the LD50 (the dose lethal to 50% of the population) and the ED50 (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio LD50/ED50. Compounds that exhibit large therapeutic indices are preferred. Although compounds that exhibit toxic side effects may be used, care should be taken to design a delivery system that targets such compounds to the site of affected tissue in order to minimize potential damage to uninfected cells and, thereby, reduce side effects.

[0438] The data obtained from the cell culture assays and animal studies can be used in formulating a range of dosage for use in humans. The dosage of such compounds lies preferably within a range of circulating concentrations that include the ED50 with little or no toxicity. The dosage may vary within this range depending upon the dosage form employed and the route of administration utilized. For any compound used in the method of the invention, the therapeutically effective dose can be estimated initially from cell culture assays. A dose may be formulated in animal models to achieve a circulating plasma concentration range that includes the EC50 (i.e., the concentration of the test compound which achieves a half-maximal response) as determined in cell culture. Such information can be used to more accurately determine useful doses in humans. Levels in plasma may be measured, for example, by high performance liquid chromatography.

[0439] The pharmaceutical compositions can be included in a container, pack or dispenser together with optional instructions for administration.

[0440] As defined herein, a therapeutically effective amount of a RNA silencing agent (i.e., an effective dosage) depends on the RNA silencing agent selected. For instance, if a plasmid encoding shRNA is selected, single dose amounts in the range of approximately 1 μg to 1000 mg may be administered; in some embodiments, 10, 30, 100 or 1000 μg may be administered. In some embodiments, 1-5 g of the compositions can be administered. The compositions can be administered one from one or more times per day to one or more times per week; including once every other day. The skilled artisan will appreciate that certain factors may influence the dosage and timing required to effectively treat a subject, including but not limited to the severity of the disease or disorder, previous treatments, the general health and/or age of the subject, and other diseases present. Moreover, treatment of a subject with a therapeutically effective amount of a protein, polypeptide, or antibody can include a single treatment or, preferably, can include a series of treatments.

[0441] The nucleic acid molecules of the invention can be inserted into expression constructs, e.g., viral vectors, retroviral vectors, expression cassettes, or plasmid viral vectors, e.g., using methods known in the art, including but not limited to those described in Xia et al., (2002), *Supra*. Expression constructs can be delivered to a subject by, for example, inhalation, orally, intravenous injection, local administration (see U.S. Pat. No. 5,328,470) or by stereotactic injection (see e.g., Chen et al. (1994), Proc. Natl. Acad. Sci. USA, 91, 3054-3057). The pharmaceutical preparation of the delivery vector can include the vector in an acceptable diluent, or can comprise a slow release matrix in which the delivery vehicle is imbedded. Alternatively, where the complete delivery vector can be produced intact from recombinant cells, e.g., retroviral vectors, the pharmaceutical preparation can include one or more cells which produce the gene delivery system.

[0442] The nucleic acid molecules of the invention can also include small hairpin RNAs (shRNAs), and expression constructs engineered to express shRNAs. Transcription of shRNAs is initiated at a polymerase III (pol III) promoter, and is thought to be terminated at position 2 of a 4-5-thymine transcription termination site. Upon expression, shRNAs are thought to fold into a stem-loop structure with 3' UU-overhangs; subsequently, the ends of these shRNAs are processed, converting the shRNAs into siRNA-like molecules of about 21 nucleotides. Brummelkamp et al. (2002), Science, 296, 550-553; Lee et al, (2002). supra;

Miyagishi and Taira (2002), Nature Biotechnol., 20, 497-500; Paddison et al. (2002), supra; Paul (2002), supra; Sui (2002) supra; Yu et al. (2002), supra.

[0443] The expression constructs may be any construct suitable for use in the appropriate expression system and include, but are not limited to retroviral vectors, linear expression cassettes, plasmids and viral or virally-derived vectors, as known in the art. Such expression constructs may include one or more inducible promoters, RNA Pol III promoter systems such as U6 snRNA promoters or H1 RNA polymerase III promoters, or other promoters known in the art. The constructs can include one or both strands of the siRNA. Expression constructs expressing both strands can also include loop structures linking both strands, or each strand can be separately transcribed from separate promoters within the same construct. Each strand can also be transcribed from a separate expression construct, Tuschl (2002), *Supra*.

[0444] In certain exemplary embodiments, a composition that includes an RNA silencing agent of the invention can be delivered to the nervous system of a subject by a variety of routes. Exemplary routes include intrathecal, parenchymal (e.g., in the brain), nasal, and ocular delivery. The composition can also be delivered systemically, e.g., by intravenous, subcutaneous or intramuscular injection, which is particularly useful for delivery of the RNA silencing agents to peripheral neurons. A preferred route of delivery is directly to the brain, e.g., into the ventricles or the hypothalamus of the brain, or into the lateral or dorsal areas of the brain. The RNA silencing agents for neural cell delivery can be incorporated into pharmaceutical compositions suitable for administration.

[0445] For example, compositions can include one or more species of an RNA silencing agent and a pharmaceutically acceptable carrier. The pharmaceutical compositions of the present invention may be administered in a number of ways depending upon whether local or systemic treatment is desired and upon the area to be treated. Administration may be topical (including ophthalmic, intranasal, transdermal), oral or parenteral. Parenteral administration includes intravenous drip, subcutaneous, intraperitoneal or intramuscular injection, intrathecal, or intraventricular (e.g., intracerebroventricular) administration. In certain exemplary embodiments, an RNA silencing agent of the invention is delivered across the Blood-Brain-Barrier (BBB) suing a variety of suitable compositions and methods described herein.

[0446] The route of delivery can be dependent on the disorder of the patient. For example, a subject diagnosed with Huntington's disease can be administered an anti-htt RNA silencing agent of the invention directly into the brain (e.g., into the globus pallidus or the corpus striatum of the basal ganglia, and near the medium spiny neurons of the corpos striatum). In addition to an RNA silencing agent of the invention, a patient can be administered a second therapy, e.g., a palliative therapy and/or disease-specific therapy. The secondary therapy can be, for example, symptomatic (e.g., for alleviating symptoms), neuroprotective (e.g., for slowing or halting disease progression), or restorative (e.g., for reversing the disease process). For the treatment of Huntington's disease, for example, symptomatic therapies can include the drugs haloperidol, carbamazepine, or valproate. Other therapies can include psychotherapy, physiotherapy, speech therapy, communicative and memory aids, social support services, and dietary advice.

[0447] An RNA silencing agent can be delivered to neural cells of the brain. Delivery methods that do not require passage of the composition across the blood-brain barrier can be utilized. For example, a pharmaceutical composition containing an RNA silencing agent can be delivered to the patient by injection directly into the area containing the disease-affected cells. For example, the pharmaceutical composition can be delivered by injection directly into the brain. The injection can be by stereotactic injection into a particular region of the brain (e.g., the substantia nigra, cortex, hippocampus, striatum, or globus pallidus). The RNA silencing agent can be delivered into multiple regions of the central nervous system (e.g., into multiple regions of the brain, and/or into the spinal cord). The RNA silencing agent can be delivered into diffuse regions of the brain (e.g., diffuse delivery to the cortex of the brain).

[0448] In one embodiment, the RNA silencing agent can be delivered by way of a cannula or other delivery device having one end implanted in a tissue, e.g., the brain, e.g., the substantia nigra, cortex, hippocampus, striatum or globus pallidus of the brain. The cannula can be connected to a reservoir of RNA silencing agent. The flow or delivery can be mediated by a pump, e.g., an osmotic pump or minipump, such as an Alzet pump (Durect, Cupertino, CA). In one embodiment, a pump and reservoir are implanted in an area distant from the tissue, e.g., in the abdomen, and delivery is effected by a conduit leading from the pump or reservoir to the site of release. Devices for delivery to the brain are described, for example, in U.S. Pat. Nos. 6,093,180, and 5,814,014.

[0449] An RNA silencing agent of the invention can be further modified such that it is capable of traversing the blood brain barrier. For example, the RNA silencing agent can be conjugated to a molecule that enables the agent to traverse the barrier. Such modified RNA silencing agents can be administered by any desired method, such as by intraventricular or intramuscular injection, or by pulmonary delivery, for example.

[0450] In certain embodiments, exosomes are used to deliver an RNA silencing agent of the invention. Exosomes can cross the BBB and deliver siRNAs, antisense oligonucleotides, chemotherapeutic agents and proteins specifically to neurons after systemic injection (*See*, Alvarez-Erviti L, Seow Y, Yin H, Betts C, Lakhal S, Wood MJ. (2011). Delivery of siRNA to the mouse brain by systemic injection of targeted exosomes. Nat Biotechnol. 2011 Apr;29(4):341-5. doi: 10.1038/nbt.1807; El-Andaloussi S, Lee Y, Lakhal-Littleton S, Li J, Seow Y, Gardiner C, Alvarez-Erviti L, Sargent IL, Wood MJ.(2011). Exosome-mediated delivery of siRNA in vitro and in vivo. Nat Protoc. 2012 Dec;7(12):2112-26. doi: 10.1038/nprot.2012.131; EL Andaloussi S, Mäger I, Breakefield XO, Wood MJ. (2013). Extracellular vesicles: biology and emerging therapeutic opportunities. Nat Rev Drug Discov. 2013 May;12(5):347-57. doi: 10.1038/nrd3978; El Andaloussi S, Lakhal S, Mäger I, Wood MJ. (2013). Exosomes for targeted siRNA delivery across biological barriers. Adv Drug Deliv Rev. 2013 Mar;65(3):391-7. doi: 10.1016/j.addr.2012.08.008).

[0451] In certain embodiments, one or more lipophilic molecules are used to allow delivery of an RNA silencing agent of the invention past the BBB (Alvarez-Ervit (2011)). The RNA silencing agent would then be activated, e.g., by enzyme degradation of the lipophilic disguise to release the drug into its active form.

[0452] In certain embodiments, one or more receptor-mediated permeablizing compounds can be used to increase the permeability of the BBB to allow delivery of an RNA silencing agent of the invention. These drugs increase the permeability of the BBB temporarily by increasing the osmotic pressure in the blood which loosens the tight junctions between the endothelial cells ((El-Andaloussi (2012)). By loosening the tight junctions normal intravenous injection of an RNA silencing agent can be performed.

[0453] In certain embodiments, nanoparticle-based delivery systems are used to deliver an RNA silencing agent of the invention across the BBB. As used herein, "nanoparticles" refer to polymeric nanoparticles that are typically solid, biodegradable, colloidal systems that have been widely investigated as drug or gene carriers (S. P.

Egusquiaguirre, M. Igartua, R. M. Hernandez, and J. L. Pedraz, "Nanoparticle delivery systems for cancer therapy: advances in clinical and preclinical research," Clinical and Translational Oncology, vol. 14, no. 2, pp. 83–93, 2012). Polymeric nanoparticles are classified into two major categories, natural polymers and synthetic polymers. Natural polymers for siRNA delivery include, but are not limited to, cyclodextrin, chitosan, and atelocollagen (Y. Wang, Z. Li, Y. Han, L. H. Liang, and A. Ji, "Nanoparticle-based delivery system for application of siRNA in vivo," Current Drug Metabolism, vol. 11, no. 2, pp. 182–196, 2010). Synthetic polymers include, but are not limited to, polyethyleneimine (PEI), poly(dl-lactide-co-glycolide) (PLGA), and dendrimers, which have been intensively investigated (X. Yuan, S. Naguib, and Z. Wu, "Recent advances of siRNA delivery by nanoparticles," Expert Opinion on Drug Delivery, vol. 8, no. 4, pp. 521–536, 2011). For a review of nanoparticles and other suitable delivery systems, *See* Jong-Min Lee, Tae-Jong Yoon, and Young-Seok Cho, "Recent Developments in Nanoparticle-Based siRNA Delivery for Cancer Therapy," BioMed Research International, vol. 2013, Article ID 782041, 10 pages, 2013. doi:10.1155/2013/782041 (incorporated by reference in its entirety.)

[0454] An RNA silencing agent of the invention can be administered ocularly, such as to treat retinal disorder, e.g., a retinopathy. For example, the pharmaceutical compositions can be applied to the surface of the eye or nearby tissue, e.g., the inside of the eyelid. They can be applied topically, e.g., by spraying, in drops, as an eyewash, or an ointment. Ointments or droppable liquids may be delivered by ocular delivery systems known in the art such as applicators or eye droppers. Such compositions can include mucomimetics such as hyaluronic acid, chondroitin sulfate, hydroxypropyl methylcellulose or poly(vinyl alcohol), preservatives such as sorbic acid, EDTA or benzylchronium chloride, and the usual quantities of diluents and/or carriers. The pharmaceutical composition can also be administered to the interior of the eye, and can be introduced by a needle or other delivery device which can introduce it to a selected area or structure. The composition containing the RNA silencing agent can also be applied via an ocular patch.

[0455] In general, an RNA silencing agent of the invention can be administered by any suitable method. As used herein, topical delivery can refer to the direct application of an RNA silencing agent to any surface of the body, including the eye, a mucous membrane, surfaces of a body cavity, or to any internal surface. Formulations for topical administration may include transdermal patches, ointments, lotions, creams, gels, drops, sprays, and liquids. Conventional pharmaceutical carriers, aqueous, powder or oily bases, thickeners and the like

may be necessary or desirable. Topical administration can also be used as a means to selectively deliver the RNA silencing agent to the epidermis or dermis of a subject, or to specific strata thereof, or to an underlying tissue.

[0456] Compositions for intrathecal or intraventricular (e.g., intracerebroventricular) administration may include sterile aqueous solutions which may also contain buffers, diluents and other suitable additives. Compositions for intrathecal or intraventricular administration preferably do not include a transfection reagent or an additional lipophilic moiety besides, for example, the lipophilic moiety attached to the RNA silencing agent.

[0457] Formulations for parenteral administration may include sterile aqueous solutions which may also contain buffers, diluents and other suitable additives. Intraventricular injection may be facilitated by an intraventricular catheter, for example, attached to a reservoir. For intravenous use, the total concentration of solutes should be controlled to render the preparation isotonic.

[0458] An RNA silencing agent of the invention can be administered to a subject by pulmonary delivery. Pulmonary delivery compositions can be delivered by inhalation of a dispersion so that the composition within the dispersion can reach the lung where it can be readily absorbed through the alveolar region directly into blood circulation. Pulmonary delivery can be effective both for systemic delivery and for localized delivery to treat diseases of the lungs. In one embodiment, an RNA silencing agent administered by pulmonary delivery has been modified such that it is capable of traversing the blood brain barrier.

[0459] Pulmonary delivery can be achieved by different approaches, including the use of nebulized, aerosolized, micellular and dry powder-based formulations. Delivery can be achieved with liquid nebulizers, aerosol-based inhalers, and dry powder dispersion devices. Metered-dose devices are preferred. One of the benefits of using an atomizer or inhaler is that the potential for contamination is minimized because the devices are self-contained. Dry powder dispersion devices, for example, deliver drugs that may be readily formulated as dry powders. An RNA silencing agent composition may be stably stored as lyophilized or spray-dried powders by itself or in combination with suitable powder carriers. The delivery of a composition for inhalation can be mediated by a dosing timing element which can include a timer, a dose counter, time measuring device, or a time indicator which when incorporated

into the device enables dose tracking, compliance monitoring, and/or dose triggering to a patient during administration of the aerosol medicament.

[0460] The types of pharmaceutical excipients that are useful as carriers include stabilizers such as human serum albumin (HSA), bulking agents such as carbohydrates, amino acids and polypeptides; pH adjusters or buffers; salts such as sodium chloride; and the like. These carriers may be in a crystalline or amorphous form or may be a mixture of the two.

[0461] Bulking agents that are particularly valuable include compatible carbohydrates, polypeptides, amino acids or combinations thereof. Suitable carbohydrates include monosaccharides such as galactose, D-mannose, sorbose, and the like; disaccharides, such as lactose, trehalose, and the like; cyclodextrins, such as 2-hydroxypropyl-.beta.-cyclodextrin; and polysaccharides, such as raffinose, maltodextrins, dextrans, and the like; alditols, such as mannitol, xylitol, and the like. A preferred group of carbohydrates includes lactose, trehalose, raffinose maltodextrins, and mannitol. Suitable polypeptides include aspartame. Amino acids include alanine and glycine, with glycine being preferred.

[0462] Suitable pH adjusters or buffers include organic salts prepared from organic acids and bases, such as sodium citrate, sodium ascorbate, and the like; sodium citrate is preferred.

[0463] An RNA silencing agent of the invention can be administered by oral and nasal delivery. For example, drugs administered through these membranes have a rapid onset of action, provide therapeutic plasma levels, avoid first pass effect of hepatic metabolism, and avoid exposure of the drug to the hostile gastrointestinal (GI) environment. Additional advantages include easy access to the membrane sites so that the drug can be applied, localized and removed easily. In one embodiment, an RNA silencing agent administered by oral or nasal delivery has been modified to be capable of traversing the blood-brain barrier.

[0464] In one embodiment, unit doses or measured doses of a composition that include RNA silencing agents are dispensed by an implanted device. The device can include a sensor that monitors a parameter within a subject. For example, the device can include a pump, such as an osmotic pump and, optionally, associated electronics.

[0465] An RNA silencing agent can be packaged in a viral natural capsid or in a chemically or enzymatically produced artificial capsid or structure derived therefrom.

XI. Kits

[0466] In certain other aspects, the invention provides kits that include a suitable container containing a pharmaceutical formulation of an RNA silencing agent, e.g., a double-stranded RNA silencing agent, or sRNA agent, (e.g., a precursor, e.g., a larger RNA silencing agent which can be processed into a sRNA agent, or a DNA which encodes an RNA silencing agent, e.g., a double-stranded RNA silencing agent, or sRNA agent, or precursor thereof). In certain embodiments the individual components of the pharmaceutical formulation may be provided in one container. Alternatively, it may be desirable to provide the components of the pharmaceutical formulation separately in two or more containers, e.g., one container for an RNA silencing agent preparation, and at least another for a carrier compound. The kit may be packaged in a number of different configurations such as one or more containers in a single box. The different components can be combined, e.g., according to instructions provided with the kit. The components can be combined according to a method described herein, e.g., to prepare and administer a pharmaceutical composition. The kit can also include a delivery device.

[0467] It will be readily apparent to those skilled in the art that other suitable modifications and adaptations of the methods described herein may be made using suitable equivalents without departing from the scope of the embodiments disclosed herein. Having now described certain embodiments in detail, the same will be more clearly understood by reference to the following example, which is included for purposes of illustration only and is not intended to be limiting.

EXAMPLES

Example 1. Reduction of Huntingtin in both primary neurons and mouse brain with unformulated, stabilized, hydrophobic siRNAs

[0468] The use of hydrophobically modified ASO-siRNA hybrids, which have the potential to offer both better efficacy and distribution *in vivo* and knockdown in primary neurons *in vitro*, was explored. The huntingtin gene was used as a target for mRNA knockdown. Huntington's disease is monogenic (Mangiarini, L. *et al.* Exon 1 of the *HTT* gene with an expanded CAG repeat is sufficient to cause a progressive neurological phenotype in transgenic mice. *Cell* **87,** 493–506 (1996)) with a number of cellular mechanisms leading to disease pathology (Zuccato, C., Valenza, M. & Cattaneo, E. Molecular Mechanisms and Potential Therapeutical Targets in Huntington's Disease.

Physiological Reviews **90,** 905–981 (2010)) making it an excellent candidate for possible future oligonucleotide therapeutics.

[0469] A panel of hydrophobically modified siRNAs targeting the Huntingtin gene was developed. Efficacy and potency in was observed both in primary neurons *in vitro*, and *in vivo* in mouse brain upon a single low dose injection without any formulation for delivery. These compounds combine a number of different chemical and structural modifications found both in earlier model siRNAs and hsiRNAs, as well as in ASOs. These properties, which include stabilizing base modifications, cholesterol conjugation, and a fully phosphorothioated single stranded tail, make these hsiRNAs excellent tools for studying gene function in hard-to-target primary cells and organs that can be adapted for use in a number of different biologically relevant systems.

1.1 hsiRNA - hydrophobically modified siRNA/antisense hybrids were efficiently internalized by primary neurons

[0470] The hsiRNAs were asymmetric compounds, with a short duplex region (15 base-pairs) and single-stranded fully phosphorothioated tail. All pyrimidines in these compounds were 2'-Fluoro and 2'-O-Methyl modified (providing stabilization), and the 3' end of the passenger strand was conjugated to TEG-Cholesterol (Figure 1A, Figure 8) 13. The cholesterol conjugate enabled quick membrane association, while the single stranded phosphorothioated tail was necessary for cellular internalization by a mechanism similar to the one used by conventional antisense oligonucleotides. Addition of Cy3-labeled hsiRNA to primary cortical neurons resulted in immediate (within minutes) cellular association (Figure 1B). Interestingly, the uptake was first observed preferentially in dendrites, followed by re-localization to the cellular body (Figure 9). The uptake was uniform across all cells in the dish, affirming efficient internalization.

1.2 Identification of hsiRNAs targeting huntingtin

[0471] A panel of 94 hsiRNA compounds (Figure 8) targeting huntingtin mRNA was designed and synthesized. These sequences spanned the gene and were selected to comply with standard siRNA design parameters (Birmingham, A. *et al.* A protocol for designing siRNAs with high functionality and specificity. *Nat Protoc* **2,** 2068–2078 (2007)) including assessment of GC content, specificity and low seed compliment frequency (Anderson, E. M. *et al.* Experimental validation of the importance of seed complement frequency to siRNA specificity. *RNA* **14,** 853–861 (2008)), elimination of sequences containing miRNA seeds,

and examination of thermodynamic bias (Khvorova, A., Reynolds, A. & Jayasena, S. D. Functional siRNAs and miRNAs Exhibit Strand Bias. *Cell* **115,** 209–216 (2003); Schwarz, D. S. *et al.* Asymmetry in the Assembly of the RNAi Enzyme Complex. *Cell* **115,** 199–208 (2003)). More than 50% of bases were chemically modified, to provide in vivo stability and minimization of immune response (Judge, A., Bola, G., Lee, A. & MacLachlan, I. Design of Noninflammatory Synthetic siRNA Mediating Potent Gene Silencing in Vivo. *Molecular Therapy* **13,** 494–505 (2006)). The modifications imposed additional restrictions on sequence space, reducing the hit rate. Impact on Huntingtin mRNA expression was measured after 72 hours exposure to 1.5 μM hsiRNA (passive uptake, no formulation) in HeLa cells by QUANTIGENE assay (Figure 2), with 7% of sequences showing more than 70% silencing. Functional target sites were spread across the gene with the exception of the distal part of the 3'UTR, later explained by preferential expression of the shorter htt isoform in HeLa cells (Li, S. H. *et al.* Huntington's disease gene (IT15) is widely expressed in human and rat tissues. *NEURON* **11,** 985–993 (1993)). IC50 values were identified for sixteen active sequences, selected based on primary screen activity and cross-species conservation (Figure 10). IC50 values ranged from 90 to 766 nM in passive uptake (no formulation) and from 4 to 91 pM in lipid-mediated uptake (Figure 8). Fully chemically-optimized active compounds were readily identified, indicating that a much smaller library should be sufficient in future screens for other genes, although hit rate is likely to be variable from target to target. The hsiRNA targeting position 10150 (HTT10150 (i.e., 5' CAGUAAAGAGAUUAA 3' (SEQ ID NO:1))) was used for further studies. To ensure that the hsiRNA chemical scaffold did not negatively impact efficacy and potency of HTT10150, the modified and unmodified versions of the compound were tested in both passive and lipid-mediated silencing assays (Figure 3). As expected, only the modified sequence was successful at cellular delivery and Htt silencing by passive uptake (IC50 = 82.2 nM), while both the modified and unmodified compounds showed similar IC50 values in lipid mediated delivery (4 pM and 13 pM respectively) suggesting that the hsiRNA scaffold modifications did not interfere with RNA-Induced Silencing Complex (RISC) loading.

1.3 Potent and specific gene silencing with unformulated hsiRNAs in primary neurons

[0472] HTT10150 was further tested for mRNA silencing in primary neurons isolated from FVBN mice. Efficacy was seen at both 72 hours and one week following simple unformulated compound addition to cortical neurons (Figure 4A) with maximum silencing (70%) observed at the 1.25 μM concentration. HTT10150 also showed similar silencing in

primary striatal neurons (Figure 4B). Protein levels were measured after one week by Western blot (Figure 14), confirming mRNA data with 85% reduction of protein upon treatment with 1.25 μM of compound (Figure 4C). The housekeeping genes (PPIB, GAPDH) and overall cell viability, measured by ALAMARBLUE Assay (Figures 11B and 14), were not affected at these concentrations. In other experiments, a slight impact on cell viability was observed at 3 μM.

[0473] To evaluate duration of effect upon a single HTT10150 treatment, the silencing was measured at one week, two week, and three week intervals (Figure 4D). The half-life of the loaded RISC complex was weeks (Song, E. *et al.* Sustained Small Interfering RNA-Mediated Human Immunodeficiency Virus Type 1 Inhibition in Primary Macrophages. *Journal of Virology* 77, 7174–7181 (2003)), and silencing was expected to be long lasting in non-dividing cells. Indeed, single treatment with hsiRNAs was sufficient to induce htt silencing at all times tested. Three weeks was the longest the primary neurons could be maintained in culture. Other systems will be used for longer-term experiments.

[0474] To demonstrate the general applicability of hsiRNAs as a tool for neuronal gene silencing, and to confirm this chemistry scaffold as valid for neuronal delivery, similar experiments were performed with several other hsiRNAs targeting HTT and with one targeting the house-keeping gene PPIB (Cyclophilin B) (Figures 11A and 13). Silencing as high as 70 and 90% was achieved with HTT and PPIB, respectively.

[0475] In summary, these data demonstrate that hydrophobically modified siRNA is a simple and straightforward approach for gene silencing in primary neurons, and can be adapted for multiple gene targets.

1.4 hsiRNA distribution *in vivo* in mouse brain upon single injection

[0476] hsiRNAs are efficiently internalized by different types of neurons *in vitro*. The selected hsiRNA, HTT10150, was further evaluated for its potential to silence gene expression in the brain *in vivo*. To determine the distribution profile of HTT10150 upon *in vivo* administration, 12.5 μg of Cy3 labelled hsiRNA (See Figure 8 for sequence) was injected intrastriatally and, after 24 hours, the brain was perfused, sectioned, and oligonucleotide distribution was visualized by fluorescence microscopy (Leica DM5500 – DFC365FX). The artificial CSF injected samples processed concurrently were used to set up microscopic imaging settings to control for background tissue epifluorescence.

[0477] The majority of compound showed a steep gradient of diffusion away from the injection site, with most of the ipsilateral striatum being covered (Figure 5A, 5B). Interestingly, hsiRNAs were detected on the non-injected side (contralateral) side of the brain (both cortex and striatum), although relative concentrations appeared much lower. Higher magnification images showed significant association of hsiRNA with fiber tracks, most likely due to the presence of a hydrophobic modification. This aspect of hsiRNA may make it useful as a labelling reagent to visualize brain signalling architecture (Figure 5C, 5D). In addition to fiber tracks and neurite labelling, hsiRNA could be detected as punctate staining in the perinuclear space of different cell types, including neurons, as evident from co-localization with NeuN (neuronal marker) stained cells (Figure 5E) only 24 hours after injection.

[0478] The effect of vitamin D on hsiRNA distribution is depicted in Figures 79 and 80.

1.5 hsiRNA efficacy *in vivo* in mouse brain upon single injection

[0479] To determine HTT10150 efficacy *in vivo*, wild type FVBN mice were dosed intrastriatally with a single injection of between 3 and 25 μg (0.1-0.9 mg/kg) of compound and mRNA silencing was examined both ipsilateral and contralateral to the injection site. Eight animals were dosed per treatment group and three individual punches were taken from each side of the striatum for mRNA and protein quantification. Level of huntingtin expression were measured by QUANTIGENE Assay and normalized to a housekeeping gene (details in Online methods).

[0480] Statistical analysis was performed by one-way ANOVA comparison against CSF or PBS control with Bonferroni corrections for repeat measures using GraphPad Prism (Online methods for details). All groups induced silencing that was significant against CSF, PBS, and non-targeting control treated animals. Raw Data from the 24 individual punches per treatment group (8 animals, 3 punches per animal) can be seen in Figure 15. At the site of administration (ipsilateral side), dose-dependent silencing reaching statistical significance was observed at all concentrations. The 25 μg treatment induced 77% silencing ($p<0.0001$), and the 12.5 μg treatment was repeated with two groups of animals on different days and showed statistically significant silencing of 66% and 42% (Figure 6).

[0481] While initial distribution studies showed a steep gradient of diffusion away from the injection site with a minimal amount of compound migrating to the contralateral

side, treatment with the higher doses of 25 μg and 12.5 μg resulted in statistically significant silencing ($p<0.0001$) on the non-injected side. However, the level of silencing was significantly less (only 36% for the 25 μg group) than on the treated side of the brain.

[0482] In summary, these data show that a single intrastriatal injection of hsiRNA is sufficient to induce potent gene silencing around the site of administration. This effect was reproducible across different treatment groups and independent experiments.

1.6 Neuronal viability following single hsiRNA injection in mouse brain

[0483] Cholesterol modification of non-modified, naked siRNA has previously been used for improvement of siRNA brain distribution, with toxicity at high doses being identified as a potential limitation. To evaluate the degree of non-specific chemistry related effects on the brain, DARPP32 expression, an established marker for dopamine receptor expression on medium spiny neurons in the striatum and representative of neuronal viability, was investigated. Additionally, potential induction of an immune response was performed by assessing the extent of microglia activation upon hsiRNA injection.

[0484] No significant impact on DARPP32 expression was observed for doses up to 12.5 μg suggesting persistent neuronal viability (Figures 7A, 7B, 16). Similarly, minimal microglial activation was visualized at the 12.5 μg dose (Figure 7C, 7D) indicative of a limited immune response in the presence of the modified hsiRNA. The 25 μg dose did induce some reduction in DARPP32 just around the site of injection indicative of toxicity and establishing the maximum dose levels for this chemical scaffold upon the indicated route of administration. A 10-12.5 μg single administration of hsiRNA efficiently silenced HTT mRNA in three, well powered, independent studies with robust silencing of 62, 42 and 52% without toxicity. These data indicate that this technology can be widely used for functional studies of other neurologically significant targets.

1.7 Further characterization in neurons

[0485] Sustained silencing was achieved for 21 days in terminally-differentiated neurons (Figure 24). A silencing plateau was observed with RNAi (cytoplasmic) but not RNaseH (predominantly nuclear) compounds (Figure 25). The observed plateau was specific to the *htt* gene. Approximately 60% of *htt* mRNA localized in the nuclei (Figure 26).

[0486] Probe sets were validated in neurons (Figure 27). A majority of the detected signal was specific to *htt* mRNA. A high fraction of yellow (co-localized staining) areas

were observed. Without intending to be bound by scientific theory, the high degree of red signal may be related to uneven concentrations of the two probed sets.

[0487] Additional probe sets were validated for intron 60-61 in neurons (Figure 28). Intron-specific probes showed one to two yellow dots in the nuclei specific to transcription sites. Exon-specific probes showed a higher degree of overlap.

[0488] *Htt* mRNA nuclear localization was specific to neurons and not to fibroblasts (Figure 29). HsiRNA^{HTT} treatment of cortical neurons preferentially eliminated cytoplasmic *htt* mRNA (Figures 30 and 31).

[0489] Close to complete HTT protein silencing was observed in primary cortical neurons (Figure 32).

[0490] Direct injection of HTT10150 caused no detectable changes in neuronal numbers (Figure 33). Cholesterol-hsiRNA exhibited a small area of toxicity adjacent to the injection site (Figure 34).

[0491] Figures 58-60 disclose hsiRNA efficacy in wild-type and Q140 primary hippocampal neurons.

1.8 Discussion

[0492] This study demonstrates that the use of hydrophobically modified siRNA for delivery to primary cells is a valuable tool to enable functional and genomic studies of neuronal pathways and neurological disorders.

[0493] The ability to cause gene silencing in primary neurons without the use of toxic formulation has a significant impact on neuroscience research, facilitating a more in depth study of neurological disorders in the context of primary cell lines, and ultimately providing a more relevant understanding of *in vivo* function and pathology. Most neuronal studies are done in stable cell lines due to ease of delivery and cell maintenance, but using artificial cell systems can lead to artifacts in the data that can be attributed to manipulation of these cell lines, a problem that can be avoided by using primary cells (Cheung, Y.-T. *et al.* Effects of all-trans-retinoic acid on human SH-SY5Y neuroblastoma as in vitro model in neurotoxicity research. *NeuroToxicology* **30,** 127–135 (2009); Gilany, K. *et al.* The proteome of the human neuroblastoma cell line SH-SY5Y: An enlarged proteome. *Biochimica et Biophysica Acta (BBA) - Proteins and Proteomics* **1784,** 983–985 (2008); Lopes, F. M. *et al.* Comparison between proliferative and neuron-like SH-SY5Y cells as an in vitro model for Parkinson

disease studies. *Brain Research* **1337,** 85–94 (2010); Zhang, W. *et al.* Cyclohexane 1,3-diones and their inhibition of mutant SOD1-dependent protein aggregation and toxicity in PC12 cells. *BIOORGANIC & MEDICINAL CHEMISTRY* 1–17 (2011). doi:10.1016/j.bmc.2011.11.039). Current methods for delivering siRNA to primary neurons include the use of lentiviral vectors, Adeno-Associated Viruses (AAV), or Lipofectamine™-mediated transfection (Karra, D. & Dahm, R. Transfection Techniques for Neuronal Cells. *Journal of Neuroscience* **30,** 6171–6177 (2010)). By conjugating a hydrophobic moiety such as cholesterol directly to the siRNA itself and by utilizing an additional single stranded phosphorothioated tail for enhanced uptake, it has been demonstrated herein that, not only can siRNA be delivered efficiently into primary neurons *in vitro* with minimal toxicity, but also remains a potent silencer of mRNA.

[0494] Without intending to be bound by scientific theory, one of the major advantages of RNAi over antisense technology is that the loaded RISC is expected to remain active for a long period of time in non-dividing cells (Bartlett, D. W. Insights into the kinetics of siRNA-mediated gene silencing from live-cell and live-animal bioluminescent imaging. *Nucleic Acids Research* **34,** 322–333 (2006)). Additionally, a limited number of loaded RISCs are sufficient for the induction of RNAi-mediated silencing (Stalder, L. *et al.* The rough endoplasmatic reticulum is a central nucleation site of siRNA-mediated RNA silencing. *The EMBO Journal* **32,** 1115–1127 (2013)). The data presented herein demonstrates silencing for up to three weeks *in vitro* in primary cortical neurons upon a single treatment with hsiRNA, supporting the notion that RNAi-mediated silencing can be both efficient and long lasting. The data presented herein also shows that these compounds can be used to target multiple regions in two different genes, which demonstrates the adaptability of hsiRNA for the study of alternative neurological pathways and diseases.

[0495] While a single intra-striatal injection of hsiRNA resulted in potent gene silencing near the injection site *in vivo*, the effect was not evenly spread throughout the brain. Although limited, spread to other areas of the brain (demonstrated by *in vivo* efficacy studies) could be happening through a number of mechanisms. These include movement in the CSF, spread via fiber tracts which were shown to have a large visual density of Cy3-labeled hsiRNA in distribution studies, or possibly through retrograde transport (Stewart, G. R. & Sah, D. Retrograde Transport of siRNA and Therapeutic Uses to Treat Neurological Disorders. *United States Patent Application Publication* US 2008/0039415 A1, 1–18 (2008)), although further studies will be conducted to determine the actual mechanism.

[0496] The technology presented herein is useful for understanding functional genomics of particular brain regions, as well as for studying relationships between brain regions. Additionally, the study of some neurological disorders (for example memory disorders (Samuelson, K. W. Post-traumatic stress disorder and declarative memory functioning: a review. *Dialogues in Clinical Neuroscience* **13,** 346–351 (2011))) can benefit from limited and regionally targeted distribution and silencing. However, due to its distribution profile, hsiRNA as it currently exists is not a viable therapeutic for general neurological disorders like Huntington's disease. Multiple injections may work to increase overall silencing in small rodents, but in order to adapt this technology for use in larger animal brains and humans, and to achieve even and widespread distribution, other chemical modifications and therapeutic methods of delivery will be utilized. There are a number of ways in which this might be approached. First, chemical adjustments to the hsiRNA composition itself can be made. These include conjugating it to a different lipid, supplementing the backbone with additional phosphorothioate groups, or by addition of hydrophobic moieties to the nucleotides themselves (Vaught, J. D., Dewey, T. & Eaton, B. E. T7 RNA Polymerase Transcription with 5-Position Modified UTP Derivatives. *J. Am. Chem. Soc.* **126,** 11231–11237 (2004)). All of these modifications could support a range of hydrophobicities that would allow for more improved distribution across a larger distance. Increased bioavailability could also be achieved with different modes of injection such as into the CSF instead of intrastriatally, increasing the likelihood of exposure to the whole brain. However, delivery via the CSF could favor localization of hsiRNA to brain regions other than the striatum, making it a less than ideal delivery method for the treatment of Huntington's disease. Another possibility is formulated delivery by packaging these hydrophobically modified siRNAs into exosomes and liposomes (less toxic than current Lipofectamine™ formulations) and using these natural and synthetic nanocarriers to deliver cargo in a more evenly distributed fashion (Alvarez-Erviti, L. *et al.* Delivery of siRNA to the mouse brain by systemic injection of targeted exosomes. *Nat Biotechnol* 1–7 (2011). doi:10.1038/nbt.1807; Marcus, M. & Leonard, J. FedExosomes: Engineering Therapeutic Biological Nanoparticles that Truly Deliver. *Pharmaceuticals* **6,** 659–680 (2013)). However, potency and efficacy of the delivered hsiRNA still needs to be validated for these methods.

[0497] In conclusion, HTT10150 was efficient for targeting huntingtin mRNA in primary neurons *in vitro* and locally in the mouse brain *in vivo*. This compound did not require any formulation for delivery to primary cells and enabled gene functional studies for

huntingtin as well as other targets, making it a very useful tool for the study of neurological disorders. Potential advances to this technology should allow for hsiRNA to function as a therapeutic treatment for Huntington's disease as well as other neurological diseases in the future.

1.9 Methods

Cell Culture

[0498] HeLa cells were maintained in DMEM (Corning Cellgro) supplemented with 10% fetal bovine serum (Gibco) and 100 U / mL penicillin / streptomycin (Invitrogen) and grown at 37°C and 5% CO_2. Cells were split every 2-5 days up to passage 15 and then discarded.

Cell Culture for Passive Uptake

[0499] Cells were plated in DMEM with 6% FBS at 10,000 cells / well in 96-well tissue culture treated plates. hsiRNA was diluted in OptiMEM (Gibco) to 2X final concentration and 50 μL diluted hsiRNA was added to 50 μL of cells for 3% FBS final. Cells were incubated for 72 hours at 37 °C and 5% CO_2.

Cell Culture for Lipid-Mediated Uptake

[0500] Cells were plated in DMEM with 6% FBS at 10,000 cells / well in 96-well tissue culture treated plates. hsiRNA was diluted in OptiMEM to 4X final concentration. LIPOFECTAMINE RNAIMAX Transfection Reagent (Invitrogen #13778150) was diluted to 4X final concentration (final = 0.3 μL/25 μL /well). RNAIMAX and hsiRNA were mixed 1:1 and 50 μL was added to 50 μL of cells for 3% FBS final. Cells were incubated for 72 hours at 37 °C and 5% CO_2.

Preparation of Primary Neurons

[0501] Primary cortical neurons were obtained from E15.5 mouse embryos of WT (FVBN) mice. Pregnant females were anesthetized by IP injection of Avertin (250 mg/kg weight) followed by cervical dislocation. Embryos were removed and transferred into a Petri dish with ice-cold DMEM/F12 medium (Invitrogen). Brains were removed and meninges were carefully detached. Cortices were isolated and transferred into a 1.5-ml tube with pre-warmed papain solution for 25 minutes at 37 °C and 5% CO_2 to dissolve tissue. Papain solution was prepared as follows: papain (Worthington #54N15251) was dissolved in 2 mL HibernateE (Brainbits) and 1 mL EBSS (Worthington). Separately, DNase (Worthington

#54M15168) was re-suspended in 0.5 mL HibernateE. Then, 0.25 mL of re-suspended DNase was transferred to re-suspended papain for the final solution. After the 25 minute incubation, papain solution was removed and 1 mL NbActiv4 (Brainbits) supplemented with 2.5% FBS was added to the tissue. The cortices were then dissociated by pipetting up and down with a fire polished, glass Pasteur pipet. Cortical neurons were counted and plated at $1x10^6$ cells / ml. For live-cell imaging studies, culture plates were pre-coated with poly-L-lysine (Sigma #P4707) and $2x10^5$ cells were added to the glass center of each dish. For silencing assays, neurons were plated on poly-L-lysine pre-coated 96-well plates (BD BIOCOAT #356515) at $1x10^5$ cells per well. After overnight incubation at 37°C and 5% CO_2 an equal volume of NbActiv4 (Brainbits) supplemented with anti-mitotics, 0.484 μL/mL of 5'UtP (Sigma #U6625) and 0.2402 μL/mL of 5'FdU (Sigma #F3503), to prevent the growth of non-neuronal cells, was added to neuronal cultures. Half of the volume of media was replaced every 48 hours (with new NbActiv4 with anti-mitotics) until the neurons were treated with siRNA. Once the cells were treated, media was not removed, only added. All subsequent media additions contained anti-mitotics.

mRNA Quantification

[0502] mRNA was quantified using the QuantiGene 2.0 Assay (Affymetrix #QS0011). Cells were lysed in 250 μL diluted lysis mixture (Affymetrix #13228), 1 part lysis mixture, 2 parts H_2O, with 0.167 μg / μL proteinase K (Affymetrix #QS0103) for 30 minutes at 55 °C. Cell lysates were mixed thoroughly and 40 μL (approximately 8000 cells) of lysate were added to the capture plate along with 40 μL additional diluted lysis mixture without proteinase K. Probe sets were diluted as specified in the Affymetrix protocol. For HeLa cells, 20 μL of human HTT or PPIB probe set (Affymetrix #SA-50339, #SA-10003) was added to appropriate wells for a final volume of 100 μL. For primary neurons, 20 μL of mouse HTT or PPIB probe set (Affymetrix #SB-14150, #SB-10002) was used.

[0503] Tissues were treated similarly, using 300 μL of Homogenizing Buffer (Affymetrix #10642) with 2 μg/μL proteinase K for a 5 mg tissue punch. Tissues were then homogenized in 96-well plate format on the QIAGEN TissueLyser II and 40 μL were added to the capture plate. Probe sets were diluted as specified in the Affymetrix protocol and 60 μL of either HTT or PPIB probe sets (Affymetrix #SB-14150, #SB-10002) were added to each well of the capture plate for a final volume of 100 μL. For DARPP32 quantification, only 10 μL of tissue sample and 30 μL of homogenizing buffer were added to each well with 60 μL of mouse Ppp1r1b probe set (Affymetrix #SB-21622). Signal was amplified according

to the Affymetrix protocol. Luminescence was detected on either the Veritas Luminometer or the Tecan M 1000.

Live Cell Staining

[0504] To monitor live cell hsiRNA uptake, cells were plated at a density of $2x10^5$ cells per 35 mm glass-bottom dish as described in the preparation of primary neurons above. Prior to imaging, cell nuclei were stained in phenol red free NbActiv4 using NUCBLUE (Molecular Probes by Life Technologies #R37605) as indicated by the manufacturer. Imaging was performed in phenol red free NbActiv4. Cells were treated with 0.5 μM of Cy3-labeled hsiRNA, and live cell imaging was performed over time. All live cell confocal images were acquired with a Zeiss confocal microscope and images were processed using ImageJ (1.47v) software.

Immunohistochemistry/Immunofluorescence

[0505] For distribution studies, brains were injected with 1 nmol (12.5 μg) of Cy3-labeled hsiRNA. After 24 hours, mice were sacrificed and brains were removed and sent to the DERC Morphology Core at UMASS Medical School to be embedded in paraffin and sliced into 4 μm sections and mounted on glass slides. Sections were de-parafinized for 8 minutes in xylene two times. Sections were then rehydrated with serial ethanol dilutions (100%, 95%, 80%) for 4 minutes each, then washed twice for two minutes with PBS. For NueN staining, slides were boiled for 5 minutes in antigen retrieval buffer and then left to sit at room temperature for 20 minutes, followed by a 5-minute wash with PBS. Slides were then blocked with 5% normal goat serum in PBS + 0.05% Tween20 for 1 hour and washed once with PBS + 0.05% Tween20 for 5 minutes. Primary antibody (1:1000 dilution in PBS + 0.05% Tween20) was added to slides for a 1 hour incubation followed by three 5-minute washes with PBS + 0.05% Tween20. Secondary antibody (1:1000 dilution in PBS + 0.05% Tween20) was added to slides for a 30-minute incubation in the dark followed by three 5-minute washes with PBS + 0.05% Tween20. Slides were then stained with DAPI (Molecular Probes by Life Technologies #D3571), diluted to 250 ng/mL in PBS, for one minute followed by three 1-minute washes with PBS. Mounting media and coverslips were applied to slides and left to dry over night before imaging on Leica DM5500 – DFC365FX microscope at indicated magnification.

[0506] For toxicity and microglia activation studies extracted, perfused brains were sliced into 40 μm sections on the Leica 2000T Vibratome in ice cold PBS.

Immunohistochemistry was performed on every 6th section against DARPP32 (Millipore, 1:10,000 dilution) and IBA-1 (Millipore, 1:500 dilution). Sections were mounted and visualized by light microscopy. Four images were taken at 20X in the striatum of both injected and non-injected sides of each section. The number of DARPP32 positive neurons was quantified using ImageJ. Activated microglia was quantified by morphology of stained cells for IBA-1.

Animals, Stereotaxic Injections

[0507] Wild-type (FVBN) mice received microinjections by stereotactic placement into the right striata (coordinates (relative to bregma) were 1.0 mm anterior, 2.0 mm lateral, and 3.0 mm ventral). Animals were deeply anesthetized prior to injection with 1.2% Avertin. For both toxicity (DARPP32) and efficacy studies, mice received injections of either PBS or artificial cerebrospinal fluid (2 μL per striata, N = 8 mice), 12.5 μg of NTC hsiRNA (2 μL of 500 μM stock solution per striata, N = 8 mice), 25 μg of HTT10150 hsiRNA (2 μL of 1 mM stock solution per striata, N = 8 mice), 12.5 μg of HTT10150 hsiRNA (2 μL of 500 μM stock solution per striata, N = 16 mice total, two sets of 8 mice on two different days), 6.3 μg of HTT10150 hsiRNA (2 μL of 250 μM stock solution per striata, N = 8 mice), or 3.1 μg of HTT10150 hsiRNA (2 μL of 125 μM stock solution per striata, N = 8 mice) and euthanized 5 days later. Brains were harvested and three 300 μm coronal sections were made. One 2 mm punch was taken per side (injected and non-injected) for each section and placed in RNAlater (Ambion #AM7020) for 24 hours at 4°C. Each punch was processed as an individual sample for the QuantiGene assay analysis. All animal procedures were approved by the University of Massachusetts Medical School Institutional Animal Care and Use Committee (IACUC, protocol number A-2411).

Statistical Analysis

[0508] Data analyses were done using GraphPad Prism 6 version 6.04 software (GraphPad Software, Inc., San Diego, CA). For concentration dependent curve IC50s, a curve was fitted using log(inhibitor) vs. response – variable slope (four parameters). The bottom of the curve was set to be no less than zero and the top of the curve was set to be no greater than 100. For each independent mouse experiment, the level of knockdown at each dose was normalized to the mean of the control group, which was the non-injected side of the

PBS or artificial CSF groups, so that all data were expressed as a proportion of the control. *In vivo* data were analyzed using the Kruskal-Wallis test (one-way ANOVA) with Bonferroni corrections for multiple comparisons. Differences in all comparisons were considered significant at P-values less than 0.05.

Cell culture for passive uptake (primary screen and dose response)

[0509] Cells were plated in DMEM (Gibco) with 6% FBS (Gibco) at 10,000 cells / well in 96-well tissue culture treated plates. HsiRNA was diluted in OptiMEM (Gibco) to 2X final concentration and 50uL diluted hsiRNA was added to 50 μL of cells for 3% FBS final. Cells were incubated for 72 hours at 37C and 5% CO_2.

Cell culture for lipid-mediated uptake

[0510] Cells were plated in DMEM (Gibco) with 6%FBS (Gibco) at 10,000 cells/well in 96-well tissue culture treated plates. HsiRNA was diluted in OptiMEM (Gibco) to 4X final concentration. LIPOFECTAMINE RNAIMAX Transfection Reagent (Invitrogen CAT#13778150) was diluted to 4X final concentration (final = 0.3 μL / 25 μL / well). RNAIMAX and hsiRNA were mixed 1:1 and 50 μL was added to 50uL of cells for 3% FBS final. Cells were incubated for 72 hours at 37C and 5% CO_2.

mRNA Quantification

[0511] mRNA was quantified using the QuantiGene 2.0 Assay (Affymetrix QS0011). Cells were lysed in 250 μL diluted lysis mixture, 1 part lysis mixture, 2 parts H2O, with 0.167 μg / μL proteinase K (Affymetrix QS0103) for 30 minutes at 55 C. Cell lysates were mixed thoroughly and 40 μL (~8000 cells) of lysate were added to capture plate along with 40 μL additional diluted lysis mixture without proteinase K. Tissues were treated similarly, using 300 μL of Homoginizing Buffer (Affymetrix) with 2 μg / μL proteinase K for a 5 mg tissue punch. Tissues were then homogenized in 96-well plate format on Qaigen TissueLyzer and 40 μL were added to capture plate. Probe sets were diluted as specified in Affymetrix protocol and 20 μL of either HTT or PPIB probes (Affymetrix: SA-50339, SA-10003) were added to each well of capture plate for final volume of 100 μL. Signal was amplified according to manufacture protocol. Luminescence was detected on either the Veritas Luminometer or the Tecan M 1000.

Live Cell staining and Brain Sections Immunostaining

[0512] For live cell uptake monitoring, cells were plated at a density of $2x10^5$ cells per 35 mm glass-bottom dish and grown overnight. Prior to imaging, cell organelles were stained in HBSS (Gibco) using staining reagents purchased from Life Technologies unless specified: cell nuclei, endoplasmic reticulum and lysosomes were respectively stained using the NUCBLUE Live READYPROBE, ER-TRACKER Green (Bodipy FL Glibenclamide) and LYSOTRACKER Deep Red reagents as indicated by the manufacturer. Imaging was performed in non-supplemented DMEM without phenol red (Invitrogen). Cells were treated with 0.5 μM of Cy3-labeled hsiRNA, and live cell imaging was performed over time.

Confocal Imaging

[0513] All confocal images were acquired with a CSU10B Spinning Disk Confocal System scan head (Solamere Technology Group) mounted on a TE-200E2 inverted microscope (Nikon) with a 60x Plan/APO oil lens and a Coolsnap HQ2 camera (Roper). Images were processed using ImageJ (1.47v) software. Number of neurons without or with hsiRNA was counted using ImageJ software. Brain sections images were acquired with a z-axis spacing of 1 μm.

Example 2. hsiRNA retention and distribution is directly related to hydrophobicity

[0514] Although FM-hsiRNAs showed improved retention and accumulation in brain and spinal cord and induce maximal silencing at 10-fold lower doses than partially stabilized hsiRNAs, they were largely retained near the injection site (Figure 102; Chol-hsiRNA). It was hypothesized that the limited distribution of hsiRNAs could result from preferential binding of hsiRNA to lipid-enriched myelin and myelinated structures due to the strong hydrophobicity of the cholesterol conjugate, and that tuning the hydrophobicity of the hsiRNA conjugates would improve distribution through the spinal cord and brain. To test the idea, a panel of naturally occurring, hydrophobic molecules capable of active neuronal trafficking, was screened including: (i) neuroactive steroids, i.e., endogenous steroids that traverse the blood-brain barrier and bind a variety of gated-ion channels and neuronal-expressed receptors (Rupprecht R. Neuroactive steroids: mechanisms of action and neuropsychopharmacological properties. *Psychoneuroendocrinology*. 2003; 28:139-68. PMID: 12510009), including GABA (Lan NC, Gee KW. Neuroactive steroid actions at the GABAA receptor. *Hormones and behavior*. 1994; 28:537-44. PMID: 7729823); (ii) gangliosides—neuroprotective glycolipids critical for neuronal plasticity and repair (Aureli

M, Mauri L, Ciampa MG, Prinetti A, Toffano G, Secchieri C, Sonnino S. GM1 Ganglioside: Past Studies and Future Potential. *Molecular neurobiology*. 2015. PMID: 25762012); and (iii) endocannabinoid-like long-chain polyunsaturated fatty acids—neuromodulatory lipids recognized by receptors involved in appetite, pain, mood, and memory (Dyall SC. Long-chain omega-3 fatty acids and the brain: a review of the independent and shared effects of EPA, DPA and DHA. *Frontiers in aging neuroscience*. 2015; 7:52. PMID: 25954194; PMCID: PMC4404917; Janssen CI, Kiliaan AJ. Long-chain polyunsaturated fatty acids (LCPUFA) from genesis to senescence: the influence of LCPUFA on neural development, aging, and neurodegeneration. *Progress in lipid research*. 2014; 53:1-17. PMID: 24334113; Figueroa JD, De Leon M. Neurorestorative targets of dietary long-chain omega-3 fatty acids in neurological injury. *Molecular neurobiology*. 2014; 50:197-213. PMID: 24740740; PMCID: PMC4183712).

[0515] The most robust approach for synthesis of oligonucleotide conjugates was to attach the activated conjugate to an amino-modified support. The structure and length of the linker were varied (e.g., branched) and the support was functionalized whenever feasible. Variations of the synthetic approaches outlined in Figures 93 and 94 were used to synthesize hsiRNAs conjugated to cortisol, docosahexaenoic acid (DHA), calciferol, cholesterol, and GM1 ganglioside (Figures 98 and 99). All compounds were HPLC-purified and their identities were confirmed by mass spectrometry. The calciferol-functionalized support was unstable, resulting in a mixture of several variants that were tested *in vivo* (described *infra*).

[0516] As expected, the compounds showed different degrees of hydrophobicity based on retention time during reverse phase chromatography. Injection of Cy3-labeled hsiRNA conjugates into striata or ICV (Figure 92) of wild-type mice revealed varying degrees of hsiRNA distribution and retention that strongly correlated with hydrophobicity. Non-conjugated or linker-only hsiRNAs showed minimal retention in the brain (similar to that of antisense oligonucleotides) and the most hydrophobic compounds, cholesterol and GM1, were primarily retained near the site of injection. Optimal retention/distribution was achieved with DHA and calciferol conjugates (*infra*), which have intermediate hydrophobicity profiles. DHA-hsiRNA was studied in detail and showed great efficacy and unprecedented safety (therapeutic index >20-fold) (Nikan et al., 2016; Molecular Therapy, in revision, see Appendix). In summary, the data presented herein show that tuning the hydrophobicity of conjugates is a valid strategy to identify conjugates that support optimal retention, distribution, and safety in brain tissues.

[0517] GM1-hsiRNA was efficiently internalized and induced huntingtin mRNA silencing in primary cortical neurons (Figure 108). GM1-hsiRNA displayed limited distribution in mouse brain upon intrastriatal injection (Figure 109).

Example 3. DHA-hsiRNA

[0518] Partially-modified hsiRNAs demonstrated a short duration of effect and no systemic exposure (Figures 35A-C). Metabolic stabilization was further explored (Figure 36). Full metabolic stabilization did not interfere with RISC entry of hsiRNAs (Figure 37). Fully metabolically stabilized hsiRNA (FM-hsiRNA) enhanced local delivery and distribution and enabled a longer duration of effect (Figures 38, 39A-B, 91, 110 and 111 The term "nucleoside").

[0519] Naturally-occurring lipids (i.e., glycosphingolipids, polyunsaturated fatty acids, secosteroids, steroid hormones and sterol lipids) were investigated as hsiRNA bioconjugates (Figure 40). Lipid bioconjugates had a pronounced effect on hsiRNA^{HTT} sense strand hydrophobicity.

[0520] A study was designed to explore *in vivo* distribution of hsiRNA conjugates. Intrastriatal unilateral injection (2 nmol/ 2 μl) of FVBN WT mice with P2-stabilized siRNA CY3 conjugates in aCSF was performed. 48 hours post-injection, animals were perfused with PBS and 10% formalin. Their brains were removed and post-fixed for 48 hours. 4 μm coronal and sagittal slices were prepared and stained with DAPI. Imaging was performed on a Leica DM 5500 fluorescent microscope (CY3 and DAPI). It was determined that hsiRNA hydrophobicity was directly correlated with brain distribution and retention (Figure 41). A key property was a balance between distribution and retention.

[0521] Docosahexaenoic acid (DHA) – hsiRNAs were synthesized (Figure 42). DHA is an omega-3 fatty acid that is a primary component of the human brain (70%). DHA crosses the bold brain barrier (BBB) and is actively internalized by neurons and other cell types. It is a non-toxic supplement clinically shown to improve cognitive function in HD and ALS patients. DHA is significantly less hydrophobic that cholesterol.

[0522] DHA-hsiRNA and chol-hsiRNA were shown to be internalized into primary cortical neurons (Figure 43). DHA-hsiRNA co-localized with neurons and astrocytes (Figure 44) and was localized to the perinuclear region of striatal neurons (chol-hsiRNA was undetectable in striatal neurons) (Figure 45). DHA-hsiRNA co-localized with neurons and astrocytes in the cortex following a single intrastriatal injection (Figure 46). DHA-hsiRNA

localized to the perinuclear region in cortical neurons, while chol-hsiRNA was undetectable (Figure 47). DHA-hsiRNA efficiently distributed throughout the brain and silenced genes in both the striatum and the cortex (Figure 57).

[0523] DHA-hsiRNA showed robust efficacy in the striatum and the cortex (Figures 48 and 49). Up to 200 μg DHA-hsiRNA had no effect on DARPP-32 levels, indicating compound safety (Figure 50). In contrast, 25 μg (1 mg/kg) was the maximum tolerated intrastriatal dose of chol-hsiRNA. Up to 200 μg DHA-hsiRNA caused no significant increase in activated microglia, indicating minimal immune stimulation (Figure 51).

[0524] hsiRNA allows for simple and efficient gene silencing in primary neurons *in vivo* in the brain. Oligonucleotide hydrophobicity defines brain tissue retention and distribution. Oligonucleotide chemistry was shown to impact cellular delivery and distribution (Figures 52-56). DHA-hsiRNA conjugates represent a new class of oligonucleotides with wide *in vivo* efficacy and a wide therapeutic index.

Example 4. PC-DHA-hsiRNA (PC-DHA-hsiRNA)

[0525] Encouraged by the wide therapeutic index of DHA-hsiRNAs, DHA and related conjugates were investigated in more detail. Circulating DHA is mostly present as a lysophosphatidylcholine ester, which is the only form actively trafficked through the blood-brain barrier via the specific transporter Mfsd2a (Nguyen LN, Ma D, Shui G, Wong P, Cazenave-Gassiot A, Zhang X, Wenk MR, Goh EL, Silver DL. Mfsd2a is a transporter for the essential omega-3 fatty acid docosahexaenoic acid. *Nature*. 2014; 509:503-6. PMID: 24828044).

[0526] The lysophosphatidylcholine ester of DHA is unstable, so a lysophosphatidylcholine (PC) amide of DHA was synthesized (Figures 93, 94, 100 and 101). PC-DHA is a metabolically stable analog compatible with solid-phase oligonucleotide synthesis. Its identity was confirmed by NMR and mass spectrometry. Testing the idea that lysophosphatidylcholine should improve trafficking of DHA-hsiRNA, it was determined that that PC-DHA-hsiRNAs showed a wider distribution and increased efficacy in brain tissue than do DHA-hsiRNAs (Figures 92 and 103). Importantly, as a class, DHA conjugates showed a wide therapeutic index with no obvious innate immune activation or neuronal

degeneration at concentrations 20-fold higher than the minimum effective dose (Figure 92). Comparatively, Chol-hsiRNA showed significant toxicity at 25 μg injections (Figure 92C). Lastly, a bolus CSF (ICV) infusion supported wide distribution in the brain, covering striatum, cortex and even reaching more posterior and ventral regions of the brain (Figure 92). Due to its exceptional characteristics, PC-DHA-hsiRNA was selected as a lead chemistry to investigate.

[0527] PC-DHA is a metabolically active analogue of DHA (Figure 62). PC-DHA-hsiRNA demonstrates enhanced neuronal silencing *in vitro*, enhanced brain distribution and enhanced *in vivo* potency (with no signs of toxicity) relative to DHA-hsiRNA.

[0528] PC-DHA-hsiRNA and chol-hsiRNA were each shown to efficiently silence both mutant and wild-type htt mRNA (Figure 61). Chol-hsiRNA demonstrated toxicity (3 out of 6 animals died). The living animals demonstrated very low (3-fold over background) human htt expression.

[0529] PC-DHA-hsiRNA, when delivered to primary neurons, demonstrated enhanced potency relative to DHA-hsiRNA (Figure 63). Although chol-hsiRNA was more effective in decreasing *htt* gene expression in primary neurons (Figure 64), PC-DHA-hsiRNA showed superior brain retention and wider distribution (Figure 65).

[0530] PC-DHA-hsiRNA showed approximately 80% silencing in mouse striatum after a single interstitial (IS) injection (Figure 66) and showed approximately 60% silencing in mouse cortex after a single IS injection (Figure 67). There was no indication of toxicity. Silencing was limited to injected side of the brain.

[0531] The kidney is the main target of PC-DHA-hsiRNA (Figure 107). PC-DHA-hsiRNA accumulated in the proximal convoluted tubules.

Example 5. Discovery of Di-hsiRNAs

[0532] Branched oligonucleotides represent a novel class of oligonucleotide therapeutics. Two to eight oligonucleotides were attached together through a hydrophobic linker, with 2-3 oligonucleotides attached together being preferred. Substantial chemical stabilization was typically used (at least 40% bases modified, fully modified preferred. Single stranded phosphorothioated tail of 2-20 was typically used (with 8-10 preferred).

[0533] The discovery of di-branched hsiRNA (di-hsiRNA) compounds was pure serendipity. Calciferol readily oxidizes and the solid support proved to be unstable, complicating QC and purification. A pool of four major byproducts were injected into striata of wild-type mice. It performed better than any compound that had been previously injected into the CNS. The products showed wide diffusion, great retention and preferential uptake into neurons in cortex, striatum, and spinal cord, which is an almost ideal profile. Detailed characterization by HPLC and mass spectrometry identified the byproducts present in the crude mixture: the desired calciferol-hsiRNA conjugate, hsiRNA capped with a triethylene glycol linker (TEG), and two hsiRNAs connected by a TEG linker. The latter compound resulted from calciferol being cleaved off during support loading, leaving two active groups on which to grow hsiRNA passenger strands (Figure 95A). After purifying each byproduct it was determined that each could efficiently enter RISC *in vitro* (Figure 95B), but only Di-hsiRNAs showed the wide distribution and preferential neuronal uptake (Figure 95C). A route to directly synthesize Di-hsiRNA with >70% yield (Figures 93 and 94), confirmed by mass spectrometry, was devised (Figures 100 and 101).

[0534] A bolus ICV infusion of Di-hsiRNAs supported delivery throughout the brain Di-branched hsiRNA (di-hsiRNA) compounds were determined to support wide distribution in the brain (Figures 68, 69 and 104A). Note the brain injected with Cy3-Di-hsiRNA in Figure 104A is pink throughout. Single injection of di-siRNA was detected on both ipsilateral and contralateral to injection site indicating that spread is not limited to the injected hemisphere but is also occurring across the midline into the non-injected side. The lesser degree of di-siRNA accumulation on the contralateral side, although significant, may necessitate bilateral injections for full brain silencing. Alternative methods of injection including intracerebral-ventricular may also facilitate bilateral distribution with only one injection.

[0535] Branching was determined to be essential for enhanced brain distribution (Figure 70). Di-hsiRNA distributed throughout the injected hemisphere of the mouse brain following intrastriatal injection. While a single non-conjugated hsiRNA can silence mRNA in primary neurons, the di-hsiRNA structure was essential for enhanced tissue distribution and tissue retention of modified oligo nucleotides. Other conjugates such as cholesterol, although retained, showed a steep gradient of diffusion away from the site of injection. The subtle hydrophobicity of the two single stranded phosphorothioated tails supported tissue

retention while also allowing for widespread and uniform distribution throughout the ipsilateral hemisphere of the injected brain.

[0536] *In vivo* gene silencing after single IS injections of di-hsiRNA was studied (Figure 71). Single injection of di-siRNA induced robust silencing in both the striatum and cortex of mouse brain (Figures 72 and 73). This level of efficacy has never been demonstrated previously for non-conjugated siRNAs. Although di-hsiRNA appears visually associated with fiber tracts in striatum, the efficacy observed clearly indicates that striatal neurons internalized di-siRNA to a significant degree.

[0537] Di-hsiRNA also supported uniform spinal cord distribution (Figure 74). A di-hsiRNA bolus IT injection supported *htt* silencing in spinal cords (Figure 75). Di-siRNA showed robust and even silencing throughout the spinal cord following intrathecal injection. A single injection of di-hsiRNA in the lumbar region of the spinal cord silenced mRNA to the same degree in the cervical, thoracic and lumbar regions indicating even and long range distribution. This accepted method of drug delivery will allow for treatment of neurodegenerative diseases affecting neurons in the spinal cord.

[0538] Di siRNA showed a very unique cellular distribution when injected intrastriatally into the brain (Figure 76). Fluorescently labeled di-siRNA appeared to localize preferentially with neurons in the cortex. This selective feature was specific to these compounds and was not true for other siRNA conjugates, such as cholesterol, which showed no cell-type preference.

[0539] Di-siRNA showed localization to fiber tracts in the striatum but was present within neuronal cell bodies in the cortex (Figure 77). Without intending to be bound by scientific theory, movement to the cortex could be through diffusion or could be the result of retrograde transport via striatal fiber tracts. The theory that retrograde transport is partially responsible is supported by the fact that some areas of the cortex showed full neuronal penetration while neurons in adjacent areas showed no di-hsiRNA association.

[0540] Intrathecal injection of di-hsiRNA produced similarly impressive results for the spinal cord (Figure 105A). Whereas chol-hsiRNA (the original conjugated hsiRNA) showed a steep gradient of distribution with a relatively small amount reaching grey matter and motor neurons, di-hsiRNAs uniformly distributed throughout the spinal cord and co-localized with the motor neurons (enlarged in Figure 105A).

[0541] The wide distribution of di-hsiRNA after a single injection was associated with greater than 85% silencing in the striatum, 70% silencing in the in cortex (Figure 104B) and approximately 50% silencing in the spinal cord (Figure 105B). While significant amounts of di-hsiRNAs accumulated over time in the striatum, cortex, liver and kidneys (Figure 104C), no evidence of inflammation or neuronal degeneration were detected at the highest doses tested (i.e., 400 μg ICV and 150 μg IT), which far exceed the minimum effective dose. At these levels, Chol-hsiRNAs are toxic. Based on these data, di-hsiRNAs have been selected as a second class of chemistry for detailed characterization, optimization, and validation. A detailed characterization of di-hsiRNAs will be performed to determine the dose-response, maximum tolerated dose and therapeutic index. Cellular, molecular and biochemical assays will be used to further measure the *in vivo* distribution and accumulation of compounds and the degree of target gene regulation.

Example 6. Evidence that axonal transport contributes to Di-hsiRNA distribution in brain

[0542] The preferential delivery of di-hsiRNAs to neurons, especially distal to the injection site, was encouraging. In mice intrastriatally injected with Cy3-di-hsiRNA (Figure 95C), we detect Di-hsiRNA in every NeuN-positive cell (neurons) of the cortex but not in other non-neuronal cell types (e.g., glia). One interpretation of this observation is that di-hsiRNAs are preferentially transported along axons to distal neurons. Why would branched oligonucleotides have such a profound effect on their distribution? It is hypothesized that a role for cooperative binding, whereby one hsiRNA weakly binds to a receptor, and a second independent binding event promotes internalization (Alves ID, Ciano KA, Boguslavski V, Varga E, Salamon Z, Yamamura HI, Hruby VJ, Tollin G. Selectivity, cooperativity, and reciprocity in the interactions between the delta-opioid receptor, its ligands, and G-proteins. *The Journal of biological chemistry*. 2004; 279:44673-82. PMID: 15317820). Cooperative binding by covalently linked hsiRNAs might dramatically enhance the rate of cellular uptake and consequently tissue retention. This and other hypotheses will be tested and detailed structure-activity relationship studies of di-hsiRNAs will be performed.

Example 7. Evidence PC-DHA and Di-hsiRNA conjugates: two novel classes of CNS active oligonucleotides.

[0543] As described in the data above, two novel, chemically distinct classes of therapeutic siRNAs, PC-DHA-hsiRNAs and Di-hsiRNAs, have been designed that support

wide distribution and potent gene silencing in CNS tissues after CSF infusion. Di-hsiRNAs appear promising but currently lack data on safety and therapeutic index. PC-DHA-hsiRNAs have a wide therapeutic window (Figure 103). This is important because antisense oligonucleotides in clinical trials for CNS indications have a narrow therapeutic index.

[0544] To mitigate potential risk, both classes of compounds will be evaluated in detail. The goal is to achieve greater than 70% target gene silencing at a dose of less than 200 μg/injection, greater than 10-fold therapeutic index, and 1-month to 3-month duration of effect with a bolus injection via CSF. The development of a simple technology platform that allows straightforward and long-lasting silencing in the brain and the spinal cord of a small animal will advance the field of neuroscience research significantly. It will enable direct functional analysis of a range of novel targets with suspected involvement in brain biology and neurodegenerative disease progression. The data described herein demonstrate that chemistry defines distribution, efficacy and safety of oligonucleotides. Chemical variants of PC-DHA-hsiRNA and di-hsiRNA will be evaluated to identify scaffolds with higher efficacy and wider therapeutic indices, features that are essential for future translation of this technology platform towards human therapeutics. Lastly, the performance of several compounds will be validated in established animal models of neurodegenerative disease, by silencing *HTT* in HD.

Example 8. Characterization of PC-DHA and Di-hsiRNA distribution, efficacy and safety in the brain and the spinal cord

Oligonucleotide Synthesis

[0545] HsiRNA and Di-hsiRNAs will be synthesized (0.2 grams, +/- Cy3) and HPLC-purified as fully metabolically stable hsiRNAs (including 5'-*E*-VP as a terminal phosphate analog), followed by characterization by mass spectrometry. A variety of linkers have been screened and optimal scaffolds for PC-DHA and di-hsiRNA conjugation have been identified. The functionalized supports will be synthesized as shown in Figures 93 and 94. The following compounds will be used: *HTT*-10150 (HD) and *PPIB*-437 (housekeeping control). Numbers denote the position of the human mRNA targeted by the 5′ nucleotide of the guide strand. All compounds have been previously identified using optimized bioinformatics parameters (Birmingham A, Anderson E, Sullivan K, Reynolds A, Boese Q, Leake D, Karpilow J, Khvorova A. A protocol for designing siRNAs with high functionality and specificity. *Nature protocols*. 2007; 2:2068-78. PMID: 17853862) and extensive

experimental screening. Each siRNA targets and silences the corresponding human, mouse and monkey mRNAs, which will simplify future clinical development.

[0546] In addition to standard oligonucleotide synthesis systems, i.e., Mermaid12 and Expedite, a mid-scale RNA-synthesis capability (funded through an S10 grant), including an OligoPilot 100, preparative HPLCs, and high-resolution LC-MS, have been established. Large batches of novel compounds required for the *in vivo* studies proposed below will be synthesized.

Optimization of Administration Route

[0547] Several routes of administration were compared and it was determined that a bolus infusion via CSF (ICV and IT infusion) supports the best degree of compound retention and distribution in CNS tissues. CSF delivery via these routes is analogous to a "spinal tap," a clinically acceptable route of administration. A side-by-side comparison of tissue retention and efficacy was compared when equivalent doses were delivered by bolus injection or by ALZET pump over a period of one week. Significantly better tissue retention and efficacy were observed with bolus injections, consistent with data reported for ASOs (Rigo F, Chun SJ, Norris DA, Hung G, Lee S, Matson J, Fey RA, Gaus H, Hua Y, Grundy JS, Krainer AR, Henry SP, Bennett CF. Pharmacology of a central nervous system delivered 2'-O-methoxyethyl-modified survival of motor neuron splicing oligonucleotide in mice and nonhuman primates. *The Journal of pharmacology and experimental therapeutics*. 2014; 350:46-55. PMID: 24784568; PMCID: PMC4056267). Without intending to be bound by scientific theory, better performance of bolus administration over pump administration could be related to the mechanism of oligonucleotide uptake. For example, non-productive oligonucleotide sinks might be saturated faster by bolus than by pump infusion, thereby allowing excess oligonucleotide to be transported more readily.

[0548] To directly quantify intact guide strand in tissues, we have developed and implemented a novel and quantitative peptide nucleic acid (PNA) hybridization assay was developed and implemented (Figure 106). The assay was highly sensitive, with a limit of detection of less than 10 fmole hsiRNA per gram tissue. HsiRNA metabolites with full-length, partially degraded, 5′-phosphorylated and 5′-dephosphorylated guide strand could be readily quantified as separate peaks or shoulders in the HPLC trace. Using this assay the

kinetics of guide strand retention in 2-mm punch biopsies taken from regions throughout spinal cord and brain will be quantified.

[0549] Based on previous experience, accumulation of 1 to 5 µg oligonucleotide per gram tissue one week after injection is usually enough to support productive target silencing (Figures 104B, 104C). The fluorescence and PNA assays allow mapping of the distribution and quantity of conjugated hsiRNA delivered. These studies will complement functional analyses and establish a foundation for silencing efficacy studies.

Identify the Maximum Tolerated Dose

[0550] In pilot studies, 200 µg DHA-hsiRNA and di-hsiRNA was established as a safe dose for intrastriatal injection (data for DHA is present in Figures 103B and 103C), 150 µg was established as a safe dose for intrathecal injection, and 400 µg was established as a safe dose for intracerebroventricular injection. Beginning at these levels, the dose will be increased in two-fold increments until animals show any indications of toxicity or until drug solubility limits of approximately 20 mM for PC-DHA-hsiRNA and approximately 50 mM for Di-hsiRNA are reached. Three weeks post-injection (optimal time required to see oligonucleotide toxicity), brain tissue will be collected and the number and viability of neurons will be assessed by staining for neuronal markers NeuN and DARPP-32 (Mullen RJ, Buck CR, Smith AM. NeuN, a neuronal specific nuclear protein in vertebrates. *Development (Cambridge, England)*. 1992; 116:201-11. PMID: 1483388; Weyer A, Schilling K. Developmental and cell type-specific expression of the neuronal marker NeuN in the murine cerebellum. *Journal of neuroscience research*. 2003; 73:400-9. PMID: 12868073; Ouimet CC, Miller PE, Hemmings HC, Jr., Walaas SI, Greengard P. DARPP-32, a dopamine- and adenosine 3':5'-monophosphate-regulated phosphoprotein enriched in dopamine-innervated brain regions. III. Immunocytochemical localization. *The Journal of neuroscience : the official journal of the Society for Neuroscience*. 1984; 4:111-24. PMID: 6319625). Microglial activation (innate immune activation) will also be assessed by staining for IBA1 (Judge AD, Bola G, Lee AC, MacLachlan I. Design of noninflammatory synthetic siRNA mediating potent gene silencing in vivo. *Molecular therapy : the journal of the American Society of Gene Therapy*. 2006; 13:494-505. PMID: 16343994; Marques JT, Williams BR. Activation of the mammalian immune system by siRNAs. *Nature biotechnology*. 2005; 23:1399-405. PMID: 16273073). To test whether compounds trigger a reversible, short-term

inflammatory response, mice will be injected with the maximum tolerated dose and glial activation will be examined at 6 hours post-administration. Completion of this study will generate data on the maximum tolerated dose for the two new classes of therapeutic hsiRNAs described herein.

Estimate PC-DHA and Di-hsiRNAs Clearance Profiles

[0551] The residence time of RNAs in CSF and blood will be determined. A repetitive CSF withdrawal in mice is unfeasible, therefore CSF clearance studies will be performed using rats, adjusting the dose accordingly. 10 µl of CSF will be drawn at 1, 6, 12 and 24 hours and at 1 week post-administration of PC-DHA- and Di-hsiRNAs using overlapping groups of animals. Similarly, 20 µL of blood will be collected at 5 and 30 min, and at 1, 4, 12, 24, 48, 72 and 96 hours post-injection. To minimize concerns related to repetitive blood draws over short time periods, and to minimize the number of animals required to obtain precise data, jugular vein catheterization will be used.

[0552] Based on previous pharmacokinetic studies with related siRNA compounds, it is expected that biphasic clearance kinetics will be observed, with the fast phase completed within four to six hours. Based on pilot studies, it could take a month(s) for complete drug clearance. However, a one-week pilot study is enough to approximate the clearance profile. Completion of this study will generate pilot data on clearance profiles for the two new classes of therapeutic hsiRNAs described herein.

Establishing the Dose Response

[0553] Dose-response studies will be performed to determine the optimal dose for silencing in areas of the brain showing significant oligonucleotide accumulation. Experiments will be performed similarly to those presented in Figures 103, 104 and 105. 3-mm punch biopsies will be harvested from the brain and spinal column of mice injected with increasing doses of PC-DHA and Di-hsiRNA, the levels of *HTT* or control mRNAs will be measured using the QUANTIGENE® assay.

[0554] QUANTIGENE® is a highly sensitive 96-well-based assay that uses signal amplification to detect mRNA in tissue or cell lysates directly. A protocol describing an automated, high-throughput (96-well) version of the assay that directly links TissueLyser and QUANTIGENE ® was recently published (Coles AH, Osborn MF, Alterman JF, Turanov AA, Godinho BM, Kennington L, Chase K, Aronin N, Khvorova A. A High-Throughput Method for Direct Detection of Therapeutic Oligonucleotide-Induced Gene Silencing In

Vivo. *Nucleic acid therapeutics*. 2015. PMID: 26595721). Thus, simultaneous quantification of *HTT* and housekeeping mRNAs can be performed for many tissues and/or animals. In pilot studies, it was determined that eight mice per group was sufficient to detect a 40% reduction in target gene expression with 80% confidence. *Id.*

[0555] *HTT* mRNA levels will be normalized to a control housekeeping mRNA. Artificial CSF and non-targeting controls (NTC) of the same chemical composition will be used to control for non-sequence-specific events. NTC hsiRNA will only be injected at the highest non-toxic concentration to limit the number of animals used. Though NTC is a better negative control, a second targeting hsiRNA (e.g., *PPIB*-targeting) will provide silencing data on two different targets with the same number of animals. Confirmation of silencing at the protein level is essential before transitioning toward animal models of disease, so Western blotting will be performed in a similar manner as has been done for chol-hsiRNAs (Alterman JF, Hall LM, Coles AH, Hassler MR, Didiot MC, Chase K, Abraham J, Sottosanti E, Johnson E, Sapp E, Osborn MF, Difiglia M, Aronin N, Khvorova A. Hydrophobically Modified siRNAs Silence Huntingtin mRNA in Primary Neurons and Mouse Brain. *Molecular therapy Nucleic acids*. 2015; 4:e266. PMID: 26623938). Completion of this study should identify doses enabling functional gene silencing in different regions of the CNS for the two new classes of therapeutic hsiRNAs described herein.

PC-DHA and Di-hsiRNA Duration of Silencing Upon Single Administration

[0556] Most neurodegenerative disorders and disease models present a late onset of symptoms (e.g., 3 to 9 months in mice). The duration of silencing from one injection and how many injections will be needed to support 6 to 9 months of silencing should be determined. In general, siRNA-induced silencing in non-dividing cells is expected to last for month(s). The half-life of loaded RISC complex is several weeks (Whitehead KA, Langer R, Anderson DG. Knocking down barriers: advances in siRNA delivery. *Nature reviews Drug discovery*. 2009; 8:129-38. PMID: 19180106) and less than 1,000 loaded RISC molecules per cell are sufficient to induce silencing (Stalder L, Heusermann W, Sokol L, Trojer D, Wirz J, Hean J, Fritzsche A, Aeschimann F, Pfanzagl V, Basselet P, Weiler J, Hintersteiner M, Morrissey DV, Meisner-Kober NC. The rough endoplasmatic reticulum is a central nucleation site of siRNA-mediated RNA silencing. *The EMBO journal*. 2013; 32:1115-27. PMID: 23511973; PMCID: 3630355; Pei Y, Hancock PJ, Zhang H, Bartz R, Cherrin C, Innocent N, Pomerantz CJ, Seitzer J, Koser ML, Abrams MT, Xu Y, Kuklin NA, Burke PA,

Sachs AB, Sepp-Lorenzino L, Barnett SF. Quantitative evaluation of siRNA delivery in vivo. *Rna*. 2010; 16:2553-63. PMID: 20940339; PMCID: 2995415).

[0557] Moreover, FM-hsiRNAs may provide another advantage. A cell usually takes up millions of hsiRNAs, but the vast majority are trapped in lysosomes. Conventional, partially modified hsiRNAs entrapped in lysosomes are degraded, but FM-hsiRNAs are not. As a result, FM-hsiRNAs transiently trapped in lysosomes form an intracellular "depot" that slowly releases FM-hsiRNAs, making them available for RISC loading. Data from the Alnylam GalNAc trials indicate that optimized delivery to the liver provides up to six-month efficacy from a single subcutaneous injection. The data presented herein are in line with this observation; a single FM-hsiRNA injection provides maximal silencing for at least a month (Figure 2D).

[0558] To measure the retention of hsiRNA and duration of silencing, three mice will be injected with the highest tolerated dose of PC-DHA- or di-hsiRNAs, and the levels of intact guide strand in tissues will be measured at 1, 2 and 4 weeks and at 2, 3, 4 and 6 months using the PNA assay (Figure 106). As soon as intact guide strand levels fall below 1 μg hsiRNA per gram tissue, the study will be terminated. *HTT* mRNA levels will be measured at time points where guide strand concentration is above one μg per gram tissue in a separate study powered (n=8) to reliably detect silencing effects. Though it is expected that the duration of silencing will be at least three months, experimental validation is desired.

Exploring Mechanisms of Cellular Uptake and Trafficking of Di-hsiRNAs

[0559] Di-branched hsiRNAs showed significantly enhanced retention and distribution in CNS tissues compared to an equal dose of linker-bound single siRNA, indicating that cooperative binding by the covalently linked siRNAs or receptor dimerization drive cellular uptake (Figure 95C). Differential uptake will be visualized and characterized using a combination of TESM microscopy (time-resolved epifluorescence structure microscopy) and mass spectrometry.

Develop "antidotes" for HTT Compounds

[0560] Gene therapy approaches (i.e., permanent gene silencing) are currently being considered for treatment of neurodegenerative disorders, so 1- to 6-month duration of silencing seems relatively safe. Nevertheless, an "antidote" to reverse the silencing would satisfy concerns about safety. An "antidote" is also be a great tool to study gene function *in*

vivo, allowing one to test how long a gene needs to be downregulated to produce associated phenotypes.

[0561] Addressing similar concerns from the FDA, Alnylam has developed a technology, called "REVERSIR®," which enables reversal of long-term silencing. The concept involves developing a high-affinity antisense (LNA and 2′-O-methyl/deoxy) MIXMER® fully complementary to the seed region of the functional hsiRNA. WA panel of hsiANTIDOTEs targeting HTT10150 (and eventually other compounds) will be designed and synthesized, and their ability to reverse silencing *in vitro* and *in vivo* will be assessed (Figure 96). Antidotes will be synthesized in the context of the PC-DHA conjugate to enable similar distribution properties as the PC-DHA-hsiRNA. Completion of this study will generate antidotes against lead hsiRNA compounds to enable reversal of their *in vivo* activity, if desired.

Alternative Approach: Test Whether PC-DHA Conjugation and Di-branched Structure Improve Antisense-Mediated Silencing in the Brain

[0562] Antisense oligonucleotides for the treatment of neurodegenerative disorders are in clinically advanced stages of development (Evers MM, Toonen LJ, van Roon-Mom WM. Antisense oligonucleotides in therapy for neurodegenerative disorders. *Advanced drug delivery reviews*. 2015. PMID: 25797014; Kordasiewicz HB, Stanek LM, Wancewicz EV, Mazur C, McAlonis MM, Pytel KA, Artates JW, Weiss A, Cheng SH, Shihabuddin LS, Hung G, Bennett CF, Cleveland DW. Sustained therapeutic reversal of Huntington's disease by transient repression of huntingtin synthesis. *Neuron*. 2012; 74:1031-44. PMID: 22726834; PMCID: PMC3383626). IONIS-HTT_{Rx} is a generation 2.5 antisense chemistry proprietary to Ionis and not generally available to the academic community.

[0563] A highly potent locked-nucleic acid (LNA) GapmeR targeting *HTT* has been developed, however. To test whether a PC-DHA conjugation and/or di-branching can improve the distribution and retention of antisense oligonucleotides in brain tissues and reduce their effective doses, PC-DHA- and di-LNA GapmeRs targeting *HTT* will be synthesized.

[0564] Completion of this example will result in the full characterization of the two novel oligonucleotide conjugates (i.e., PC-DHA and di-hsiRNAs) described herein in CNS (brain and spinal cord), including optimal delivery route, drug clearance and retention, safety, dose response and duration of effect. The experimentation described herein will enable use

of these chemistries for gene silencing and target validation studies in CNS *in vivo*, as well as provide a solid foundation toward development of novel therapies for HD.

Example 9. Synthesis and Characterization of a panel of PC-DHA and Di-hsiRNA Chemical Variants to Improve Distribution and Therapeutic Index

[0565] The data presented herein (Figures 92, 103, 104 and 105) indicates that PC-DHA- and di-hsiRNA chemistry will be sufficient to reach a target of 1-month to 3-month duration of effect in spinal cord, striatum, and cortex, which is sufficient for functional genomics studies *in vivo*. This alone is a significant achievement, but future translation of this technology platform toward human therapeutics represents another level of complexity. Before we translate the technology, we will optimize the chemistry for (i) the widest possible therapeutic index and (ii) enhanced distribution to support delivery to large brains.

[0566] Slight changes in the chemical scaffold of a conjugate can profoundly affect tissue distribution as was demonstrated by functionalizing DHA with phosphatidylcholine (Figure 92). Capitalizing on these synthetic platforms, a panel of PC-DHA- and di-hsiRNA variants will be synthesized to further optimize therapeutic index and wide tissue distribution.

PC-DHA Optimization

[0567] There are two essential components to the PC-DHA structure: phosphatidylcholine and DHA (see structure in Figure 92). The synthesis approach described herein (Figures 93 and 94) will allow these chemistries to be varied independently. Little to no information exists in the literature on the structure-function relationship of oligonucleotide conjugates, but a large body of information exists describing how polymer structures and lipid compounds affect lipid-particle formation[48] . The studies indicate that the length of lipid has a major impact on overall formulation efficacy.

[0568] Polyunsaturated bonds are essential for enhanced hsiRNA distribution in CNS tissues. Conjugation of DCA, a fully saturated analog of DHA, does not promote wide distribution in CNS. Conjugation of EPA, two carbons shorter than DHA, leads to an interesting distribution profile, but efficacy has not yet been tested. A panel of phosphatidylcholine-modified polyunsaturated fatty acid variants, changing the length of the lipid tail from 10 to 22 carbons and the number of polyunsaturated bonds from 0 to 4 will be synthesized. The precursors for these synthesis reactions are all commercially available.

These compounds will reveal how the length of the lipid tail length and number of polyunsaturated bonds affects oligonucleotide distribution in the CNS.

[0569] Second, a systematic substitution of the choline entity will be performed for a range of modifications, mostly favoring naturally occurring chemical scaffolds, e.g., phosphatidylserine, phosphatidylinositol and phosphatidyl amine. Most of these syntheses can be performed in parallel, creating a library of compounds with fixed lipid tail composition and a variety of head groups. This library will be used to define the importance of head groups on the *in vivo* performance of hsiRNA conjugates.

[0570] It is well known that, regardless of the nature of the chemistry or formulation for delivery, the vast majority of internalized oligonucleotides are not biologically available. Endosomolytic, peptide-modified polymers have been used by Arrowhead Research Corporation (Wong SC, Klein JJ, Hamilton HL, Chu Q, Frey CL, Trubetskoy VS, Hegge J, Wakefield D, Rozema DB, Lewis DL. Co-injection of a targeted, reversibly masked endosomolytic polymer dramatically improves the efficacy of cholesterol-conjugated small interfering RNAs in vivo. *Nucleic acid therapeutics*. 2012; 22:380-90. PMID: 23181701) to enhance systemic efficacy of co-administered cholesterol-modified siRNA compounds. Building on this concept, a library of linkers varying the number and composition of endosomolytic peptides was synthesized. Most variants had no impact on chol-hsiRNA efficacy, but the best lead (Figure 97A) enhanced silencing upon passive uptake greater than 10-fold (Figure 97B). Without intending to be bound by scientific theory, the enhanced activity likely results from the increased bioavailability of internalized chol-hsiRNA, as the modified linker did not increase the efficacy by lipid-mediated uptake (Figure 97B) or the overall amount of oligonucleotide internalized. This linker will be combined with the most optimal combination of lipid length and head group.

Di-hsiRNA Optimization

[0571] The two hsiRNAs in the di-hsiRNA compound are connected asymmetrically: one by a phosphate bond and the other by a phosphoramidate bond (Figures 93, 94 and 103A). To establish whether the phosphoramidate bond is necessary, a di-branched compound in which the hsiRNAs are both connected to the linker via phosphates is being currently tested (modified synthesis scheme based on that in Figures 93 and 94). Showing that the phosphoramidate bond is not essential would simplify structure-activity relationship studies, as a large number of commercially available precursors can be used. Nevertheless, a

requirement for the phosphoamide bond would be interesting because phosphoamide is much less stable in acidic conditions and is expected to promote release of compounds from endosomes.

[0572] A panel of variants will be synthesized, testing the following parameters: number of hsiRNAs (2, 3, 4 or 6, using two- and three-branch dividers available from Glen Research); and the chemical nature of the linker connecting the oligonucleotides (TEG, saturated and non-saturated alkyl chain, charged, non-charged, lengths from 3 to 30 carbons and proton sponges). The minimal number of phosphorothioate bonds required for uptake will also be identified. It was already determined that phosphorothioate bonds were essential for passive uptake and efficacy of hsiRNA, so it is suspected that cooperative binding of two phosphorothioate tails drives the enhanced distribution and uptake of Di-hsiRNAs. However, phosphorothioate bonds also drive the toxicity of antisense oligonucleotides (Geary RS, Norris D, Yu R, Bennett CF. Pharmacokinetics, biodistribution and cell uptake of antisense oligonucleotides. *Advanced drug delivery reviews*. 2015;87:46-51). Thus, optimizing and reducing the number of phosphorothioates is a reasonable path toward enhancing the therapeutic index.

Evaluation of Efficacy of PC-DHA- and Di-hsiRNA Variants

[0573] Tissue culture experiments will be used to confirm safety and efficient entry into the RISC complex. Each compound will then be injected ICV at the minimum effective and maximum tolerated doses established as described herein. Compounds that are efficacious at lower concentrations or/and nontoxic at higher concentrations will be selected for detailed studies. Lastly, the ability of the most promising compounds to distribute through a large brain (e.g., sheep) will be assessed. A sheep model has been designed to evaluate the distribution of AAV-htt vectors. The PNA assay described herein will be used to measure the levels of compound in biopsies from different regions of the sheep brain after a bolus ICV infusion.

[0574] Completion of this example is expected to: (i) inform on chemical structures that define *in vivo* efficacy of PC-DHA- and Di-hsiRNAs; and (ii) generate versions of these compounds with enhanced distribution, efficacy and therapeutic index.

Example 10. Development of Candidate Pre-Clinical Compounds for Huntington's Disease Models

Evaluation of Novel Conjugate of hsiRNA HTT10150 in Huntington's Disease Animal Models

[0575] The hyper-functional, FM-hsiRNA for *Htt* , HTT-10150, is described herein. Multiple HD animal models currently running in his lab (Chang R, Liu X, Li S, Li XJ. Transgenic animal models for study of the pathogenesis of Huntington's disease and therapy. *Drug design, development and therapy*. 2015; 9:2179-88. PMID: 25931812; PMCID: PMC4404937), including YAC128 (Hodgson JG, Agopyan N, Gutekunst CA, Leavitt BR, LePiane F, Singaraja R, Smith DJ, Bissada N, McCutcheon K, Nasir J, Jamot L, Li XJ, Stevens ME, Rosemond E, Roder JC, Phillips AG, Rubin EM, Hersch SM, Hayden MR. A YAC mouse model for Huntington's disease with full-length mutant huntingtin, cytoplasmic toxicity, and selective striatal neurodegeneration. *Neuron*. 1999; 23:181-92. PMID: 10402204), BACHD (Hult S, Soylu R, Bjorklund T, Belgardt BF, Mauer J, Bruning JC, Kirik D, Petersen A. Mutant huntingtin causes metabolic imbalance by disruption of hypothalamic neurocircuits. *Cell metabolism*. 2011;13:428-39. PMID: 21459327; Hult Lundh S, Nilsson N, Soylu R, Kirik D, Petersen A. Hypothalamic expression of mutant huntingtin contributes to the development of depressive-like behavior in the BAC transgenic mouse model of Huntington's disease. *Human molecular genetics*. 2013; 22:3485-97. PMID: 23697793; Gray M, Shirasaki DI, Cepeda C, Andre VM, Wilburn B, Lu XH, Tao J, Yamazaki I, Li SH, Sun YE, Li XJ, Levine MS, Yang XW. Full-length human mutant huntingtin with a stable polyglutamine repeat can elicit progressive and selective neuropathogenesis in BACHD mice. *The Journal of neuroscience : the official journal of the Society for Neuroscience*. 2008; 28:6182-95. PMID: 18550760; PMCID: PMC2630800), and recently established allelic series including Q140 (Website: chdifoundation.org), will be used.

[0576] Based on optimal parameters identified as described herein, a bolus ICV injection of HTT-10150 will be administered into each Huntington's mouse model and the mice will be assayed for *Htt* silencing, Huntington's behavior and/or onset of Huntington's-associated phenotypes, and validated histological parameters. A set of validated assays have been designed to detect differential expression of YAC128 and Q140 mutant mRNAs and wild-type *Htt* mRNA. A panel of behavioral assays has been designed to assess motor function, including rotarod, elevated platform, and open field assays (Sah DW, Aronin N. Oligonucleotide therapeutic approaches for Huntington disease. *The Journal of clinical investigation*. 2011; 121:500-7. PMID: 21285523; PMCID: 3026739; Kordasiewicz HB, Stanek LM, Wancewicz EV, Mazur C, McAlonis MM, Pytel KA, Artates JW, Weiss A,

Cheng SH, Shihabuddin LS, Hung G, Bennett CF, Cleveland DW. Sustained therapeutic reversal of Huntington's disease by transient repression of huntingtin synthesis. *Neuron.* 2012; 74:1031-44. PMID: 22726834; PMCID: PMC3383626). One group of mice will be treated at age three months to assess disease prevention, and another group of mice will be treated at age six months to assess disease reversal. HTT aggregates will be assessed by immunohistochemical staining using a commercially available anti-polyglutamine antibody (3B5H10). HTT-10150 hsiRNA conjugates will be re-administered, if necessary. Control groups will include mice injected with PBS and non-targeting control compound having identical chemistry as the HTT-10150 hsiRNA conjugate. The best lead will be re-test them independently by another group in several behavioral models of Huntington's disease.

[0577] Completion of this example, together with efficacy, safety and duration-of-effect studies, will generate a set of data sufficient to move the optimized hsiRNA HTT-10150 into pre-clinical development. Currently, the best available program for oligonucleotide-based treatment of Huntington's disease is the 2′-O-methoxyethyl GapmeR (Id.), which will be used as a benchmark. The IONIS-HTT_{Rx} compound has recently initiated Phase 1 clinical trials in which patients receive a bolus spinal tap injection and a reduction in HTT levels in CSF serves as a biomarker for proof of concept. This establishes a clinical path forward in the development of oligonucleotide therapeutics to treat Huntington's disease. Initially, it is planned to silence both mutant and wild-type *Htt*, similar to the IONIS approach. The scaffold described herein will also be used to generate compounds that selectively silence mutant HTT by SNP discrimination. Indeed, five SNP alleles are linked to toxic CAG expansion in 75% of Huntington's disease patient mutations (Pfister EL, Kennington L, Straubhaar J, Wagh S, Liu W, DiFiglia M, Landwehrmeyer B, Vonsattel JP, Zamore PD, Aronin N. Five siRNAs targeting three SNPs may provide therapy for three-quarters of Huntington's disease patients. *Current biology : CB.* 2009;19:774-8. PMID: 19361997; PMCID: PMC2746439).

Incorporation by Reference

[0578] The contents of all cited references (including literature references, patents, patent applications, and websites) that maybe cited throughout this application are hereby expressly incorporated by reference in their entirety for any purpose, as are the references cited therein. The disclosure will employ, unless otherwise indicated, conventional techniques of immunology, molecular biology and cell biology, which are well known in the art.

[0579] The present disclosure also incorporates by reference in their entirety techniques well known in the field of molecular biology and drug delivery. These techniques include, but are not limited to, techniques described in the following publications:

Atwell et al. J. Mol. Biol. 1997, 270: 26-35;

Ausubel et al. (eds.), CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, John Wiley &Sons, NY (1993);

Ausubel, F.M. et al. eds., SHORT PROTOCOLS IN MOLECULAR BIOLOGY (4th Ed. 1999) John Wiley & Sons, NY. (ISBN 0-471-32938-X);

CONTROLLED DRUG BIOAVAILABILITY, DRUG PRODUCT DESIGN AND PERFORMANCE, Smolen and Ball (eds.), Wiley, New York (1984);

Giege, R. and Ducruix, A. Barrett, CRYSTALLIZATION OF NUCLEIC ACIDS AND PROTEINS, a Practical Approach, 2nd ea., pp. 20 1-16, Oxford University Press, New York, New York, (1999);

Goodson, in MEDICAL APPLICATIONS OF CONTROLLED RELEASE, vol. 2, pp. 115-138 (1984);

Hammerling, et al., in: MONOCLONAL ANTIBODIES AND T-CELL HYBRIDOMAS 563-681 (Elsevier, N.Y., 1981;

Harlow et al. , ANTIBODIES: A LABORATORY MANUAL, (Cold Spring Harbor Laboratory Press, 2nd ed. 1988);

Kabat et al., SEQUENCES OF PROTEINS OF IMMUNOLOGICAL INTEREST (National Institutes of Health, Bethesda, Md. (1987) and (1991);

Kabat, E.A., *et al.* (1991) SEQUENCES OF PROTEINS OF IMMUNOLOGICAL INTEREST, Fifth Edition, U.S. Department of Health and Human Services, NIH Publication No. 91-3242;

Kontermann and Dubel eds., ANTIBODY ENGINEERING (2001) Springer-Verlag. New York. 790 pp. (ISBN 3-540-41354-5).

Kriegler, Gene Transfer and Expression, A Laboratory Manual, Stockton Press, NY (1990);

Lu and Weiner eds., CLONING AND EXPRESSION VECTORS FOR GENE FUNCTION ANALYSIS (2001) BioTechniques Press. Westborough, MA. 298 pp. (ISBN 1-881299-21-X).

MEDICAL APPLICATIONS OF CONTROLLED RELEASE, Langer and Wise (eds.), CRC Pres., Boca Raton, Fla. (1974);

Old, R.W. & S.B. Primrose, PRINCIPLES OF GENE MANIPULATION: AN INTRODUCTION TO GENETIC ENGINEERING (3d Ed. 1985) Blackwell Scientific Publications, Boston. Studies in Microbiology; V.2:409 pp. (ISBN 0-632-01318-4).

Sambrook, J. et al. eds., MOLECULAR CLONING: A LABORATORY MANUAL (2d Ed. 1989) Cold Spring Harbor Laboratory Press, NY. Vols. 1-3. (ISBN 0-87969-309-6).

SUSTAINED AND CONTROLLED RELEASE DRUG DELIVERY SYSTEMS, J.R. Robinson, ed., Marcel Dekker, Inc., New York, 1978

Winnacker, E.L. FROM GENES TO CLONES: INTRODUCTION TO GENE TECHNOLOGY (1987) VCH Publishers, NY (translated by Horst Ibelgaufts). 634 pp. (ISBN 0-89573-614-4).

Equivalents

[0580] The disclosure may be embodied in other specific forms without departing from the spirit or essential characteristics thereof. The foregoing embodiments are therefore to be considered in all respects illustrative rather than limiting of the disclosure. Scope of the disclosure is thus indicated by the appended claims rather than by the foregoing description, and all changes that come within the meaning and range of equivalency of the claims are therefore intended to be embraced herein.

Claims

What is claimed:

1. An RNA molecule that is between 15 and 35 bases in length, comprising a region of complementarity which is substantially complementary to 5' CAGUAAAGAGAUUAA 3' (SEQ ID NO:1).

2. The RNA molecule of claim 1, wherein said RNA molecule is single stranded (ss) RNA or double stranded (ds) RNA.

3. The dsRNA of claim 2 comprising a sense strand and an antisense strand, wherein the antisense strand comprises the region of complementarity which is substantially complementary to 5' CAGUAAAGAGAUUAA 3' (SEQ ID NO:1).

4. The dsRNA of claim 2, wherein said dsRNA is between 30 and 35 base pairs in length.

5. The dsRNA of claim 2, wherein said region of complementarity is complementary to at least 10, 11, 12 or 13 contiguous nucleotides of SEQ ID NO:1.

6. The dsRNA of claim 2, wherein said region of complementarity contains no more than 3 mismatches with SEQ ID NO:1.

7. The dsRNA of claim 3, wherein said region of complementarity is fully complementary to SEQ ID NO:1.

8. The dsRNA of claim 2, wherein said dsRNA is blunt-ended.

9. The dsRNA of claim 2, wherein said dsRNA comprises at least one single stranded nucleotide overhang.

10. The dsRNA of claim 2, wherein said dsRNA comprises naturally occurring nucleotides.

11. The dsRNA of claim 2, wherein said dsRNA comprises at least one modified nucleotide.

12. The dsRNA of claim 11, wherein said modified nucleotide is chosen from the group of: a 2'-O-methyl modified nucleotide, a nucleotide comprising a 5'phosphorothioate group, and a terminal nucleotide linked to a cholesteryl derivative or dodecanoic acid bisdecylamide group.

13. The dsRNA of claim 11, wherein said modified nucleotide is chosen from the group of: a 2'-deoxy-2'-fluoro modified nucleotide, a 2'-deoxy-modified nucleotide, a locked nucleotide, an abasic nucleotide, 2'-amino-modified nucleotide, 2'-alkyl-modified nucleotide, morpholino nucleotide, a phosphoramidate, and a non-natural base comprising nucleotide.

14. The dsRNA of claim 2, wherein said dsRNA comprises at least one 2'-O-methyl modified nucleotide and at least one nucleotide comprising a 5'phosphorothioate group.

15. A pharmaceutical composition for inhibiting the expression of Huntingtin (*HTT*) gene in an organism, comprising the dsRNA of claim 2 and a pharmaceutically acceptable carrier.

16. The dsRNA of claim 2, wherein said dsRNA comprises a cholesterol moiety.

17. The RNA molecule of claim 1, wherein the RNA molecule comprises a 5' end, a 3' end and has complementarity to a target, wherein:

(1) the RNA molecule comprises alternating 2'-methoxy-ribonucleotides and 2'-fluoro-ribonucleotides;

(2) the nucleotides at positions 2 and 14 from the 5' end are not 2'-methoxy-ribonucleotides;

(3) the nucleotides are connected via phosphodiester or phosphorothioate linkages; and

(4) the nucleotides at positions 1-6 from the 3' end, or positions 1-7 from the 3' end, are connected to adjacent nucleotides via phosphorothioate linkages.

18. The dsRNA of claim 2, said dsRNA having a 5' end, a 3' end and complementarity to a target, and comprising a first oligonucleotide and a second oligonucleotide, wherein:

(1) the first oligonucleotide comprises a sequence set forth as SEQ ID NO:1;

(2) a portion of the first oligonucleotide is complementary to a portion of the second

oligonucleotide;

(3) the second oligonucleotide comprises alternating 2'-methoxy-ribonucleotides and 2'-fluoro-ribonucleotides;

(4) the nucleotides at positions 2 and 14 from the 3' end of the second oligonucleotide are 2'-methoxy-ribonucleotides; and

(5) the nucleotides of the second oligonucleotide are connected via phosphodiester or phosphorothioate linkages.

19. The nucleic acid of claim 18, wherein the second oligonucleotide is linked to a hydrophobic molecule at the 3' end of the second oligonucleotide.

20. The nucleic acid of claim 19, wherein the linkage between the second oligonucleotide and the hydrophobic molecule comprises polyethylene glycol or triethylene glycol.

21. The nucleic acid of claim 18, wherein the nucleotides at positions 1 and 2 from the 3' end of second oligonucleotide are connected to adjacent nucleotides via phosphorothioate linkages.

22. The nucleic acid of claim 18, wherein the nucleotides at positions 1 and 2 from the 3' end of second oligonucleotide, and the nucleotides at positions 1 and 2 from the 5' end of second oligonucleotide, are connected to adjacent ribonucleotides via phosphorothioate linkages.

23. A method for inhibiting expression of *HTT* gene in a cell, the method comprising:

(a) introducing into the cell a double-stranded ribonucleic acid (dsRNA) of claim 2; and

(b) maintaining the cell produced in step (a) for a time sufficient to obtain degradation of the mRNA transcript of the *HTT* gene, thereby inhibiting expression of the *HTT* gene in the cell.

24. A method of treating or managing Huntington's disease comprising administering to a patient in need of such treatment or management a therapeutically effective amount of said dsRNA of claim 2.

25. The method of claim 24, wherein said dsRNA is administered to the brain of the patient.

26. The method of claim 24, wherein said dsRNA is administered by intrastriatal infusion.

27. The method of claim 25, wherein administering the dsRNA of claim 2 to the brain causes a decrease in *HTT* gene mRNA in the striatum.

28. The method of claim 24, wherein administering the dsRNA of claim 2 to the brain causes a decrease in *HTT* gene mRNA in the cortex.

29. A vector for inhibiting the expression of *HTT* gene in a cell, said vector comprising a regulatory sequence operably linked to a nucleotide sequence that encodes an RNA molecule substantially complementary to 5' CAGUAAAGAGAUUAA 3' (SEQ ID NO:1), wherein said RNA molecule is between 10 and 35 bases in length, and wherein said RNA molecule, upon contact with a cell expressing said *HTT* gene, inhibits the expression of said *HTT* gene by at least 20%.

30. The vector of claim 29, wherein said RNA molecule is ssRNA or dsRNA.

31. The dsRNA of claim 30 comprising a sense strand and an antisense strand, wherein the antisense strand comprises the region of complementarity which is substantially complementary to 5' CAGUAAAGAGAUUAA 3' (SEQ ID NO:1).

32. A cell comprising the vector of claim 30.

33. An RNA molecule that is between 15 and 35 base pairs in length, comprising a region of complementarity which is substantially complementary to 5' AUAUCAGUAAAGAGA 3' (SEQ ID NO:2) or 5' CUCAGGAUUUAAAAU 3' (SEQ ID NO:3).

34. The RNA molecule of claim 33, wherein said RNA molecule is ssRNA or dsRNA.

35. The dsRNA of claim 34 comprising a sense strand and an antisense strand, wherein the antisense strand comprises the region of complementarity which is substantially

complementary to 5' AUAUCAGUAAAGAGA 3' (SEQ ID NO:2) or 5' CUCAGGAUUUAAAAU 3' (SEQ ID NO:3).

36. The dsRNA of claim 34, wherein said dsRNA is between 30 and 35 base pairs in length.

37. The dsRNA of claim 34, wherein said region of complementarity is complementary to at least 10, 11, 12 or 13 contiguous nucleotides of SEQ ID NO:2 or 3.

38. The dsRNA of claim 34, wherein said region of complementarity contains no more than 3 mismatches with SEQ ID NO: 2 or 3.

39. The dsRNA of claim 35, wherein said region of complementarity is fully complementary to SEQ ID NO: 2 or 3.

40. The dsRNA of claim 34, wherein said dsRNA is blunt-ended.

41. The dsRNA of claim 34, wherein said dsRNA comprises at least one single stranded nucleotide overhang.

42. The dsRNA of claim 34, wherein said dsRNA comprises naturally occurring nucleotides.

43. The dsRNA of claim 34, wherein said dsRNA comprises at least one modified nucleotide.

44. The dsRNA of claim 43, wherein said modified nucleotide is chosen from the group of: a 2'-O-methyl modified nucleotide, a nucleotide comprising a 5' phosphorothioate group, and a terminal nucleotide linked to a cholesteryl derivative or dodecanoic acid bisdecylamide group.

45. The dsRNA of claim 43, wherein said modified nucleotide is chosen from the group of: a 2'-deoxy-2'-fluoro modified nucleotide, a 2'-deoxy-modified nucleotide, a locked nucleotide, an abasic nucleotide, 2'-amino-modified nucleotide, 2'-alkyl-modified nucleotide, morpholino nucleotide, a phosphoramidate, and a non-natural base comprising nucleotide.

46. The dsRNA of claim 34, wherein said dsRNA comprises at least one 2'-O-methyl modified nucleotide and at least one nucleotide comprising a 5' phosphorothioate group.

47. A pharmaceutical composition for inhibiting the expression of the *HTT* gene in an organism, comprising the dsRNA of claim 34 and a pharmaceutically acceptable carrier.

48. The dsRNA of claim 34, wherein said dsRNA comprises a cholesterol moiety.

49. The RNA molecule of claim 33, wherein the RNA molecule comprises a 5' end, a 3' end and has complementarity to a target, wherein:

(1) the RNA molecule comprises alternating 2'-methoxy-ribonucleotides and 2'-fluoro-ribonucleotides;

(2) the nucleotides at positions 2 and 14 from the 5' end are not 2'-methoxy-ribonucleotides;

(3) the nucleotides are connected via phosphodiester or phosphorothioate linkages; and

(4) the nucleotides at positions 1-6 from the 3' end, or positions 1-7 from the 3' end, are connected to adjacent nucleotides via phosphorothioate linkages.

50. The dsRNA of claim 34, said dsRNA having a 5' end, a 3' end and complementarity to a target, and comprising a first oligonucleotide and a second oligonucleotide, wherein:

(1) the first oligonucleotide comprises a sequence set forth as SEQ ID NO:2 or SEQ ID NO:3;

(2) a portion of the first oligonucleotide is complementary to a portion of the second oligonucleotide;

(3) the second oligonucleotide comprises alternating 2'-methoxy-ribonucleotides and 2'-fluoro-ribonucleotides;

(4) the nucleotides at positions 2 and 14 from the 3' end of the second oligonucleotide are 2'-methoxy-ribonucleotides; and

(5) the nucleotides of the second oligonucleotide are connected via phosphodiester or phosphorothioate linkages.

51. The nucleic acid of claim 50, wherein the second oligonucleotide is linked to a hydrophobic molecule at the 3' end of the second oligonucleotide.

52. The nucleic acid of claim 50, wherein the linkage between the second oligonucleotide and the hydrophobic molecule comprises an alkyl chain, polyethylene glycol or triethylene glycol.

53. The nucleic acid of claim 51, wherein the nucleotides at positions 1 and 2 from the 3' end of second oligonucleotide are connected to adjacent nucleotides via phosphorothioate linkages.

54. The nucleic acid of claim 50, wherein the nucleotides at positions 1 and 2 from the 3' end of second oligonucleotide, and the nucleotides at positions 1 and 2 from the 5' end of second oligonucleotide, are connected to adjacent ribonucleotides via phosphorothioate linkages.

55. A method for inhibiting expression of *HTT* gene in a cell, the method comprising:

(a) introducing into the cell a dsRNA of claim 34; and

(b) maintaining the cell produced in step (a) for a time sufficient to obtain degradation of the mRNA transcript of the *HTT* gene, thereby inhibiting expression of the *HTT* gene in the cell.

56. A method of treating or managing Huntington's disease comprising administering to a patient in need of such treatment or management a therapeutically effective amount of said dsRNA of claim 34.

57. The method of claim 56, wherein said dsRNA is administered to the brain of the patient.

58. The method of claim 56, wherein said dsRNA is administered by intrastriatal infusion.

59. The method of claim 58, wherein administering the dsRNA of claim 30 to the brain causes a decrease in *HTT* gene mRNA in the striatum.

60. The method of claim 58, wherein administering the dsRNA of claim 30 to the brain causes a decrease in *HTT* gene mRNA in the cortex.

61. A vector for inhibiting the expression of *HTT* gene in a cell, said vector comprising a regulatory sequence operably linked to a nucleotide sequence that encodes an RNA molecule substantially complementary to 5' AUAUCAGUAAAGAGA 3' (SEQ ID NO:2) or 5' CUCAGGAUUUAAAAU 3' (SEQ ID NO:3), wherein said RNA molecule is between 15 and 35 bases in length, and wherein said RNA molecule, upon contact with a cell expressing said *HTT* gene, inhibits the expression of said *HTT* gene by at least 20%.

62. The vector of claim 61, wherein said RNA molecule is ssRNA or dsRNA.

63. The dsRNA of claim 62, comprising a sense strand and an antisense strand, wherein the antisense strand comprises the region of complementarity which is substantially complementary to 5' AUAUCAGUAAAGAGA 3' (SEQ ID NO:2) or 5' CUCAGGAUUUAAAAU 3' (SEQ ID NO:3).

64. The dsRNA of claim 62, wherein said dsRNA is between 30 and 35 base pairs in length.

65. A cell comprising the vector of claim 62.

66. An RNA molecule that is between 15 and 35 bases in length, comprising a region of complementarity which is substantially complementary to 5' CAGUAAAGAGAUUAA 3' (SEQ ID NO:1), 5' AUAUCAGUAAAGAGA 3' (SEQ ID NO:2) or 5' CUCAGGAUUUAAAAU 3' (SEQ ID NO:3), wherein the RNA molecule targets a 3' untranslated region (UTR) of *HTT* gene short mRNA.

67. The RNA molecule of claim 66, wherein said RNA molecule is ssRNA or dsRNA.

68. The dsRNA of claim 67 comprising a sense strand and an antisense strand, wherein the antisense strand comprises the region of complementarity which is substantially complementary to 5' CAGUAAAGAGAUUAA 3' (SEQ ID NO:1), 5' AUAUCAGUAAAGAGA 3' (SEQ ID NO:2) or 5' CUCAGGAUUUAAAAU 3' (SEQ ID NO:3).

69. A dsRNA molecule that is between 15 and 35 bases in length, comprising a region of complementarity which is substantially complementary to 5' CAGUAAAGAGAUUAA 3'

(SEQ ID NO:1), 5' AUAUCAGUAAAGAGA 3' (SEQ ID NO:2) or 5' CUCAGGAUUUAAAAU 3' (SEQ ID NO:3), wherein the RNA molecule targets an *HTT* mRNA and comprises at least one modified nucleotide.

70. The dsRNA of claim 69, wherein said modified nucleotide is a terminal nucleotide linked to a phosphatidylcholine derivative.

71. A di-branched RNA compound comprising two RNA molecules that are between 15 and 35 bases in length, comprising a region of complementarity which is substantially complementary to 5' CAGUAAAGAGAUUAA 3' (SEQ ID NO:1), 5' AUAUCAGUAAAGAGA 3' (SEQ ID NO:2) or 5' CUCAGGAUUUAAAAU 3' (SEQ ID NO:3), wherein the two RNA molecules are connected to one another by one or more moieties independently selected from a linker, a spacer and a branching point.

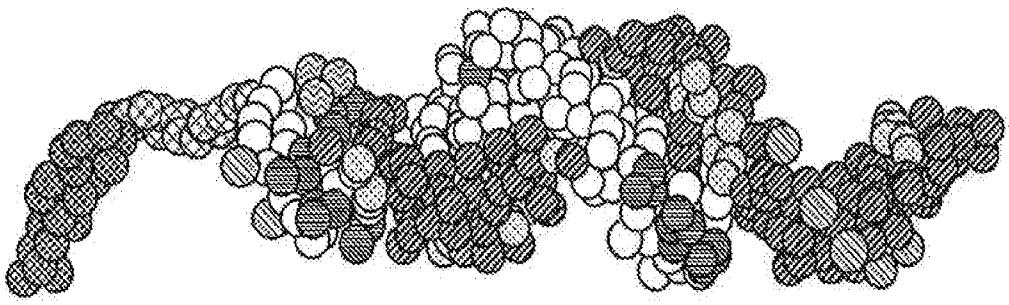
72. The RNA molecule of claim 1, wherein said RNA molecule is an antisense molecule or a GAPMER molecule.

73. The RNA molecule of claim 72, wherein said antisense molecule is an antisense oligonucleotide.

74. The RNA molecule of claim 73, wherein said antisense molecule enhances degradation of the region of complementarity.

75. The RNA molecule of claim 74, wherein said degradation is nuclease degradation.

76. The RNA molecule of claim 75, wherein said nuclease degradation is mediated by RNase H.



2'-O-Methyl RNA
2'-Fluoro RNA
Sense strand
Antisense strand
Cholesterol
TEG linker
Phosphorothioate
5'-Phosphate

Fig. 1A

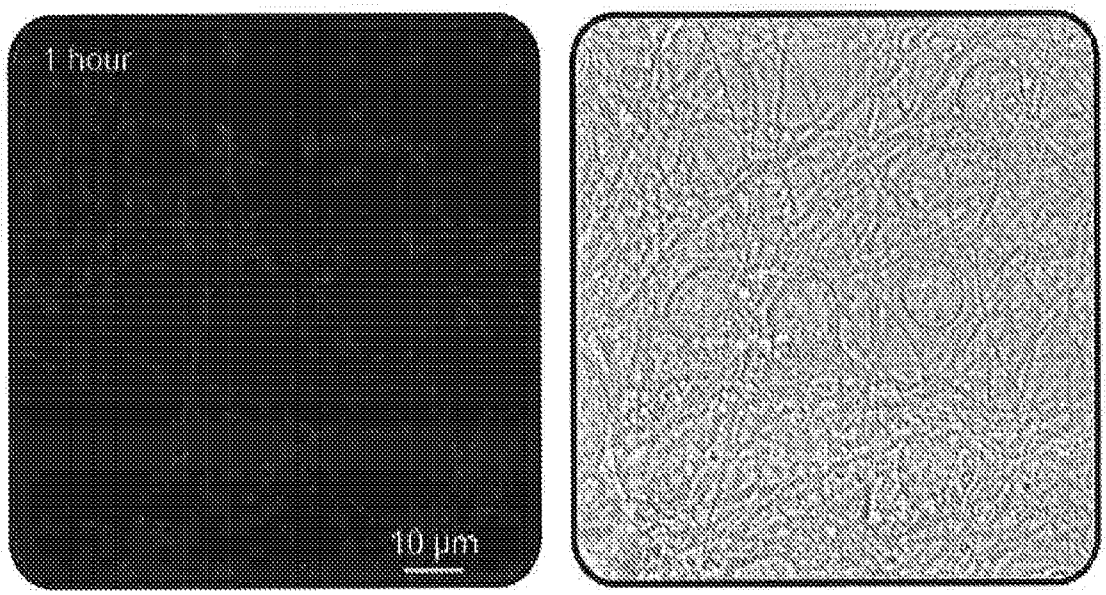


Fig. 1B

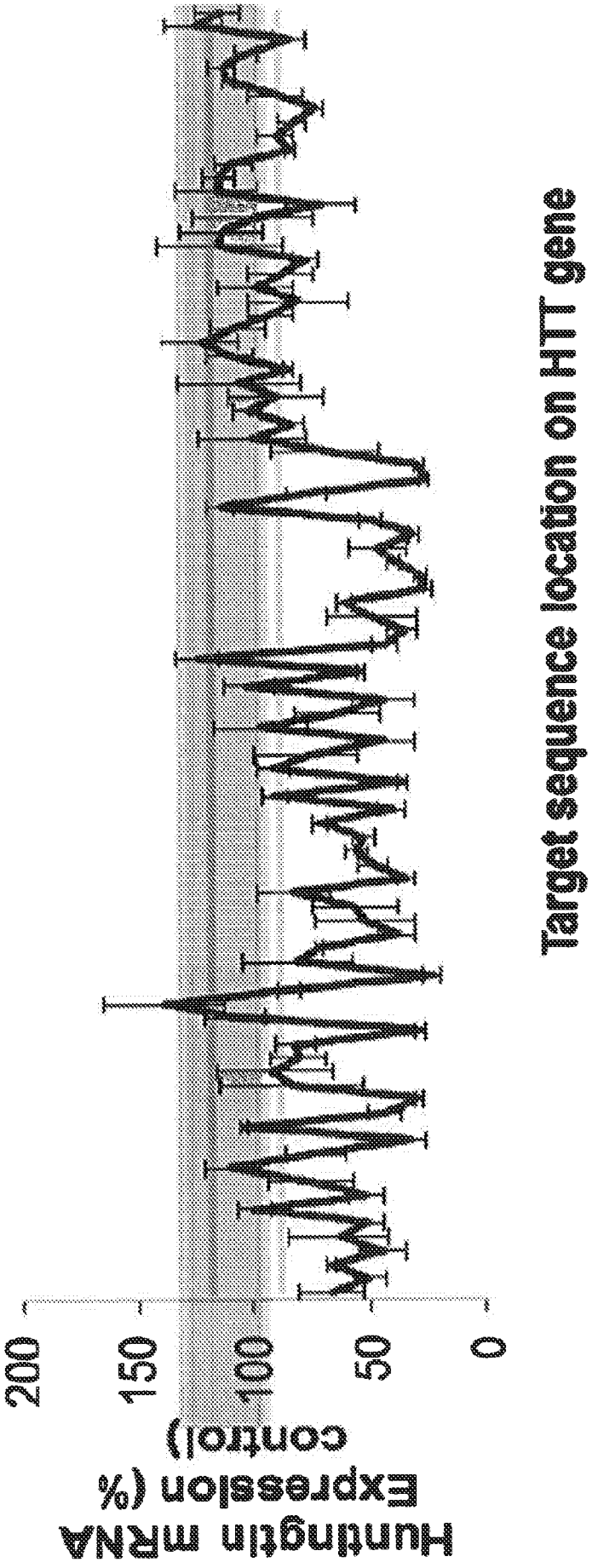

Huntingtin mRNA Expression (% control)
200
150
100
50
0
Target sequence location on HTT gene

Fig. 2A

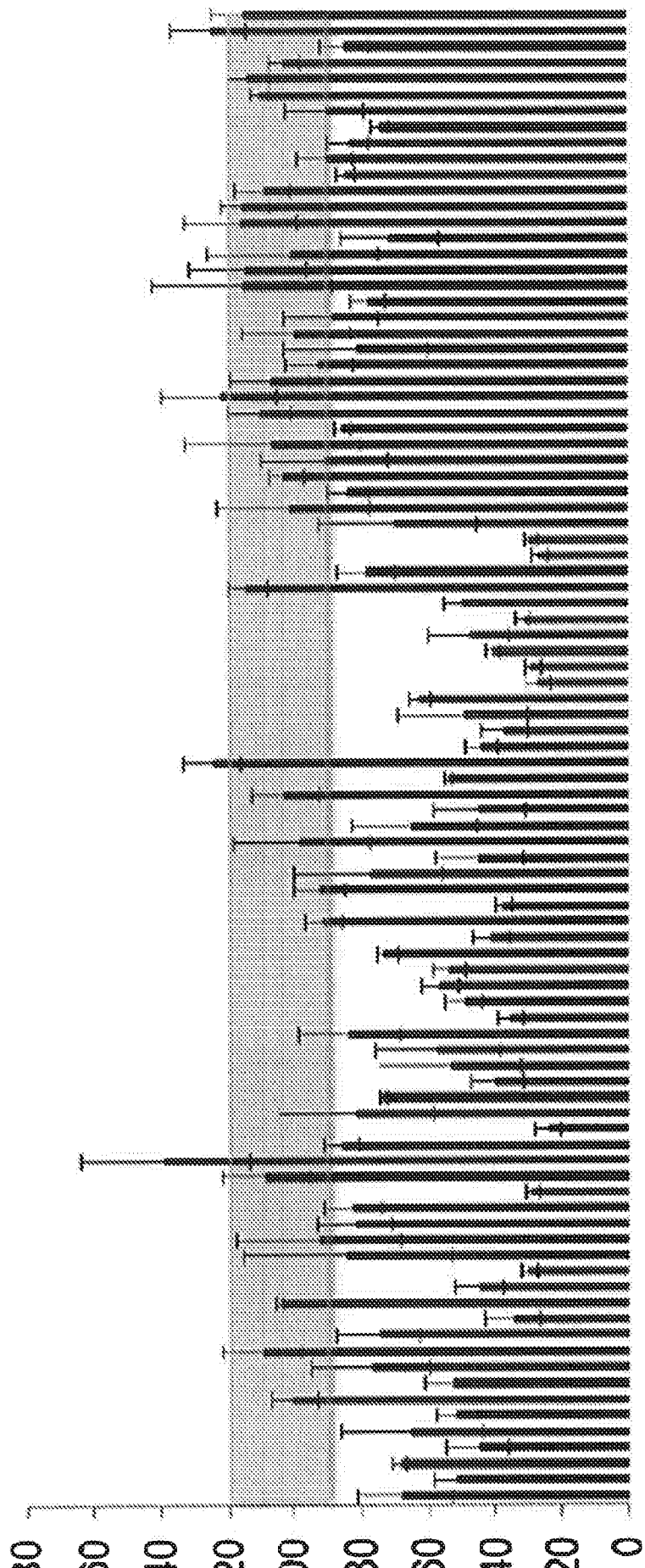

Huntingtin mRNA
Expression (% control)
180
160
140
120
100
80
60
40
20
0
Target sequence location on HTT gene

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Fig. 2B

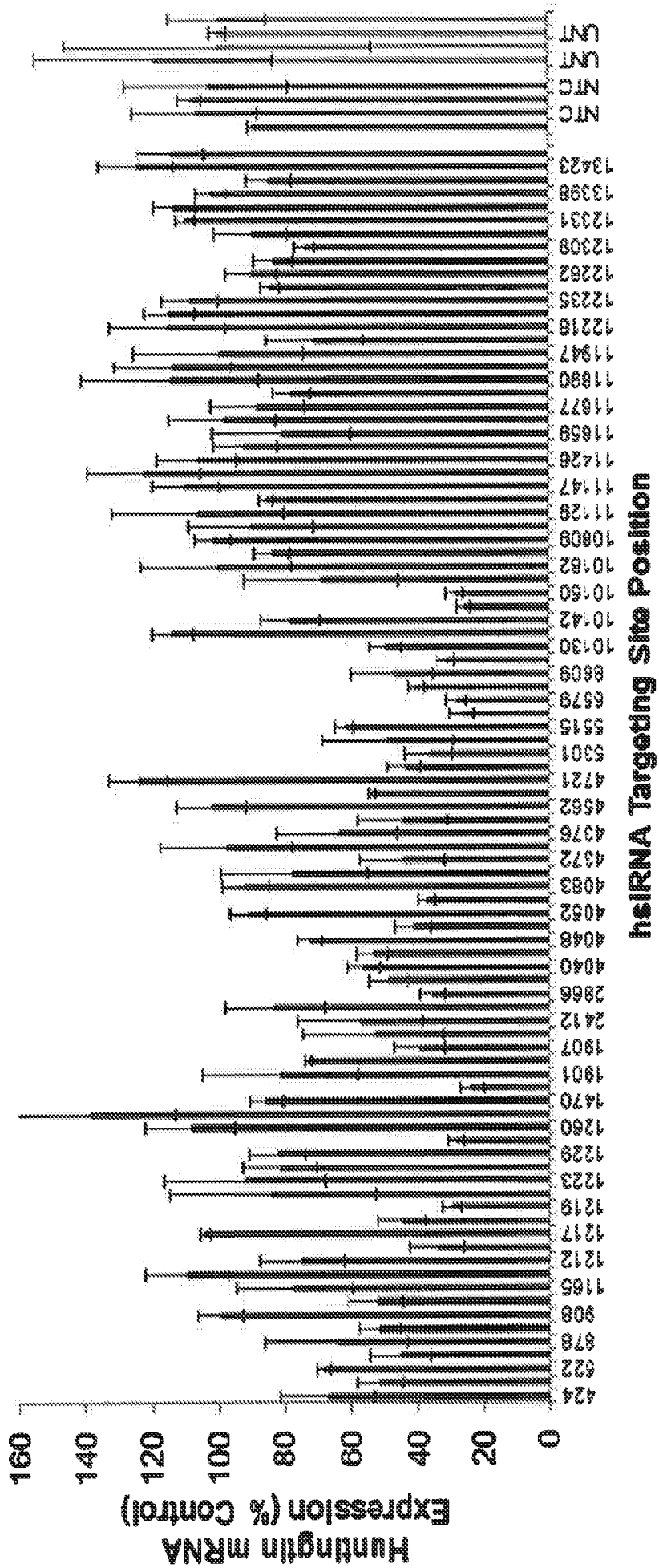
Huntingtin mRNA Expression (% control)
0
20
40
60
80
100
120
140
160
hsiRNA Targeting Site Position
424
622
678
908
1165
1212
1217
1219
1223
1229
1260
1470
1901
1907
2412
2866
4040
4048
4052
4083
4372
4376
4562
4721
5301
5515
6579
8609
10130
10142
10150
10162
10809
11129
11147
11428
11659
11677
11890
11947
12218
12235
12262
12309
12331
13398
13423
NTC
NTC
UNT
UNT

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Fig. 2C

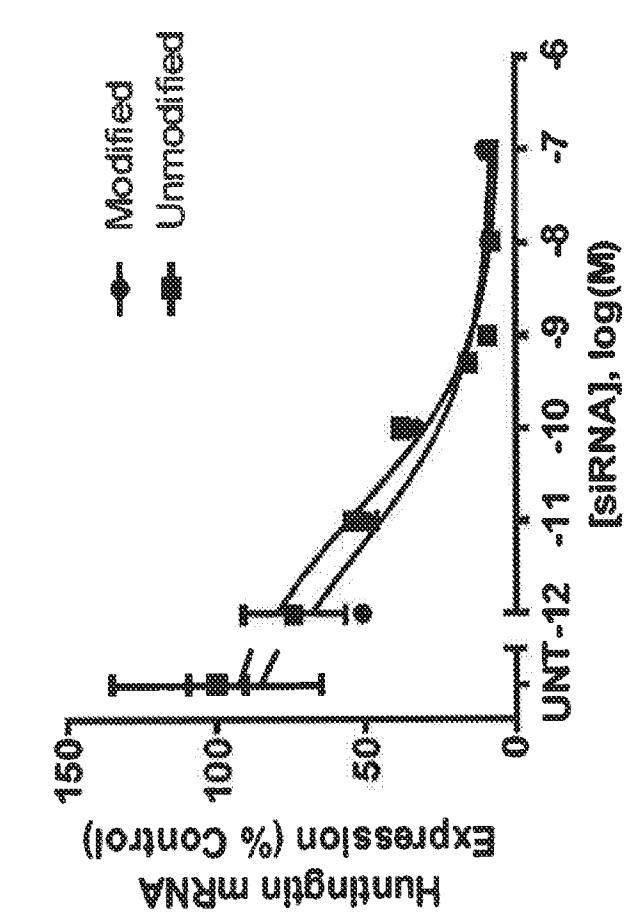

Fig. 3A

Fig. 3B

	Passive Uptake	Lipid-Mediated Uptake
IC50 Modified	82.2 nM	4.4 pM
IC50 Unmodified	N/A	13.3 pM

Fig. 3C

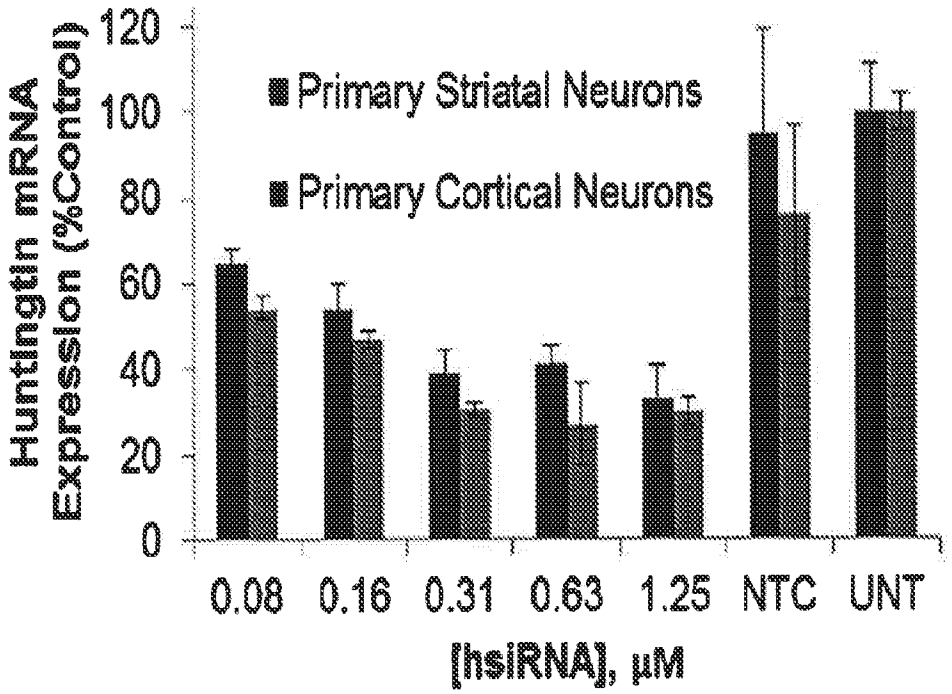
Huntingtin mRNA Expression (%Control)
120
100
80
60
40
20
0
Primary Striatal Neurons
Primary Cortical Neurons
0.08
0.16
0.31
0.63
1.25
NTC
UNT
[hsiRNA], µM

Fig. 4A

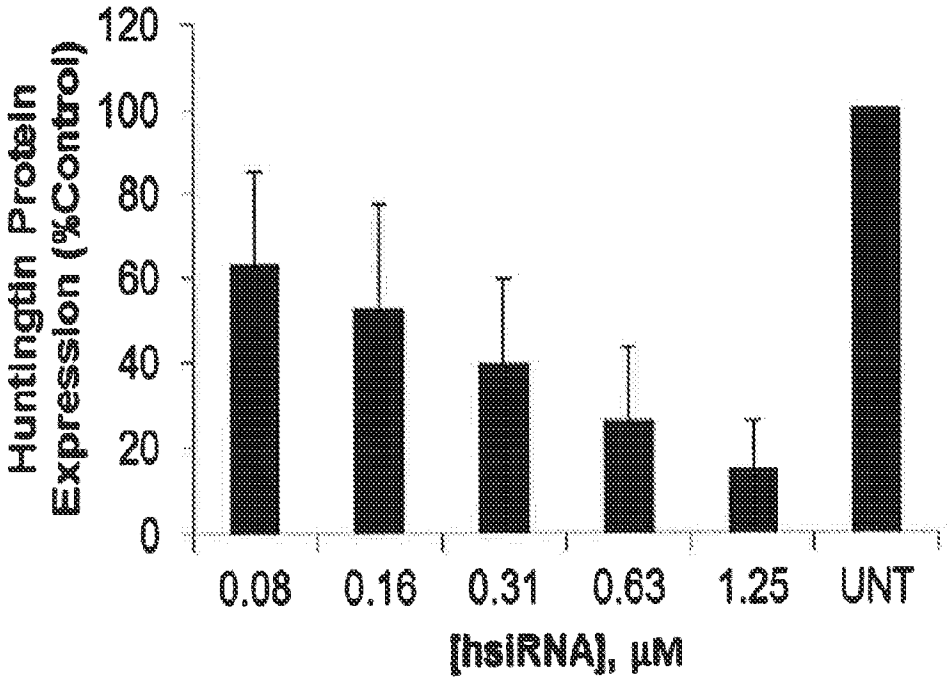
Huntingtin Protein Expression (%Control)
120
100
80
60
40
20
0
0.08
0.16
0.31
0.63
1.25
UNT
[hsiRNA], µM

Fig. 4B

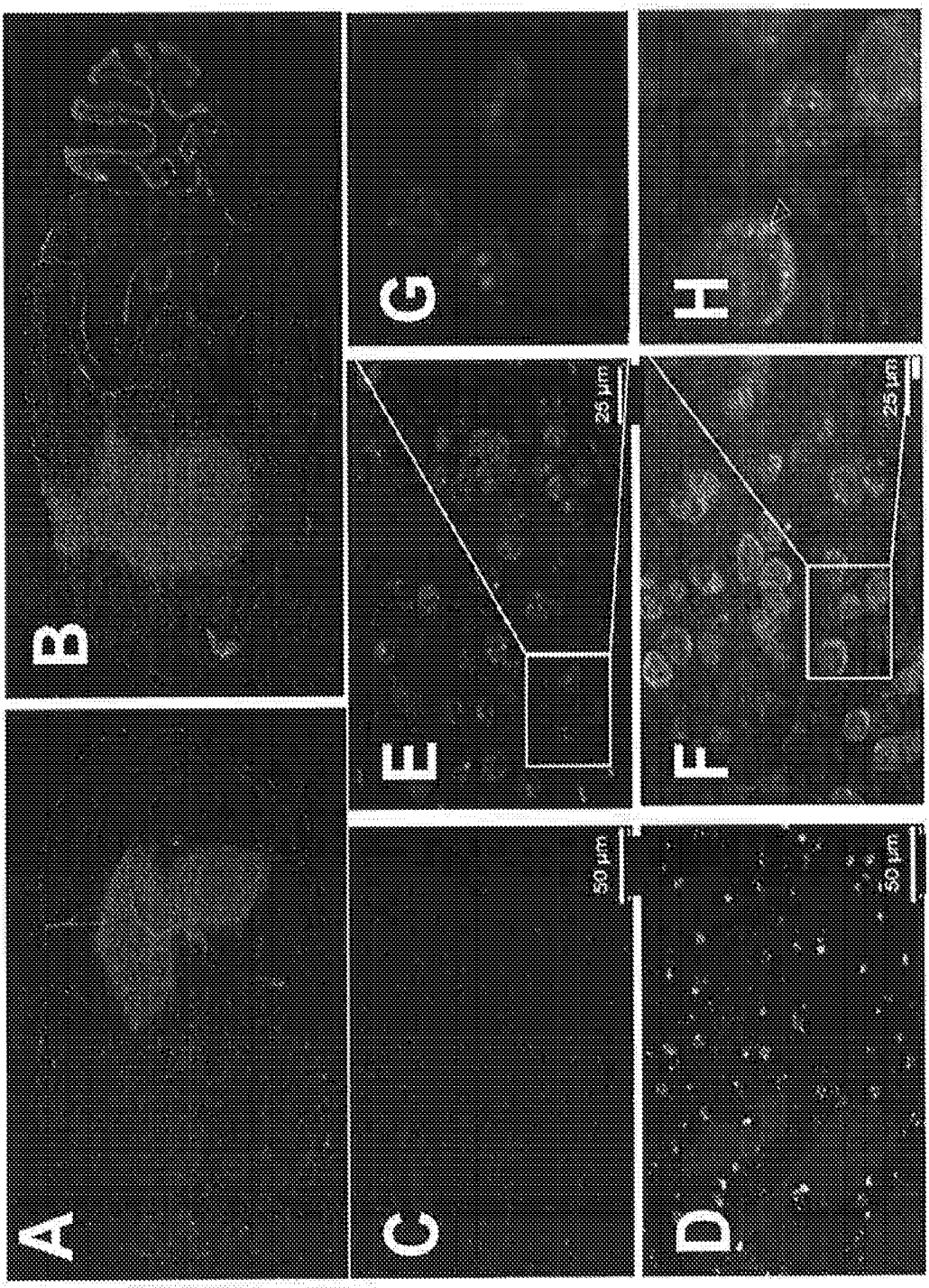

Fig. 5A-5H

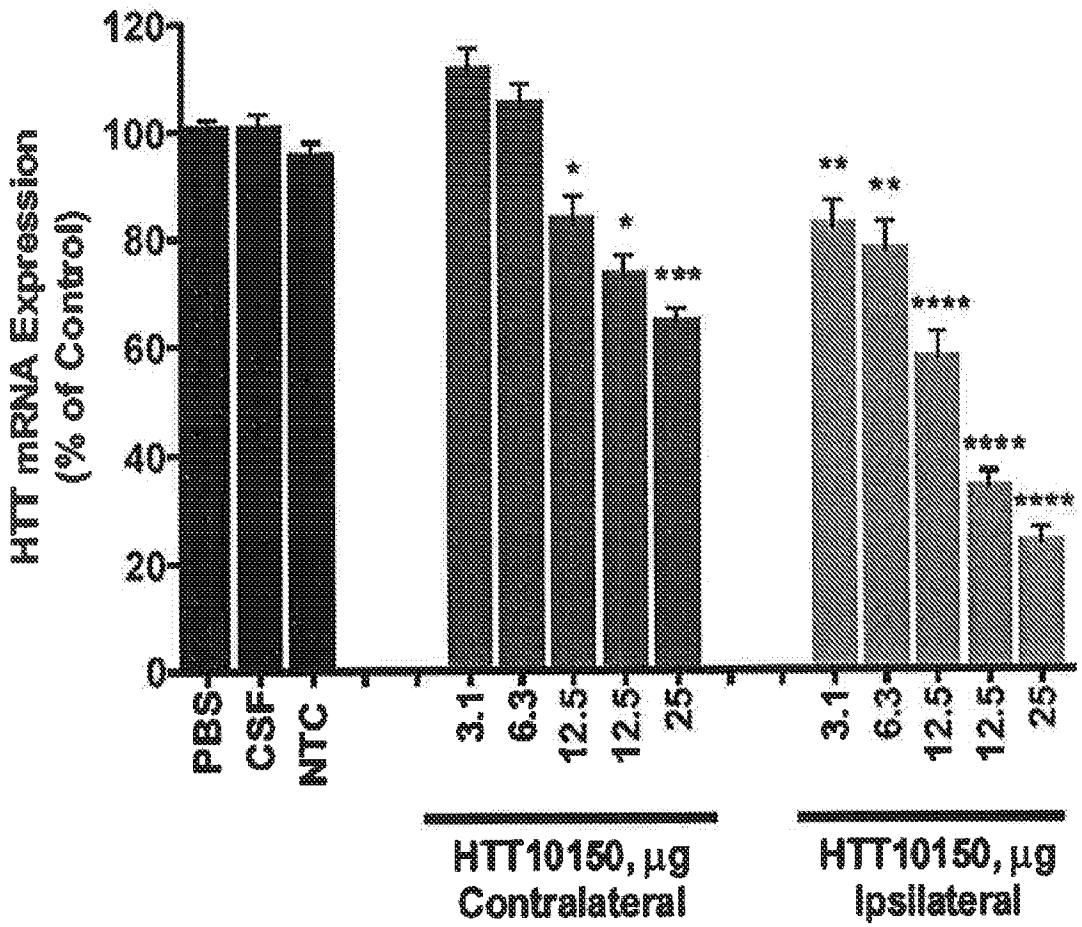
HTT mRNA Expression
(% of Control)
120
100
80
60
40
20
0
PBS
CSF
NTC
3.1
6.3
12.5
12.5
25
3.1
6.3
12.5
12.5
25
HTT10150, µg
Contralateral
HTT10150, µg
Ipsilateral

Fig. 6

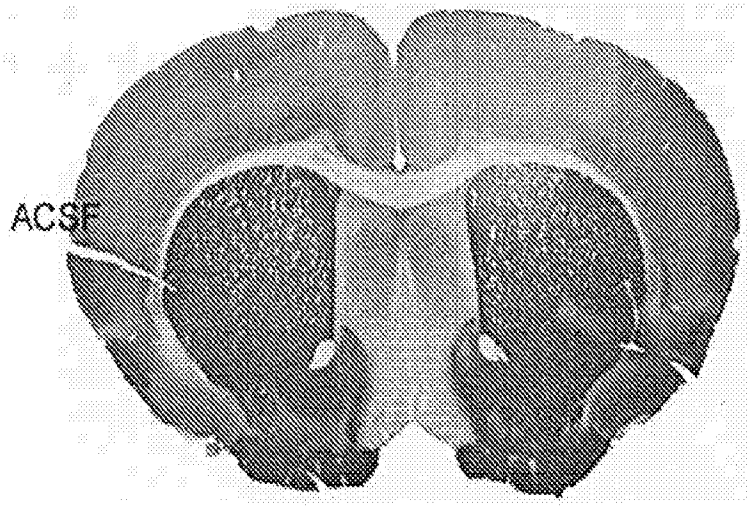

Fig. 7A

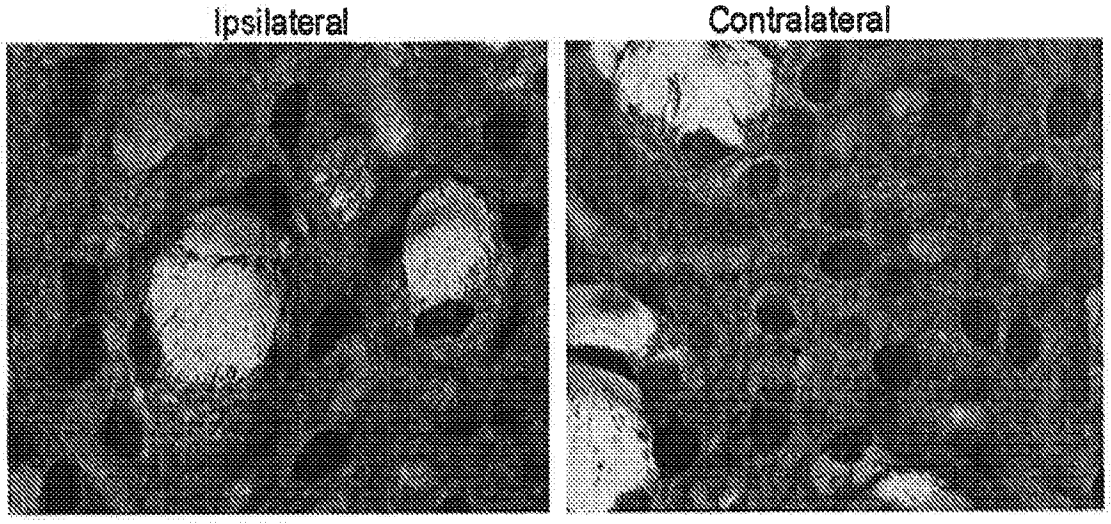

Fig. 7B

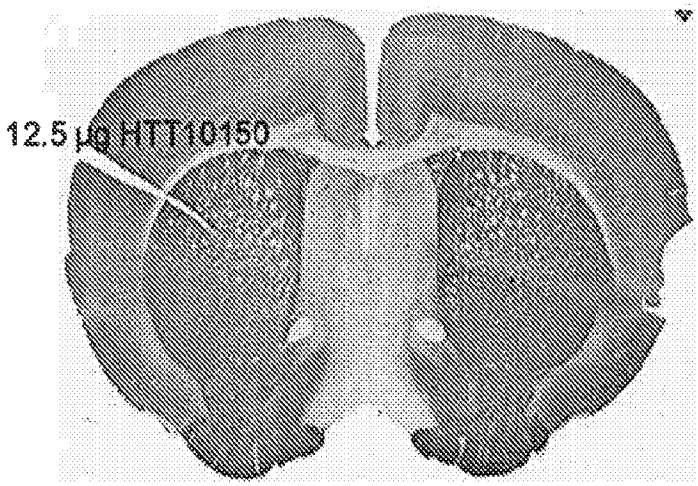

Fig. 7C

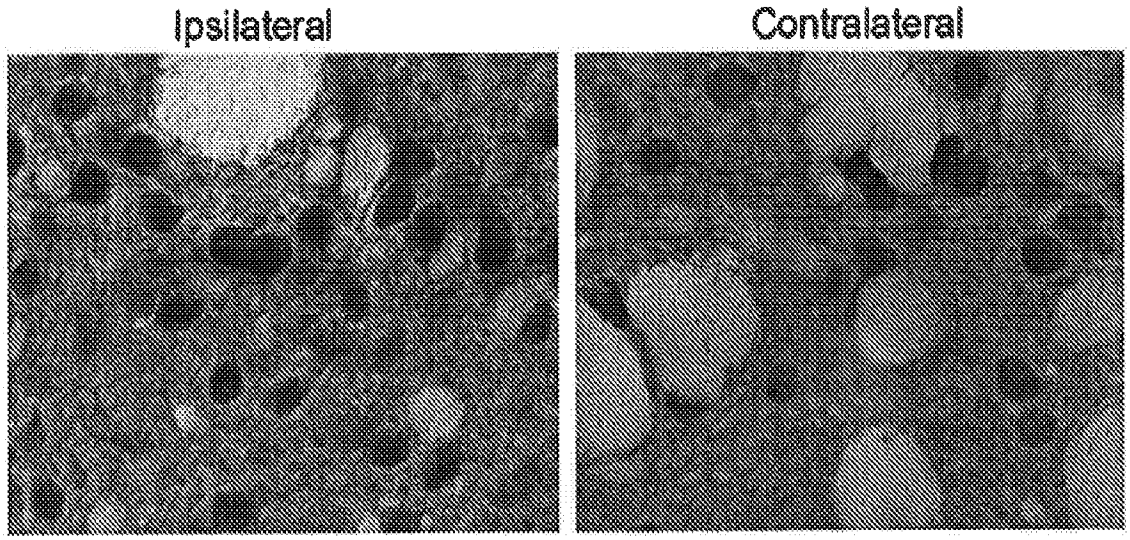
Ipsilateral
Contralateral

Fig. 7D

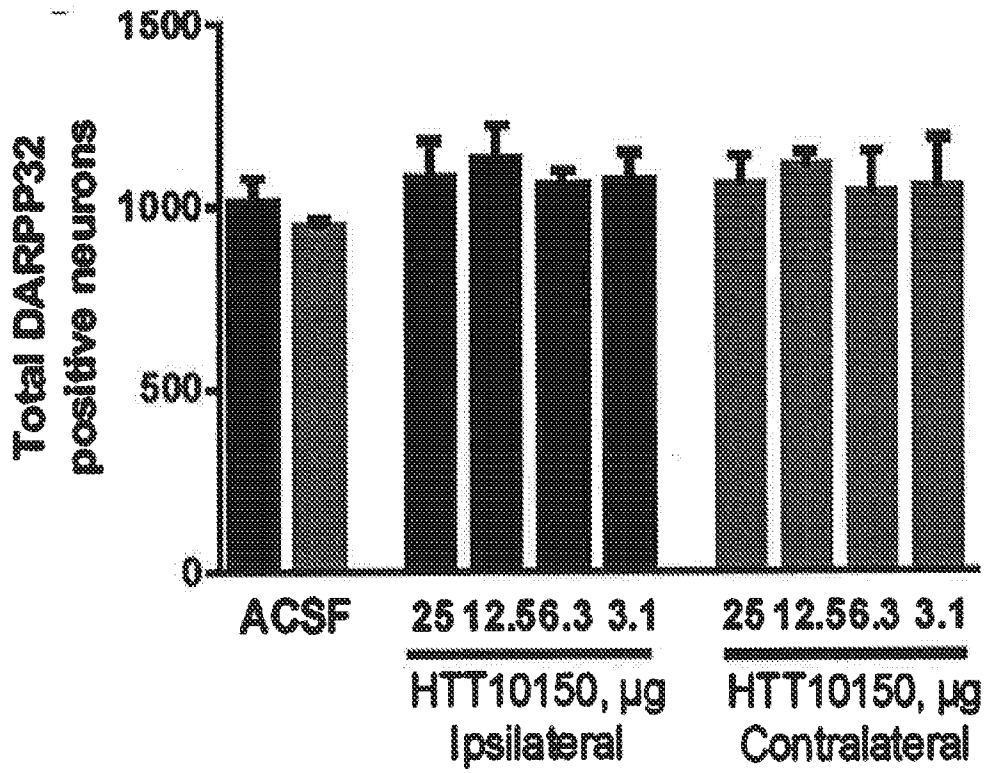
Total DARPP32 positive neurons
1500
1000
500
0
ACSF
25 12.5 6.3 3.1
HTT10150, µg
Ipsilateral
25 12.5 6.3 3.1
HTT10150, µg
Contralateral

Fig. 7E

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Active hsiRNAs				Strand Modifications	
Gene	Accession Number	Position	Target Sequence	Sense Strand	
HTT	NM_002111.6	1214	GGUUUAUGAACUGAC	mG.mG.mU.mU.mU.A.mU.G.A.A.mC.mU.G#mA#mAtegChol	
HTT	NM_002111.6	1218	UAUGAACUGACGUUA	mU.mA.mU.G.A.A.mC.mU.G.A.mC.G.mU#mU#mAtegChol	
HTT	NM_002111.6	1219	AUGAACUGACGUUAC	mA.mU.G.A.A.mC.mU.G.A.mC.G.mU.mU#mA#mAtegChol	
HTT	NM_002111.6	1257	AAUGUUGUGACCGGA	mA.mA.mU.G.mU.mU.G.mU.G.A.mC.mC.G#mG#mAtegChol	
HTT	NM_002111.6	1894	UAGACGGUACCGACA	mU.mA.G.A.mC.G.G.mU.A.mC.mC.G.A#mC#mAtegChol	
HTT	NM_002111.6	1907	CAACCAGUAUUUGGG	mC.mA.A.mC.mC.A.G.mU.A.mU.mU.mU.G#mG#mAtegChol	
HTT	NM_002111.6	2866	UGCUCAAUAAUGUUG	mU.mG.mC.mU.mC.A.A.mU.A.A.mU.G.mU#mU#mAtegChol	
HTT	NM_002111.6	4041	UCCUGCUUUAGUCGA	mU.mC.mC.mU.G.mC.mU.mU.mU.mU.A.G.mU.mC#mG#mAtegChol	
HTT	NM_002111.6	4049	UAGUCGAGAACCAAU	mU.mA.G.mU.mC.G.A.mG.A.A.mC.mC.A#mA#mAtegChol	
HTT	NM_002111.6	5301	AGUACUUCAACGCUA	mA.mG.mU.A.mC.mU.mU.mC.A.A.mC.G.mC#mU#mAtegChol	
HTT	NM_002111.6	6016	UUCAGUCUCGUUGUG	mU.mU.mC.A.G.mU.mC.mU.mC.G.mU.mU.G#mU#mAtegChol	
HTT	NM_002111.6	6579	CUAGCUCCAUGCUUA	mC.mU.A.G.mC.mU.mC.mC.A.mU.G.mC.mU#mU#mAtegChol	
HTT	NM_002111.6	8603	CUGCGUGAACAUUCA	mC.mU.G.mC.G.mU.G.A.A.mC.A.mU.mU#mC#mAtegChol	
HTT	NM_002111.6	10125	CUCAGGAUUUAAAAU(SEQ ID NO:3)	mC.mU.mC.A.G.G.A.mU.mU.mU.A.A.A#mA#mAtegChol	
HTT	NM_002111.6	10146	AUAUCAGUAAAGAGA (SEQ ID NO:2)	mA.mU.A.mU.mC.A.G.mU.A.A.A.G.A#mG#mAtegChol	
HTT	NM_002111.6	10150	CAGUAAAGAGAUUAA (SEQ ID NO:1)	mC.mA.G.mU.A.A.A.mG.A.G.A.mU.mU#mA#mAtegChol	
HTT	NM_002111.6	424	CAGCUACCAAGAAAG	mC.mA.G.mC.mU.A.mC.mC.A.A.G.A.A#mA#mAtegChol	
HTT	NM_002111.6	456	CUGACAAUAUGUGAA	mC.mU.G.A.mC.A.A.mU.A.mU.G.mU.G#mA#mAtegChol	
HTT	NM_002111.6	522	GGCAUCGCUAUGGAA	mG.mG.mC.A.mU.mC.G.mC.mU.A.mU.G.G#mA#mAtegChol	
HTT	NM_002111.6	527	CGCUAUGGAACUUUU	mC.mG.mC.mU.A.mU.G.G.mA.A.mC.mU.mU#mU#mAtegChol	
HTT	NM_002111.6	878	UGACAAUGAAAUUAA	mU.mG.A.mC.A.A.mU.G.A.mA.A.mU.mU#mA#mAtegChol	
HTT	NM_002111.6	879	GACAAUGAAAUUAAG	mG.mA.mC.A.A.mU.G.A.mA.A.mU.A#mA#mAtegChol	
HTT	NM_002111.6	908	CUUCAUAGCGAACCU	mC.mU.mU.mC.A.mU.A.G.mC.G.A.A.mC#mC#mAtegChol	
HTT	NM_002111.6	1024	AUGUGCUCUUAGGCU	mA.mU.G.mU.G.mC.mU.mC.mU.mU.A.G.G#mC#mAtegChol	
HTT	NM_002111.6	1165	UGACAAGGAAAGAAA	mU.mG.A.mC.A.A.G.mG.A.A.mA.G.A#mA#mAtegChol	

Continued on sheet 12/136

Fig. 8

Continued on sheet 15/136

Active hsiRNAs				Strand Modifications
Gene	Accession Number	Position	Target Sequence	Sense Strand
HTT	NM_002111.6	1207	UUGUCCAGGUUUAUG	mU.mU.G.mU.mC.mC.A.G.G.mU.mU.mU.A#mU#mAtegChol
HTT	NM_002111.6	1212	CAGGUUUAUGAACUG	mC.mA.G.G.mU.mU.mU.A.mU.G.A.A.mC#mU#mAtegChol
HTT	NM_002111.6	1217	UUAUGAACUGACGUU	mU.mU.A.mU.G.A.A.mC.mU.G.A.mC.G#mU#mAtegChol
HTT	NM_002111.6	1220	UGAACUGACGUUACA	mU.mG.A.A.mC.mU.G.A.mC.G.mU.mU.A#mC#mAtegChol
HTT	NM_002111.6	1223	ACUGACGUUACAUCA	mA.mC.mU.G.A.mC.G.mU.mU.A.mC.A.mU#mC#mAtegChol
HTT	NM_002111.6	1227	ACGUUACAUCAUACA	mA.mC.G.mU.mU.A.mC.A.mU.mC.A.mU.A#mC#mAtegChol
HTT	NM_002111.6	1229	GUUACAUCAUACACA	mG.mU.mU.A.mC.A.mU.mC.A.mU.A.mC.A#mC#mAtegChol
HTT	NM_002111.6	1260	GUUGUGACCGGAGCC	mG.mU.mU.G.mU.G.A.mC.mC.G.mG.A.G#mC#mAtegChol
HTT	NM_002111.6	1403	UAUUGUGGAACUUAU	mU.mA.mU.mU.G.mU.G.G.A.A.mC.mU.mU#mA#mAtegChol
HTT	NM_002111.6	1470	AAAGUGCUCUUAGGA	mA.mA.A.G.mU.G.mC.mU.mC.mU.mU.A.G#mG#mAtegChol
HTT	NM_002111.6	1901	UACCGACAACCAGUA	mU.mA.mC.mC.G.A.mC.A.A.mC.mC.A.G#mU#mAtegChol
HTT	NM_002111.6	1903	CCGACAACCAGUAUU	mC.mC.G.A.mC.A.A.mC.mC.A.G.mU.A#mU#mAtegChol
HTT	NM_002111.6	2411	CUACAUCGAUCAUGG	mC.mU.A.mC.A.mU.mC.G.A.mU.mC.A.mU#mG#mAtegChol
HTT	NM_002111.6	2412	UACAUCGAUCAUGGA	mU.mA.mC.A.mU.mC.G.A.mU.mC.A.mU.G#mG#mAtegChol
HTT	NM_002111.6	2865	GUGCUCAAUAAUGUU	mG.mU.G.mC.mU.mC.A.A.mU.A.A.mU.G#mU#mAtegChol
HTT	NM_002111.6	3801	GUUACAACAAGUAAA	mG.mU.mU.A.mC.A.A.mC.A.A.G.mU.A#mA#mAtegChol
HTT	NM_002111.6	4040	AUCCUGCUUUAGUCG	mA.mU.mC.mC.mU.G.mC.mU.mU.mU.A.G.mU#mC#mAtegChol
HTT	NM_002111.6	4048	UUAGUCGAGAACCAA	mU.mU.A.G.mU.mC.G.A.mG.A.A.mC.mC#mA#mAtegChol
HTT	NM_002111.6	4052	UCGAGAACCAAUGAU	mU.mC.G.A.mG.A.A.mC.mC.A.A.mU.G#mA#mAtegChol
HTT	NM_002111.6	4055	AGAACCAAUGAUGGC	mA.mG.A.A.mC.mC.A.A.mU.G.A.mU.G#mG#mAtegChol
HTT	NM_002111.6	4083	CAACAAUUGUUGAAG	mC.mA.A.mC.A.A.mU.mU.G.mU.mU.G.A#mA#mAtegChol
HTT	NM_002111.6	4275	AACAUGGUGCAGGCG	mA.mA.mC.A.mU.G.G.mU.G.mC.A.G.G#mC#mAtegChol
HTT	NM_002111.6	4372	CAAAGAACCGUGCAG	mC.mA.A.A.G.A.A.mC.mC.G.mU.G.mC#mA#mAtegChol
HTT	NM_002111.6	4374	AAGAACCGUGCAGAU	mA.mA.G.A.A.mC.mC.G.mU.G.mC.A.G#mA#mAtegChol
HTT	NM_002111.6	4376	GAACCGUGCAGAUAA	mG.mA.A.mC.mC.G.mU.G.mC.A.G.A.mU#mA#mAtegChol

Continued on sheet 13/136

Fig. 8 (Cont.)

Continued on sheet 16/136

Active hsiRNAs				Strand Modifications
Gene	Accession Number	Position	Target Sequence	Sense Strand
HTT	NM_002111.6	4425	CCUCUUGUUAUAAAA	mC.mC.mU.mC.mU.mU.G.mU.mU.A.mU.A.A#mA#mAtegChol
HTT	NM_002111.6	4562	UGGCUUUUGUAUUGAA	mU.mG.G.mC.mU.mU.mU.mU.G.mU.A.mU.mU.G#mA#mAtegChol
HTT	NM_002111.6	4692	GGAAUUCCUAAAAUC	mG.mG.A.A.mU.mU.mU.mC.mC.mU.A.A.A.A#mU#mAtegChol
HTT	NM_002111.6	4721	UGGCAUCAUGGCCAG	mU.mG.G.mC.A.mU.mC.A.mU.G.G.mC.mC#mA#mAtegChol
HTT	NM_002111.6	5200	CCCAGUCAACUGAAG	mC.mC.mC.A.G.mU.mC.A.A.mC.mU.G.A#mA#mAtegChol
HTT	NM_002111.6	5443	AGCAGCAACAUACUU	mA.mG.mC.A.G.mC.A.A.mC.A.mU.A.mC#mU#mAtegChol
HTT	NM_002111.6	5515	GAAUGUUCCGGAGAA	mG.mA.A.mU.G.mU.mU.mC.mC.G.G.A.G#mA#mAtegChol
HTT	NM_002111.6	8609	GAACAUUCACAGCCA	mG.mA.A.mC.A.mU.mU.mC.A.mC.A.G.mC#mC#mAtegChol
HTT	NM_002111.6	10130	GAUUUAAAAUUUAAU	mG.mA.mU.mU.mU.A.A.A.A.A.mU.mU.A#mA#mAtegChol
HTT	NM_002111.6	10134	UAAAAUUUAAUUAUA	mU.mA.A.A.A.mU.mU.mU.A.A.mU.mU.A#mU#mAtegChol
HTT	NM_002111.6	10142	AAUUAUAUCAGUAAA	mA.mA.mU.mU.A.mU.A.mU.mC.A.G.mU.A#mA#mAtegChol
HTT	NM_002111.6	10169	AACGUAACUCUUUCU	mA.mA.mC.G.mU.A.A.mC.mU.mC.mU.mU.mU#mC#mAtegChol
HTT	NM_002111.6	10182	CUAUGCCCGUGUAAA	mC.mU.A.mU.G.mC.mC.mC.G.mU.G.mU.A#mA#mAtegChol
HTT	NM_002111.6	10186	GCCCGUGUAAAGUAU	mG.mC.mC.mC.G.mU.G.mU.A.A.A.G.mU#mA#mAtegChol
HTT	NM_002111.6	10809	AGUCAGGAGAGUGCA	mA.mG.mU.mC.A.G.G.A.G.A.G.mU.G#mC#mAtegChol
HTT	NM_002111.6	11116	UGGGUAUUGAAUGUG	mU.mG.G.G.mU.A.mU.mU.G.A.A.mU.G#mU#mAtegChol
HTT	NM_002111.6	11129	UGGUAAGUGGAGGAA	mU.mG.G.mU.A.A.G.mU.G.G.A.G.G#mA#mAtegChol
HTT	NM_002111.6	11134	AGUGGAGGAAAUGUU	mA.mG.mU.G.G.A.G.G.A.A.A.mU.G#mU#mAtegChol
HTT	NM_002111.6	11147	UUGGAACUCUGUGCA	mU.mU.G.G.A.A.mC.mU.mC.mU.G.mU.G#mC#mAtegChol
HTT	NM_002111.6	11412	UGAGGAGGCCCUUAA	mU.mG.A.G.G.mA.G.G.mC.mC.mC.mU.mU#mA#mAtegChol
HTT	NM_002111.6	11426	AGGGAAGCUACUGAA	mA.mG.G.G.A.A.G.mC.mU.A.mC.mU.G#mA#mAtegChol
HTT	NM_002111.6	11443	AUAACACGUAAGAAA	mA.mU.A.A.mC.A.mC.G.mU.A.A.G.A#mA#mAtegChol
HTT	NM_002111.6	11659	UACAUUUGUAAGAAA	mU.mA.mC.A.mU.mU.mU.G.mU.A.A.G.A#mA#mAtegChol
HTT	NM_002111.6	11666	GUAAGAAAUAACACU	mG.mU.A.A.G.mA.A.A.mU.A.A.mC.A#mC#mAtegChol
HTT	NM_002111.6	11677	CACUGUGAAUGUAAA	mC.mA.mC.mU.G.mU.G.A.A.mU.G.mU.A#mA#mAtegChol

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Fig. 8 (Cont.)

Continued on sheet 17/136

Active hsiRNAs				Strand Modifications	
Gene	Accession Number	Position	Target Sequence	Sense Strand	
HTT	NM_002111.6	11863	GAGCUCAUUAGUAAA	mG.mA.G.mC.mU.mC.A.mU.mU.A.G.mU.A#mA#mAtegChol	
HTT	NM_002111.6	11890	CACGCAUAUACAUAA	mC.mA.mC.G.mC.A.mU.A.mU.A.mC.A.mU#mA#mAtegChol	
HTT	NM_002111.6	11927	GACACAUCUAUAAUU	mG.mA.mC.A.mC.A.mU.mC.mU.A.mU.A.A#mU#mAtegChol	
HTT	NM_002111.6	11947	CACACACCUCUCAAG	mC.mA.mC.A.mC.A.mC.mC.mU.mC.mU.mC.A.#mA#mAtegChol	
HTT	NM_002111.6	12163	UAUCAUGUUCCUAAA	mU.mA.mU.mC.A.mU.G.mU.mU.mC.mC.mC.mU.A#mA#mAtegChol	
HTT	NM_002111.6	12218	GCAAAUGUGAUUAAU	mG.mC.A.A.A.mU.G.mU.G.A.mU.mU.A#mA#mAtegChol	
HTT	NM_002111.6	12223	UGUGAUUAAUUUGGU	mU.mG.mU.G.A.mU.mU.A.A.mU.mU.mU.G#mG#mAtegChol	
HTT	NM_002111.6	12235	GGUUGUCAAGUUUUG	mG.mG.mU.mU.G.mU.mC.A.A.G.mU.mU.mU.mU#mU#mAtegChol	
HTT	NM_002111.6	12279	UUUCCUGCUGGUAAU	mU.mU.mU.mC.mC.mU.G.mC.mU.G.G.mU.A#mA#mAtegChol	
HTT	NM_002111.6	12282	CCUGCUGGUAAUAUC	mC.mC.mU.G.mC.mU.G.G.mU.A.A.mU.A#mU#mAtegChol	
HTT	NM_002111.6	12297	GGGAAAGAUUUUAAU	mG.mG.G.A.A.A.G.A.mU.mU.mU.mU.A#mA#mAtegChol	
HTT	NM_002111.6	12309	AAUGAAACCAGGGUA	mA.mA.mU.G.A.A.A.mC.mC.A.G.G.G.G#mU#mAtegChol	
HTT	NM_002111.6	12313	AAACCAGGGUAGAAU	mA.mA.A.mC.mC.A.G.G.G.G.mU.A.G.A#mA#mAtegChol	
HTT	NM_002111.6	12331	UUGGCAAUGCACUGA	mU.mU.G.G.mC.A.A.mU.G.mC.A.mC.mU#mG#mAtegChol	
HTT	NM_002111.6	13136	CAGUUGUUUCUAAGA	mC.mA.G.mU.mU.G.mU.mU.mU.mC.mU.A.A#mG#mAtegChol	
HTT	NM_002111.6	13398	GACGAGAGAUGUAUA	mG.mA.mC.G.A.G.A.G.A.mU.G.mU.A#mU#mAtegChol	
HTT	NM_002111.6	13403	GAGAUGUAUAUUUAA	mG.mA.G.A.mU.G.mU.A.mU.A.mU.mU.mU#mA#mAtegChol	
HTT	NM_002111.6	13423	UAACUGCUGCAAACA	mU.mA.A.mC.mU.G.mC.mU.G.mC.A.A.A#mC#mAtegChol	
HTT	NM_002111.6	13428	GCUGCAAACAUUGUA	mG.mC.mU.G.mC.A.A.A.mC.A.mU.mU.G#mU#mAtegChol	
NTC	N/A	N/A	ACAAAUACGAUUA	mA.mC.A.A.A.mU.A.mC.G.A.mU#mU#mA#tegChol	

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Fig. 8 (Cont.)

SUBSTITUTE SHEET (RULE 26)

Continued from sheet 11/136

Gene	Accession Number	Position	Strand Modifications	Homology			Primary Screen	IC50 (nM)	
			Antisense Strand	H. sapien	M. musculus	M. mulatta	Huntingtin mRNA Expression (% control)	Passive Uptake	Lipid-Mediated Uptake
HTT	NM_002111.6	1214	PmU.fU.fC.A.G.fU.fU.fC.A.fU.A.A.mA.fC#fC#fU#G#G#mA#C	yes	yes	yes	34.3	197.4	N/A
HTT	NM_002111.6	1218	PmU.A.A.fC.G.fU.fC.A.G.fU.fU.fC.A.fU#A#A#mA#fC#fC#U	yes		yes	44.8	293.2	N/A
HTT	NM_002111.6	1219	PmU.fU.A.A.fC.G.fU.fC.A.G.fU.fU.fC.A#fU#A#A#mA#fC#C	yes		yes	29.6	163.6	0.053
HTT	NM_002111.6	1257	PmU.fC.fC.G.G.fU.fC.A.fC.A.A.fC.A.fU#fU#G#fU#G#G#U	yes		yes	28.5	156.7	N/A
HTT	NM_002111.6	1894	PmU.G.fU.fC.G.G.fU.A.fC.fC.G.fU.fC.fU#A#A#fC#A#fC#A	yes		yes	23.7	95.53	0.048
HTT	NM_002111.6	1907	PmU.fC.fC.A.A.mA.fU.A.fC.fU.G.G.fU.fU#G#fU#fC#G#G#U	yes		yes	39.3	217.9	N/A
HTT	NM_002111.6	2866	PmU.A.A.fC.A.fU.fU.A.fU.fU.G.A.mG.fC#A#fC#fU#fC#G#U	yes		yes	35.3	191.7	0.091
HTT	NM_002111.6	4041	PmU.fC.G.A.fC.fU.A.A.mA.G.fC.A.G.mG#A#fU#fU#fU#fC#A	yes	yes	yes	53.5	765.7	N/A
HTT	NM_002111.6	4049	PmU.fU.fU.G.G.fU.fU.fC.fU.fC.G.A.fC.fU#A#A#mA#G#fC#A	yes	yes	yes	41.2	217.8	N/A
HTT	NM_002111.6	5301	PmU.A.G.fC.G.fU.fU.G.A.mA.G.fU.A.fC#fU#G#fU#fC#fC#C	yes			36.6	230.2	0.080
HTT	NM_002111.6	6016	PmU.A.fC.A.A.fC.G.A.mG.A.fC.fU.G.A#mA#fU#fU#G#fC#C	yes		yes	26.4	147.9	N/A
HTT	NM_002111.6	6579	PmU.A.A.mG.fC.A.fU.G.G.mA.G.fC.fU.A#G#fC#A#G#mG#C	yes		yes	28.3	89.8	0.056
HTT	NM_002111.6	8603	PmU.G.A.mA.fU.G.fU.fU.fC.A.fC.G.fC.A#G#fU#G#G#mG#C	yes	yes	yes	40.0	236.1	N/A
HTT	NM_002111.6	10125	PmU.fU.fU.fU.fU.A.A.mA.fU.fC.fC.fU.G.A#mG#A#A#mG#A#A	yes		yes	31.1	158.7	0.059
HTT	NM_002111.6	10146	PmU.fC.fU.fC.fU.fU.fU.A.fC.fU.G.A.fU.A#fU#A#A#fU#fU#A	yes	yes	yes	25.9	217.7	0.052
HTT	NM_002111.6	10150	PmU.fU.A.A.fU.fC.fU.fC.fU.fU.fU.A.fC.fU#G#A#fU#A#fU#A	yes	yes	yes	28.6	82.2	0.004
HTT	NM_002111.6	424	PmU.fU.fU.fU.fC.fU.fU.G.G.fU.A.G.fC.fU#G#A#mA#A#G#U	yes		yes	67.4	N/A	N/A
HTT	NM_002111.6	456	PmU.fU.fC.A.fC.A.fU.A.fU.fU.G.fU.fC.A#G#mA#fC#A#A#U	yes		yes	51.5	N/A	N/A
HTT	NM_002111.6	522	PmU.fU.fC.fC.A.fU.A.G.fC.G.A.fU.G.fC#fC#fC#A#G#mA#A	yes		yes	68.2	N/A	N/A
HTT	NM_002111.6	527	PmU.A.A.mA.G.fU.fU.fC.fC.A.fU.A.G.fC#G#A#fU#G#fC#C	yes		yes	45.5	N/A	N/A
HTT	NM_002111.6	878	PmU.fU.A.A.fU.fU.fU.fC.A.fU.fU.G.fU.fC#A#fU#fU#fU#G#C	yes		yes	64.8	N/A	N/A
HTT	NM_002111.6	879	PmU.fU.fU.A.A.fU.fU.fU.fC.A.fU.fU.G.fU#fC#A#fU#fU#fU#G	yes		yes	51.5	N/A	N/A
HTT	NM_002111.6	908	PmU.G.G.fU.fU.fC.G.fC.fU.A.fU.G.A.mA#G#G#fC#fC#fU#U	yes		yes	99.6	N/A	N/A
HTT	NM_002111.6	1024	PmU.G.fC.fC.fU.A.A.mG.A.G.fC.A.fC.A#fU#fU#fU#A#G#U	yes		yes	52.9	N/A	N/A
HTT	NM_002111.6	1165	PmU.fU.fU.fC.fU.fU.fU.fC.fC.fU.fU.G.fU.fC#A#fC#fU#fC#fC#G	yes			77.0	N/A	N/A

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Fig. 8 *(Cont.)*

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SUBSTITUTE SHEET (RULE 26)

Gene	Accession Number	Position	Strand Modifications	Homology			Primary Screen	IC50 (nM)	
			Antisense Strand	H. sapien	M. musculus	M. mulatta	Huntingtin mRNA Expression (% control)	Passive Uptake	Lipid-Mediated Uptake
HTT	NM_002111.6	1207	PmU.A.fU.A.A.mA.fC.fC.fU.G.G.mA.fC.A#A#mG#fC#fU#G#C	yes	yes	yes	109.5	N/A	N/A
HTT	NM_002111.6	1212	PmU.A.G.fU.fU.fC.A.fU.A.A.mA.fC.fC.fU#G#G#mA#fC#A#A	yes	yes	yes	74.9	N/A	N/A
HTT	NM_002111.6	1217	PmU.A.fC.G.fU.fC.A.G.fU.fU.fC.A.fU.A#A#mA#fC#fC#fU#G	yes		yes	104.0	N/A	N/A
HTT	NM_002111.6	1220	PmU.G.fU.A.A.fC.G.fU.fC.A.G.fU.fU.fC#A#fU#A#A#mA#C	yes		yes	83.9	N/A	N/A
HTT	NM_002111.6	1223	PmU.G.A.fU.G.fU.A.A.fC.G.fU.fC.A.G#fU#fU#fC#A#fU#A	yes		yes	92.2	N/A	N/A
HTT	NM_002111.6	1227	PmU.G.fU.A.fU.G.A.fU.G.fU.A.A.fC.G#fU#fC#A#G#fU#U	yes		yes	81.4	N/A	N/A
HTT	NM_002111.6	1229	PmU.G.fU.G.fU.A.fU.G.A.fU.G.fU.A.A#fC#G#fU#fC#A#G	yes		yes	82.2	N/A	N/A
HTT	NM_002111.6	1260	PmU.G.fC.fU.fC.fC.G.G.fU.fC.A.fC.A.A#fC#A#fU#fU#G#U	yes		yes	108.4	N/A	N/A
HTT	NM_002111.6	1403	PmU.fU.A.A.mG.fU.fU.fC.fC.A.fC.A.A.fU#A#fC#fU#fC#fC#C	yes		yes	138.6	N/A	N/A
HTT	NM_002111.6	1470	PmU.fC.fC.fU.A.A.mG.A.G.fC.A.fC.fU.fU#fU#G#fC#fC#fU#U	yes	yes	yes	85.6	N/A	N/A
HTT	NM_002111.6	1901	PmU.A.fC.fU.G.G.fU.fU.G.fU.fC.G.G.fU#A#fC#fC#G#fU#C	yes		yes	81.4	N/A	N/A
HTT	NM_002111.6	1903	PmU.A.fU.A.fC.fU.G.G.fU.fU.G.fU.fC.G#G#fU#A#fC#fC#G	yes		yes	72.7	N/A	N/A
HTT	NM_002111.6	2411	PmU.fC.A.fU.G.A.fU.fC.G.A.fU.G.fU.A#G#fU#fU#fC#A#A	yes		yes	53.0	N/A	N/A
HTT	NM_002111.6	2412	PmU.fC.fC.A.fU.G.A.fU.fC.G.A.fU.G.fU#A#G#fU#fU#fC#A	yes		yes	57.1	N/A	N/A
HTT	NM_002111.6	2865	PmU.A.fC.A.fU.fU.A.fU.fU.G.A.mG.fC.A#fC#fU#fC#G#fU#U	yes		yes	83.1	N/A	N/A
HTT	NM_002111.6	3801	PmU.fU.fU.A.fC.fU.fU.G.fU.fU.G.fU.A.A#fC#A#G#mG#A#C	yes		yes	48.9	N/A	N/A
HTT	NM_002111.6	4040	PmU.G.A.fC.fU.A.A.mA.G.fC.A.G.mG.A#fU#fU#fU#fC#A#G	yes	yes	yes	56.2	N/A	N/A
HTT	NM_002111.6	4048	PmU.fU.G.G.fU.fU.fC.fU.fC.G.A.fC.fU.A#A#mA#G#fC#A#G	yes	yes	yes	72.2	N/A	N/A
HTT	NM_002111.6	4052	PmU.fU.fC.A.fU.fU.G.G.fU.fU.fC.fU.fC.G#A#fC#fU#A#A#A	yes	yes	yes	90.8	N/A	N/A
HTT	NM_002111.6	4055	PmU.fC.fC.A.fU.fC.A.fU.fU.G.G.fU.fU.fC#fU#fC#G#A#fC#U	yes	yes	yes	37.2	N/A	N/A
HTT	NM_002111.6	4083	PmU.fU.fU.fC.A.A.fC.A.A.fU.fU.G.fU.fU#G#A#mA#fC#A#C	yes		yes	91.7	N/A	N/A
HTT	NM_002111.6	4275	PmU.G.fC.fC.fU.G.fC.A.fC.fC.A.fU.G.fU#fU#fC#fC#fU#fC#A	yes	yes	yes	77.2	N/A	N/A
HTT	NM_002111.6	4372	PmU.fU.G.fC.A.fC.G.G.fU.fU.fC.fU.fU.fU#G#fU#G#A#fC#A	yes		yes	44.5	N/A	N/A
HTT	NM_002111.6	4374	PmU.fU.fC.fU.G.fC.A.fC.G.G.fU.fU.fC.fU#fU#fU#G#fU#G#A	yes		yes	97.5	N/A	N/A
HTT	NM_002111.6	4376	PmU.fU.A.fU.fC.fU.G.fC.A.fC.G.G.fU.fU#fC#fU#fU#fU#G#U	yes		yes	64.1	N/A	N/A

Continued on sheet 16/136

Fig. 8 *(Cont.)*

Continued from sheet 13/136

Gene	Accession Number	Position	Strand Modifications	Homology			Primary Screen	IC50 (nM)	
			Antisense Strand	H. sapien	M. musculus	M. mulatta	Huntingtin mRNA Expression (% control)	Passive Uptake	Lipid-Mediated Uptake
HTT	NM_002111.6	4425	PmU.fU.fU.fU.A.fU.A.A.fC.A.A.mG.A.G#mG#fU#fU#fC#A#A	yes	yes	yes	44.6	N/A	N/A
HTT	NM_002111.6	4562	PmU.fU.fC.A.A.fU.A.fC.A.A.mA.G.fC.fC#A#A#fU#A#A#A	yes		yes	102.1	N/A	N/A
HTT	NM_002111.6	4692	PmU.A.fU.fU.fU.fU.A.G.mG.A.A.fU.fU.fC#fC#A#A#fU#G#A	yes	yes	yes	53.8	N/A	N/A
HTT	NM_002111.6	4721	PmU.fU.G.G.fC.fC.A.fU.G.A.fU.G.fC.fC#A#fU#fC#A#fC#A	yes		yes	124.2	N/A	N/A
HTT	NM_002111.6	5200	PmU.fU.fU.fC.A.G.fU.fU.G.A.fC.fU.G.G#mG#A#A#mA#fU#C	yes		yes	43.8	N/A	N/A
HTT	NM_002111.6	5443	PmU.A.G.fU.A.fU.G.fU.fU.G.fC.fU.G.fC#fU#fC#A#fC#fU#C	yes		yes	48.9	N/A	N/A
HTT	NM_002111.6	5515	PmU.fU.fC.fU.fC.fC.G.G.mA.A.fC.A.fU.fU#fC#fC#A#G#mA#	yes	yes	yes	62.0	N/A	N/A
HTT	NM_002111.6	8609	PmU.G.G.fC.fU.G.fU.G.A.mA.fU.G.fU.fU#fC#A#fC#G#fC#A	yes	yes	yes	47.4	N/A	N/A
HTT	NM_002111.6	10130	PmU.fU.fU.A.A.mA.fU.fU.fU.fU.A.A.mA.fU#fC#fC#fU#G#A#G	yes		yes	49.6	N/A	N/A
HTT	NM_002111.6	10134	PmU.A.fU.A.A.fU.fU.A.A.mA.fU.fU.fU.fU#A#A#mA#fU#fC#C	yes	yes	yes	113.7	N/A	N/A
HTT	NM_002111.6	10142	PmU.fU.fU.A.fC.fU.G.A.fU.A.fU.A.A.fU#fU#A#A#mA#fU#U	yes	yes	yes	78.0	N/A	N/A
HTT	NM_002111.6	10169	PmU.G.A.mA.A.G.mA.G.fU.fU.A.fC.G.fU#fU#A#A#mA#A#U	yes		yes	69.0	N/A	N/A
HTT	NM_002111.6	10182	PmU.fU.fU.A.fC.A.fC.G.G.mG.fC.A.fU.A#G#mA#A#A#mG#A	yes	yes	yes	100.1	N/A	N/A
HTT	NM_002111.6	10186	PmU.fU.A.fC.fU.fU.fU.A.fC.A.fC.G.G.mG#fC#A#fU#A#G#A	yes		yes	83.5	N/A	N/A
HTT	NM_002111.6	10809	PmU.G.fC.A.fC.fU.fC.fU.fC.fC.fU.G.A.fC#fU#A#A#mA#A#G	yes			101.7	N/A	N/A
HTT	NM_002111.6	11116	PmU.A.fC.A.fU.fU.fC.A.A.fU.A.fC.fC.fC#A#A#mA#A#fC#A	yes			90.0	N/A	N/A
HTT	NM_002111.6	11129	PmU.fU.fC.fC.fU.fC.fC.A.fC.fU.fU.A.fC.fC#A#fC#A#fU#fU#C	yes			105.9	N/A	N/A
HTT	NM_002111.6	11134	PmU.A.fC.A.fU.fU.fU.fC.fC.fU.fC.fC.A.fC#fU#fU#A#fC#fC#A	yes			85.1	N/A	N/A
HTT	NM_002111.6	11147	PmU.G.fC.A.fC.A.G.mA.G.fU.fU.fC.fC.A#A#fC#A#fU#fU#U	yes			109.9	N/A	N/A
HTT	NM_002111.6	11412	PmU.fU.A.A.mG.G.G.fC.fC.fU.fC.fC.fU.fC#A#A#mA#fC#A#U	yes			122.0	N/A	N/A
HTT	NM_002111.6	11426	PmU.fU.fC.A.G.fU.A.G.fC.fU.fU.fC.fC.fC#fU#fU#A#A#mG#G	yes	yes		106.3	N/A	N/A
HTT	NM_002111.6	11443	PmU.fU.fU.fC.fU.fU.A.fC.G.fU.G.fU.fU.A#fU#A#A#fU#fU#C	yes			91.7	N/A	N/A
HTT	NM_002111.6	11659	PmU.fU.fU.fC.fU.fU.A.fC.A.A.mA.fU.G.fU#A#A#mA#fC#A#U	yes			80.7	N/A	N/A
HTT	NM_002111.6	11666	PmU.G.fU.G.fU.fU.A.fU.fU.fU.fC.fU.fU.A#fC#A#A#mA#fU#G	yes			98.5	N/A	N/A
HTT	NM_002111.6	11677	PmU.fU.fU.A.fC.A.fU.fU.fC.A.fC.A.G.fU#G#fU#fU#A#fU#U	yes			87.8	N/A	N/A

Continued on sheet 18/136

Fig. 8 (Cont.)

SUBSTITUTE SHEET (RULE 26)

Continued from sheet 14/136

Gene	Accession Number	Position	Strand Modifications	Homology			Primary Screen	IC50 (nM)	
			Antisense Strand	H. sapien	M. musculus	M. mulatta	Huntingtin mRNA Expression (% control)	Passive Uptake	Lipid-Mediated Uptake
HTT	NM_002111.6	11863	PmU.fU.fU.A.fC.fU.A.A.fU.G.A.mG.fC.fU#fC#A#fU#A#fU#U	yes			77.4	N/A	N/A
HTT	NM_002111.6	11890	PmU.fU.A.fU.G.fU.A.fU.A.fU.G.fC.G.fU#G#G#mG#fU#G#A	yes			114.3	N/A	N/A
HTT	NM_002111.6	11927	PmU.A.fU.fU.A.fU.A.G.mA.fU.G.fU.G.fU#fC#fU#A#fU#A#U	yes			113.3	N/A	N/A
HTT	NM_002111.6	11947	PmU.fU.fU.G.A.mG.A.G.mG.fU.G.fU.G.fU#G#fU#G#fU#A#A	yes			99.8	N/A	N/A
HTT	NM_002111.6	12163	PmU.fU.fU.A.G.mG.A.A.fC.A.fU.G.A.fU#A#A#mA#G#fU#C	yes			70.7	N/A	N/A
HTT	NM_002111.6	12218	PmU.fU.fU.A.A.fU.fC.A.fC.A.fU.fU.fU.G#fC#A#A#fC#A#A	yes			115.3	N/A	N/A
HTT	NM_002111.6	12223	PmU.fC.fC.A.A.mA.fU.fU.A.A.fU.fC.A.fC#A#fU#fU#fU#G#C	yes			114.6	N/A	N/A
HTT	NM_002111.6	12235	PmU.A.A.mA.A.fC.fU.fU.G.A.fC.A.A.fC#fC#A#A#mA#fU#U	yes			108.3	N/A	N/A
HTT	NM_002111.6	12279	PmU.fU.fU.A.fC.fC.A.G.fC.A.G.mG.A.A#mA#A#fC#A#A#A	yes			83.9	N/A	N/A
HTT	NM_002111.6	12282	PmU.A.fU.A.fU.fU.A.fC.fC.A.G.fC.A.G#mG#A#A#mA#A#C	yes			89.9	N/A	N/A
HTT	NM_002111.6	12297	PmU.fU.fU.A.A.mA.A.fU.fC.fU.fU.fU.fC.fC#fC#G#A#fU#A#U	yes			82.9	N/A	N/A
HTT	NM_002111.6	12309	PmU.A.fC.fC.fC.fU.G.G.fU.fU.fU.fC.A.fU#fU#A#A#mA#A#U	yes			73.4	N/A	N/A
HTT	NM_002111.6	12313	PmU.fU.fU.fC.fU.A.fC.fC.fC.fU.G.G.fU.fU#fU#fC#A#fU#fU#A	yes			89.8	N/A	N/A
HTT	NM_002111.6	12331	PmU.fC.A.G.fU.G.fC.A.fU.fU.G.fC.fC.A#A#mA#fC#A#A#U	yes			109.9	N/A	N/A
HTT	NM_002111.6	13136	PmU.fC.fU.fU.A.G.mA.A.A.fC.A.A.fC.fU#G#A#mG#G#G#G	yes			113.2	N/A	N/A
HTT	NM_002111.6	13398	PmU.A.fU.A.fC.A.fU.fC.fU.fC.fU.fC.G.fU#fC#A#G#fU#fC#C	yes			102.1	N/A	N/A
HTT	NM_002111.6	13403	PmU.fU.A.A.mA.fU.A.fU.A.fC.A.fU.fC.fU#fC#fU#fC#G#fU#C	yes			84.1	N/A	N/A
HTT	NM_002111.6	13423	PmU.G.fU.fU.fU.G.fC.A.G.fC.A.G.fU.fU#A#A#mA#A#A#A	yes			124.8	N/A	N/A
HTT	NM_002111.6	13428	PmU.A.fC.A.A.fU.G.fU.fU.fU.G.fC.A.G#fC#A#G#fU#fU#A	yes			114.1	N/A	N/A
NTC	N/A	N/A	P.mU.A.A.fU.fC.G.fU.A.fU.fU.fU.GU#mC#A#A#mU#mC#A	yes			102.0	N/A	N/A

Fig. 8 *(Cont.)*

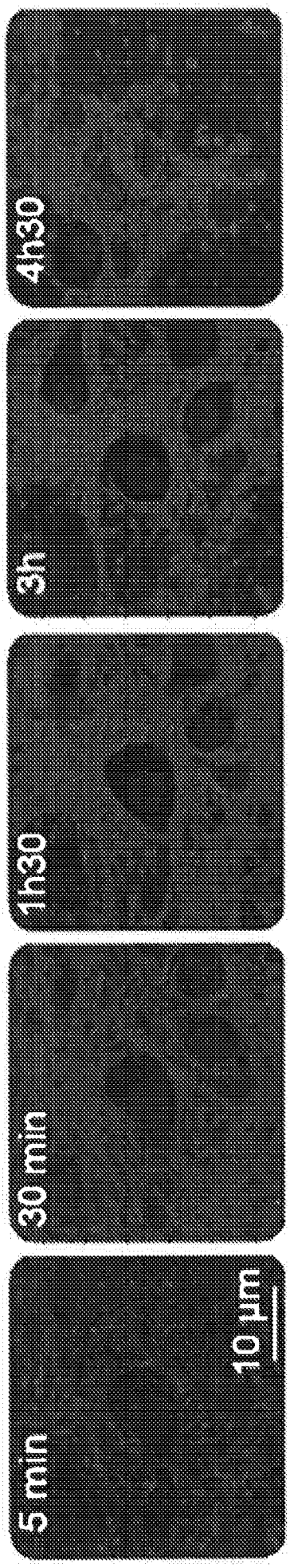

Fig. 9

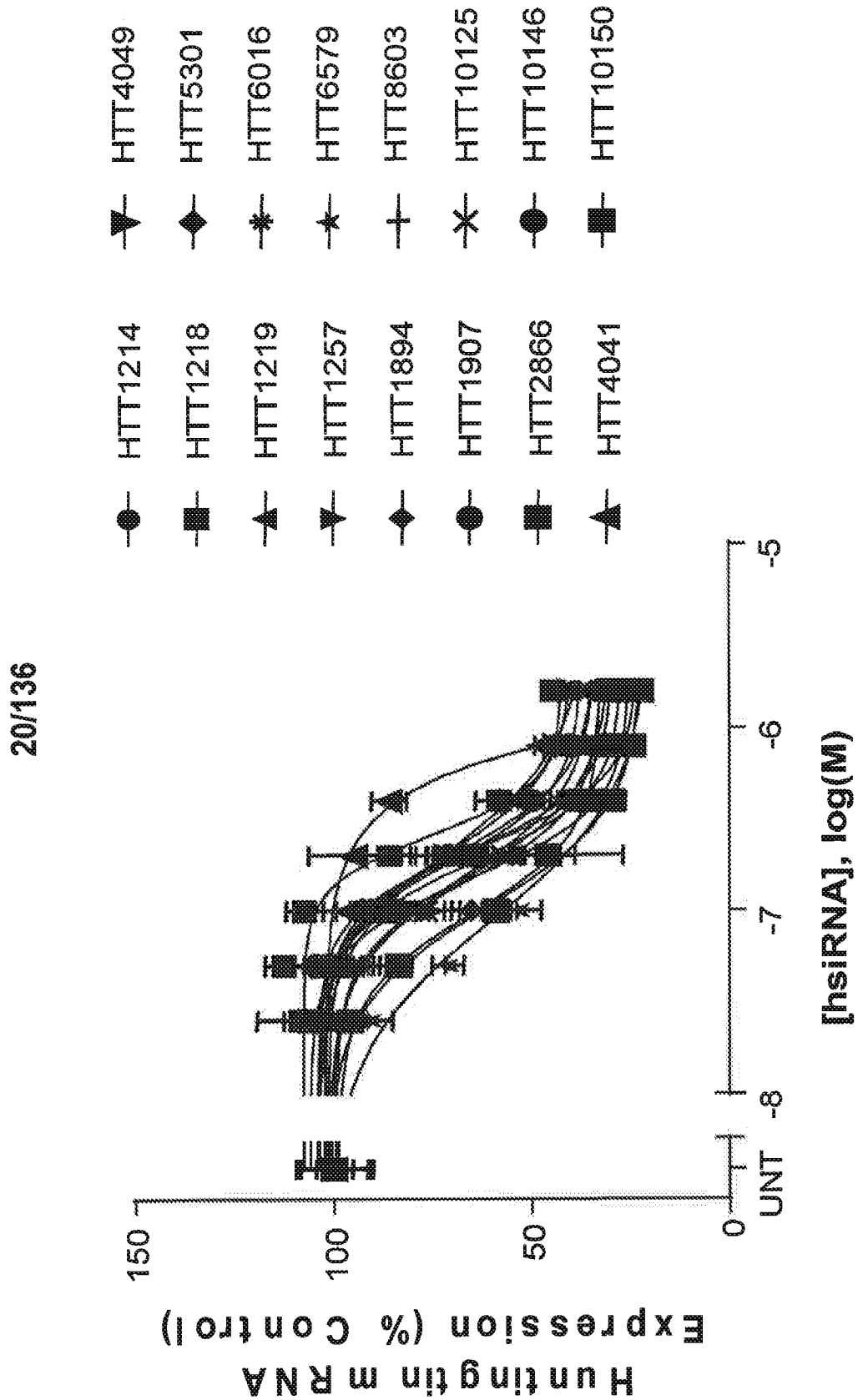
20/136
HTT1214
HTT1218
HTT1219
HTT1257
HTT1894
HTT1907
HTT2866
HTT4041
HTT4049
HTT5301
HTT6016
HTT6579
HTT8603
HTT10125
HTT10146
HTT10150
Huntingtin mRNA Expression (% Control)
150
100
50
0
UNT
-8
-7
-6
-5
[hsiRNA], log(M)

Fig. 10A

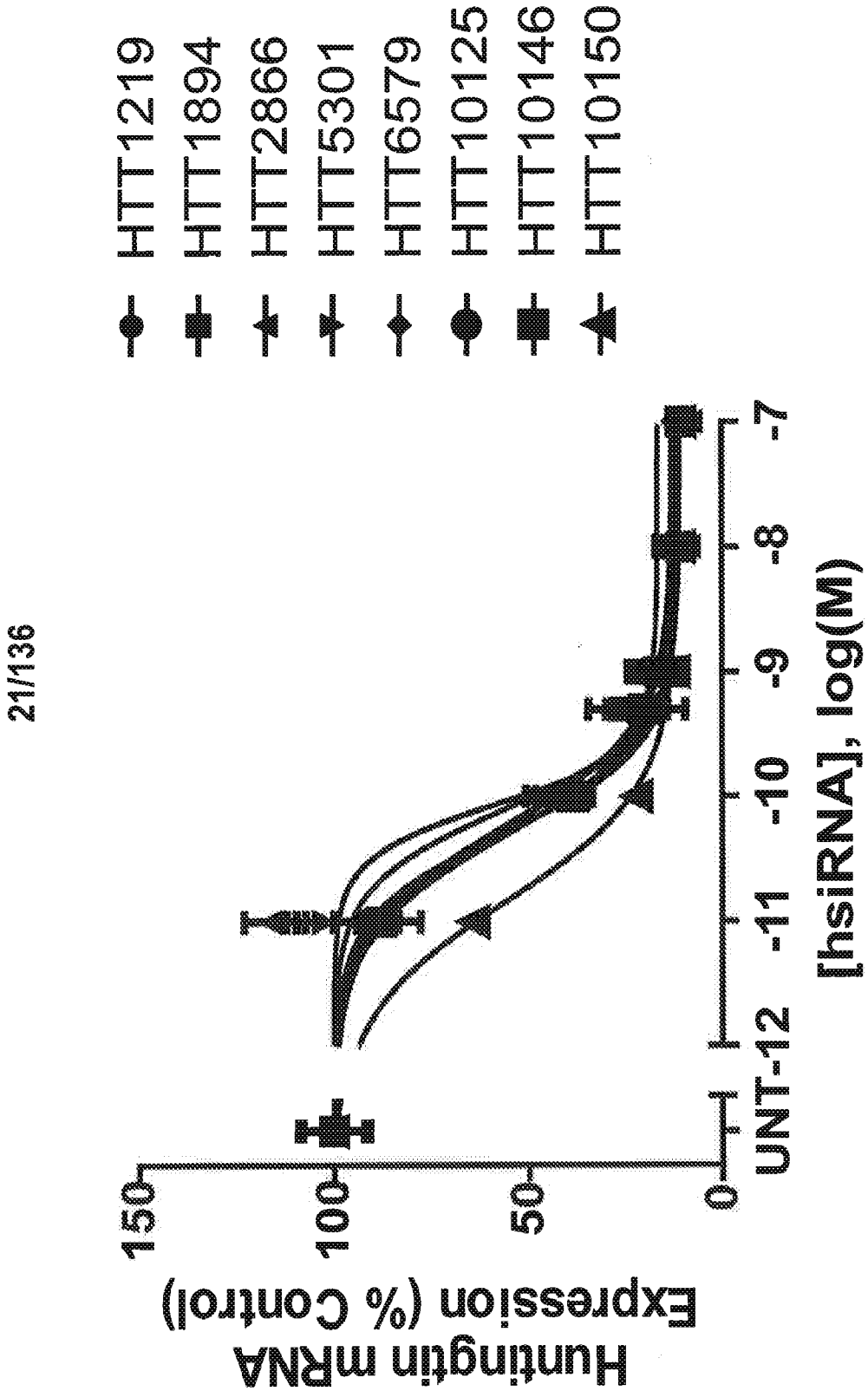

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Fig. 10B

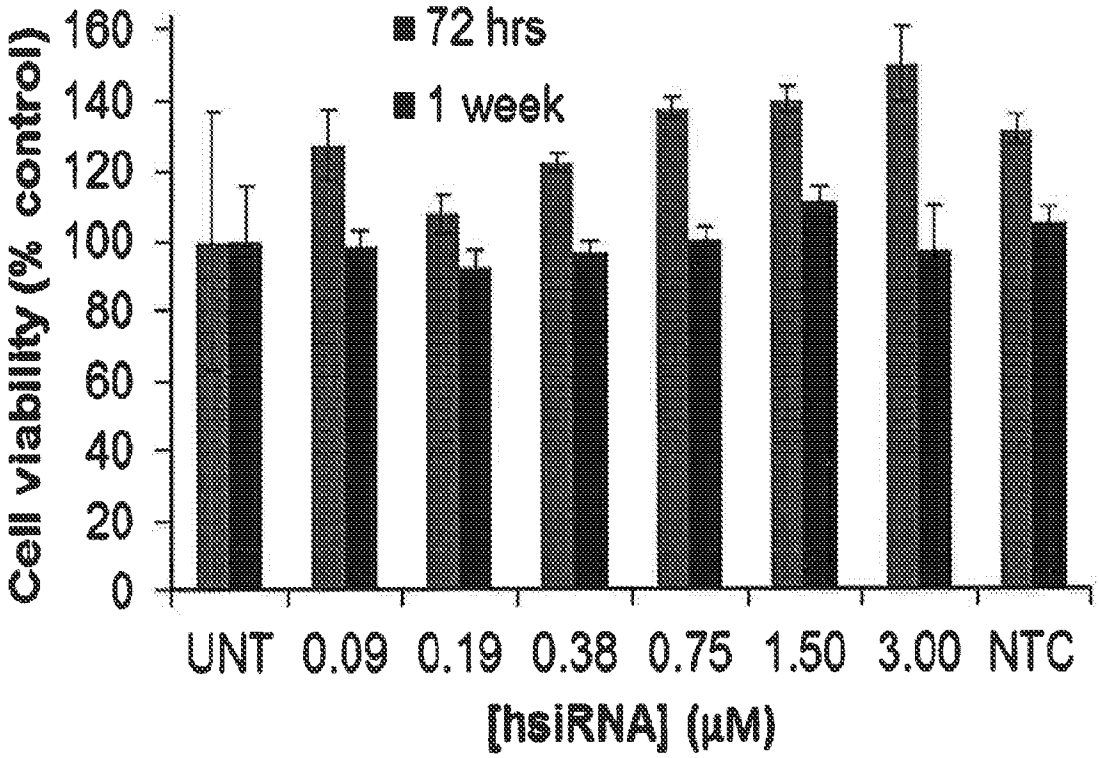


Fig. 11A

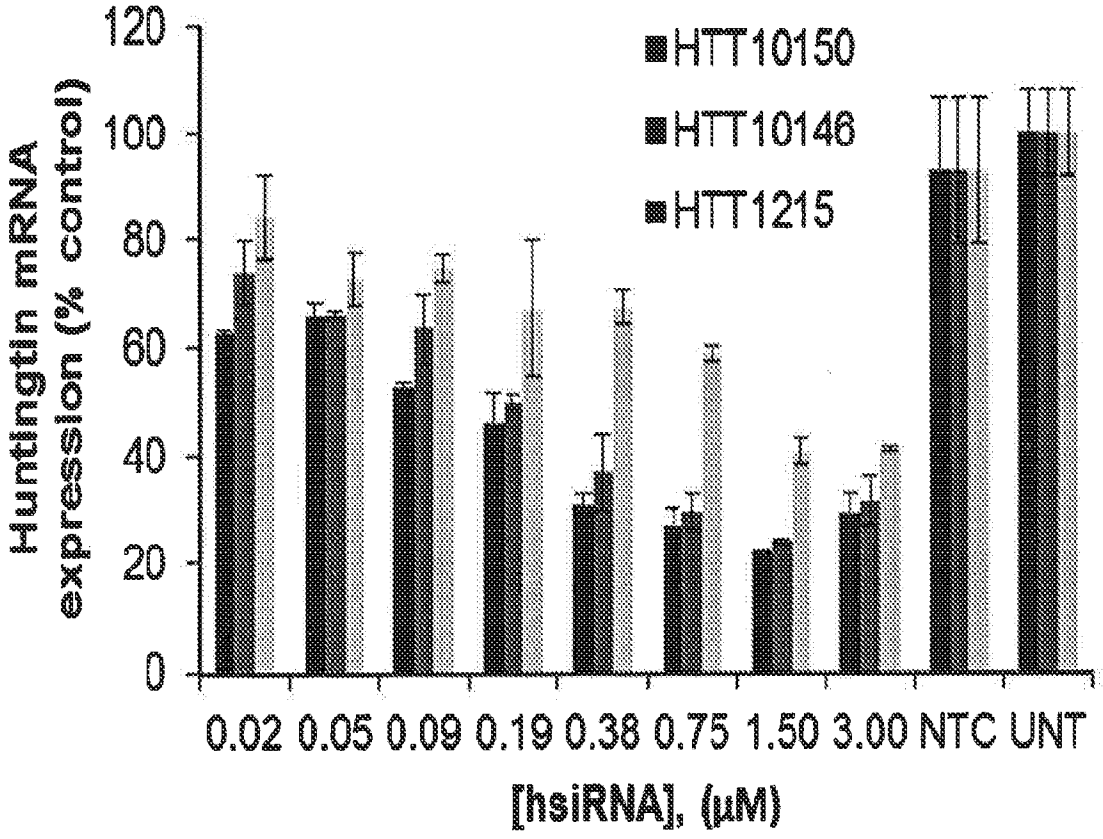


Fig. 11B

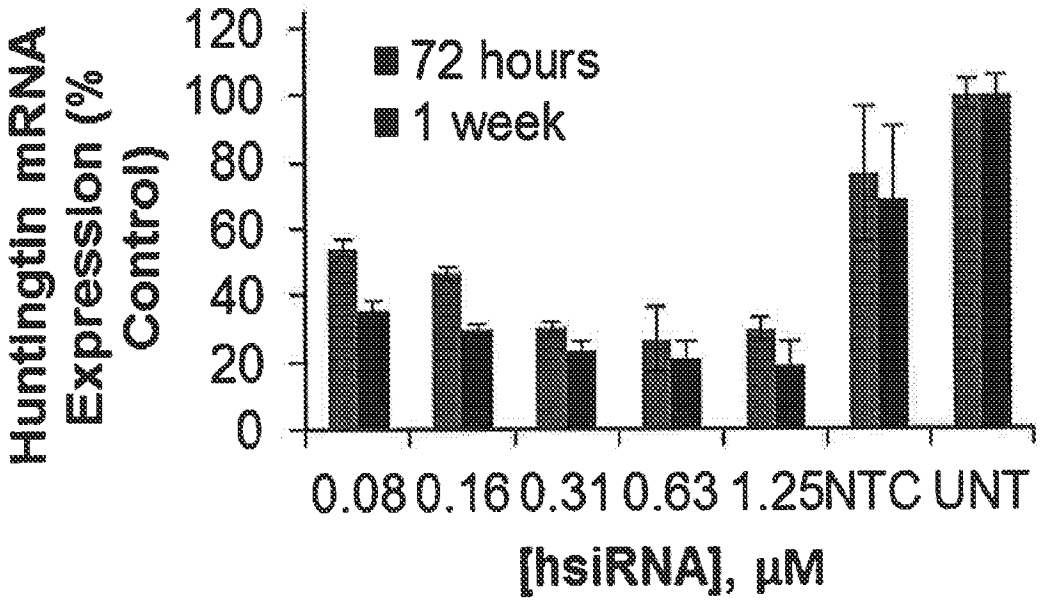


Fig. 12A

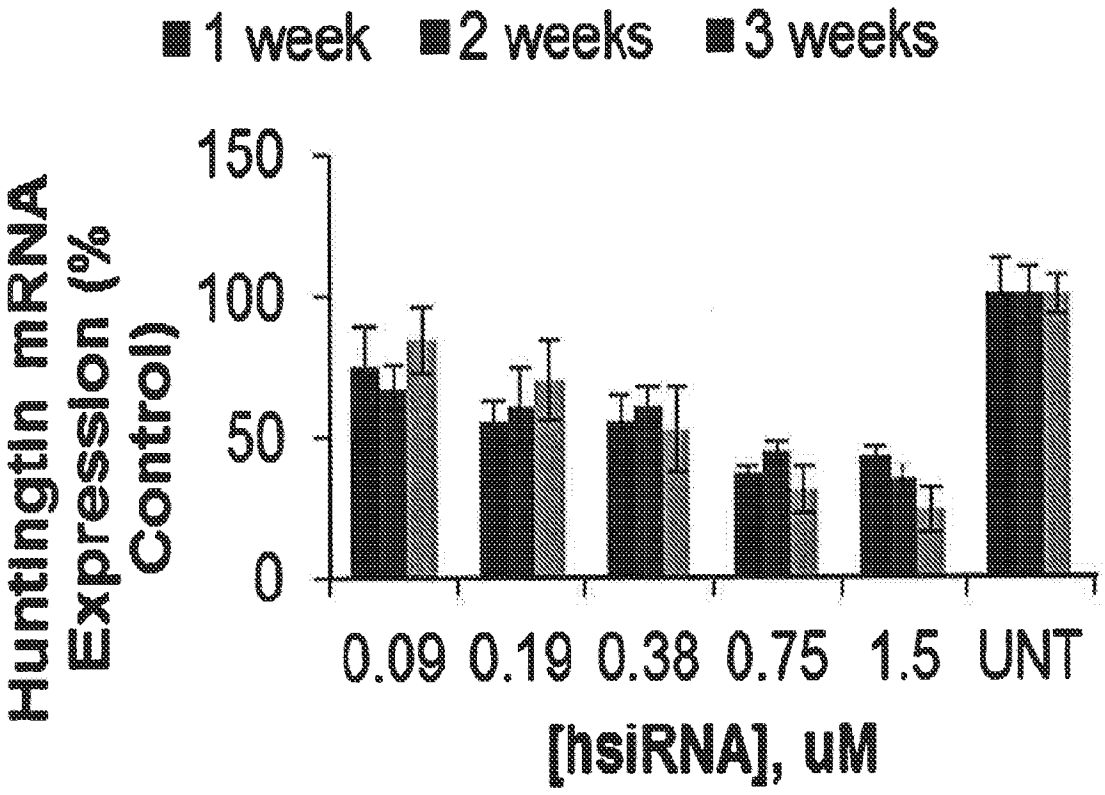


Fig. 12B

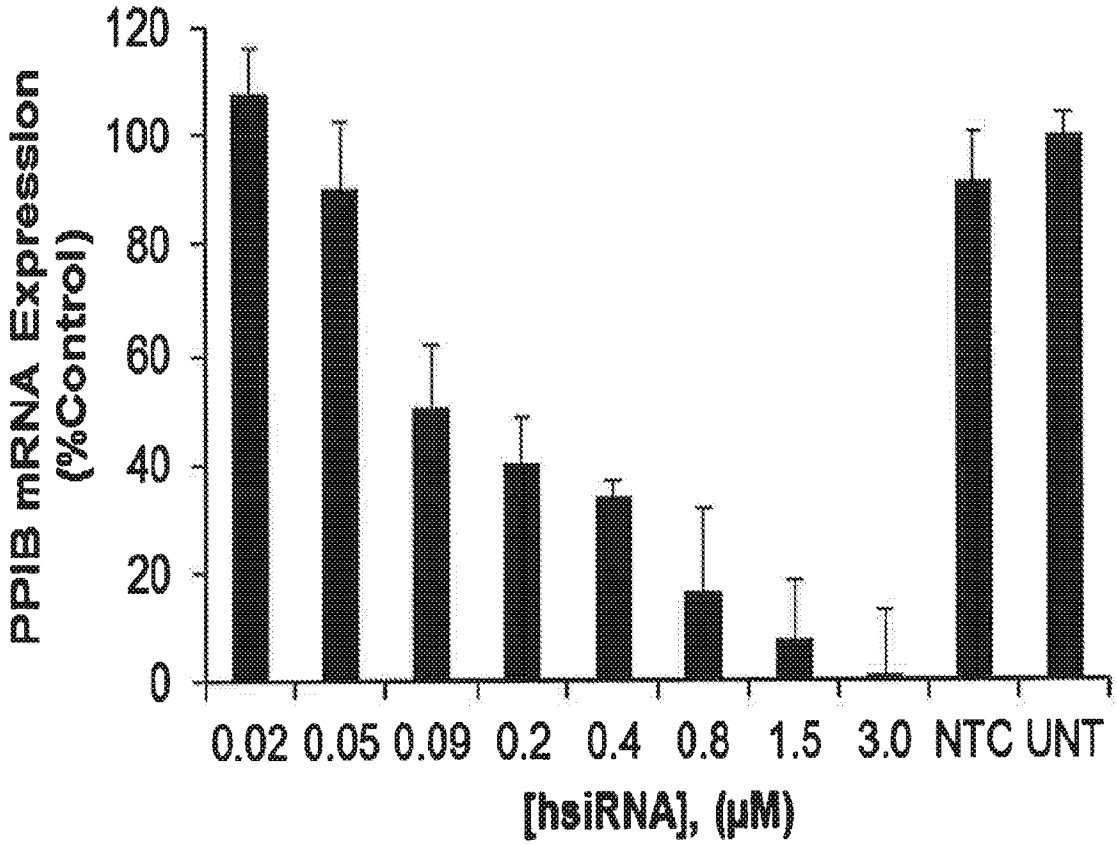

PPIB mRNA Expression
(%Control)
120
100
80
60
40
20
0
0.02 0.05 0.09 0.2 0.4 0.8 1.5 3.0 NTC UNT
[hsiRNA], (μM)

Fig. 13

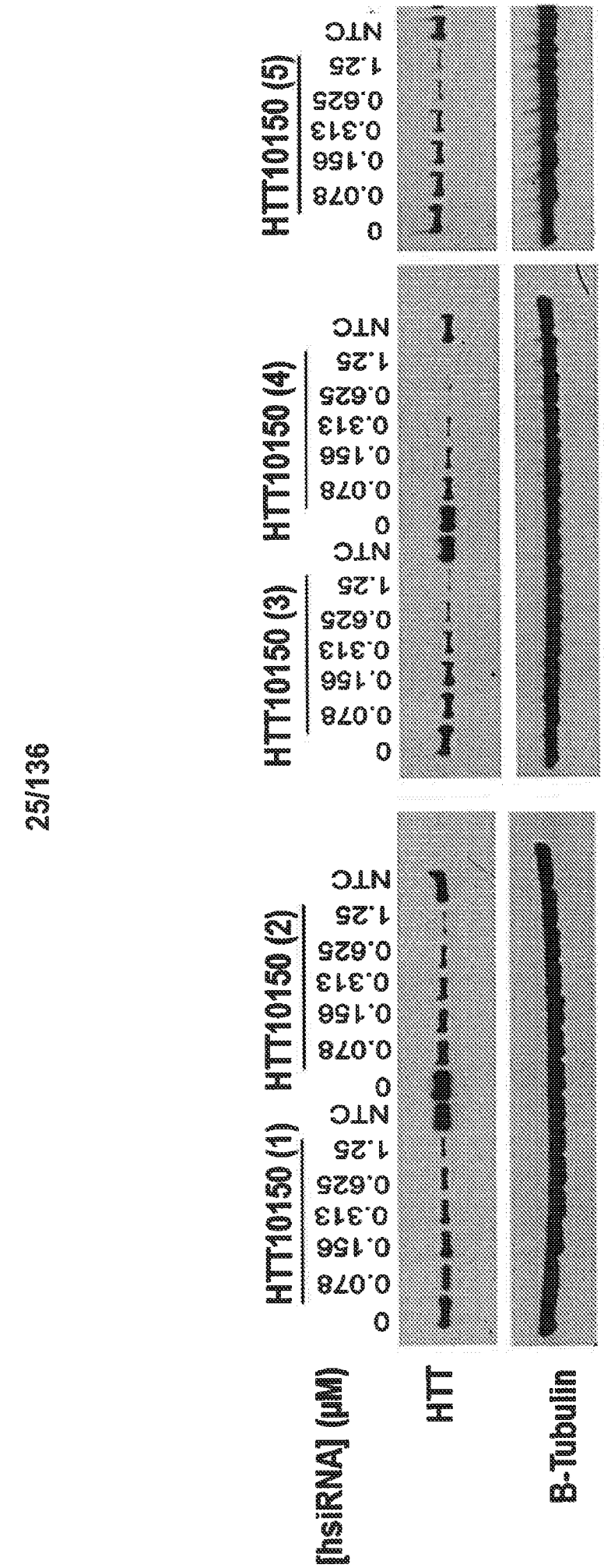

[hsiRNA] (μM)
HTT10150 (1)
0
0.078
0.156
0.313
0.625
1.25
NTC
HTT10150 (2)
0
0.078
0.156
0.313
0.625
1.25
NTC
HTT
B-Tubulin
HTT10150 (3)
0
0.078
0.156
0.313
0.625
1.25
NTC
HTT10150 (4)
0
0.078
0.156
0.313
0.625
1.25
NTC
HTT10150 (5)
0
0.078
0.156
0.313
0.625
1.25
NTC

Fig. 14

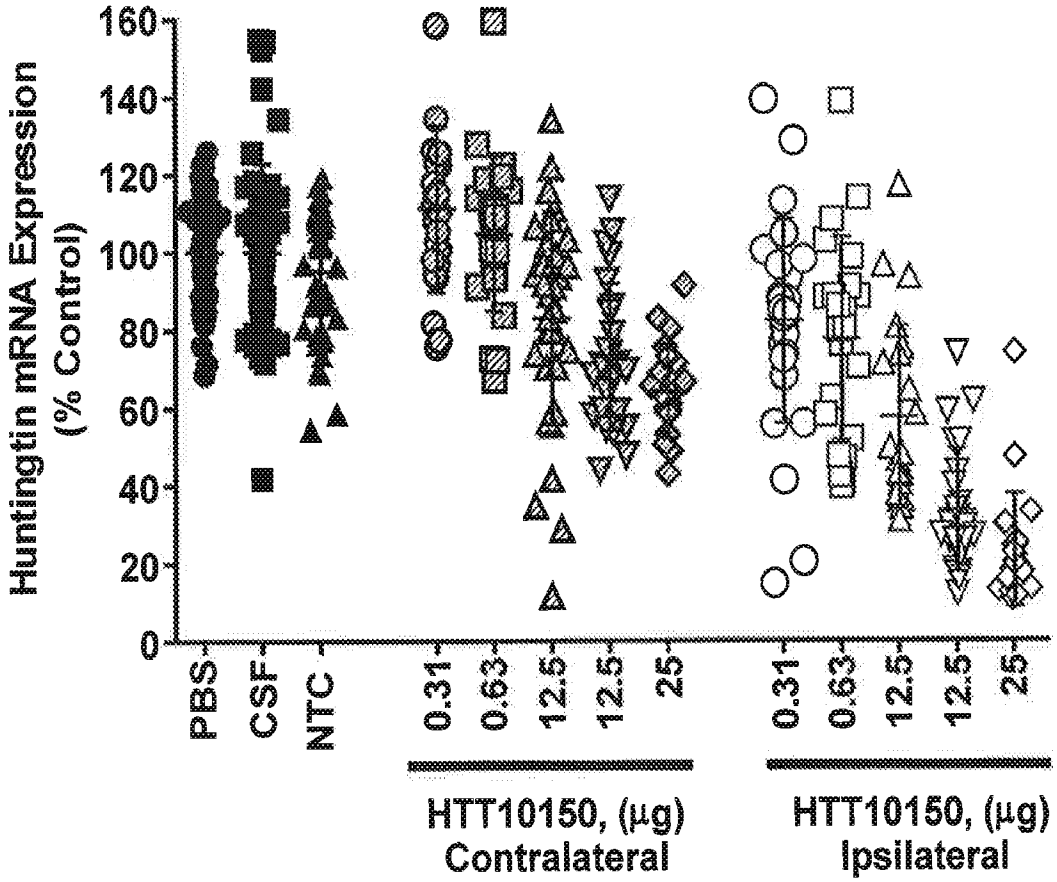


Fig. 15A

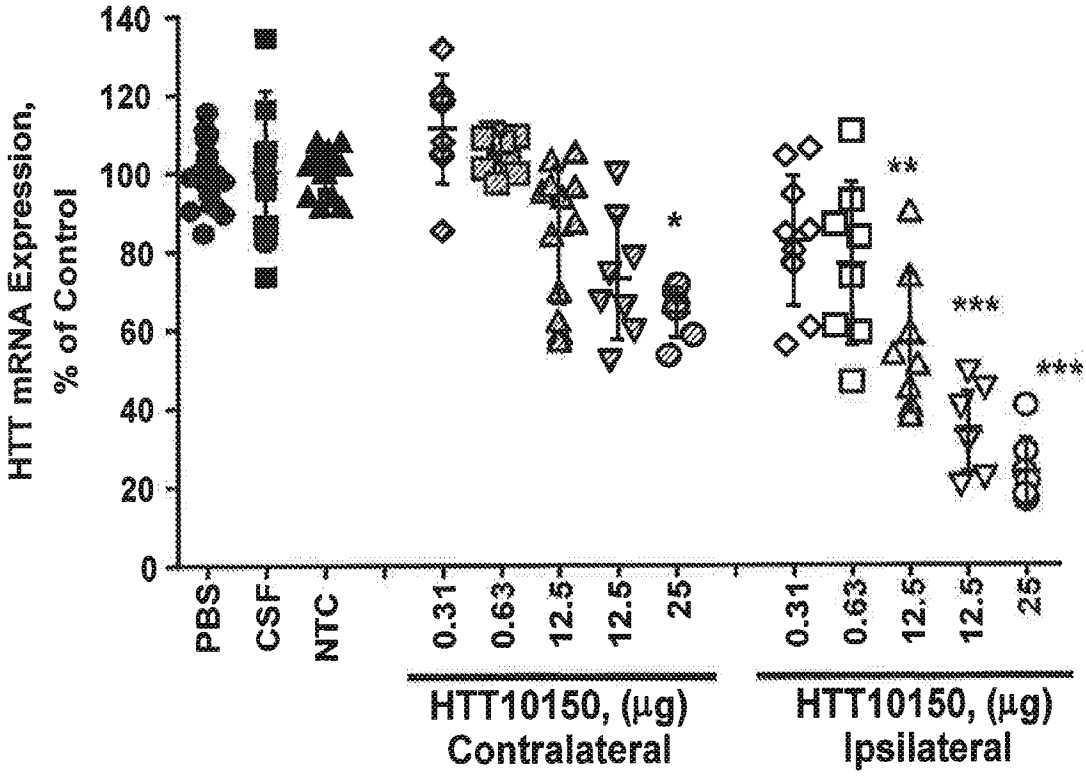


Fig. 15B

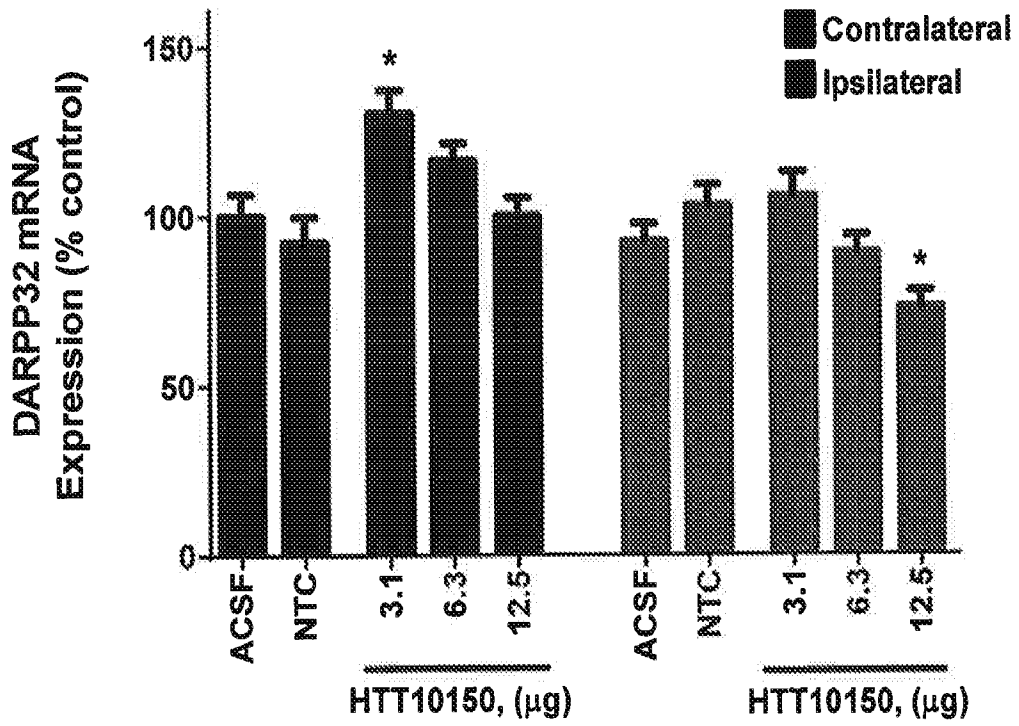
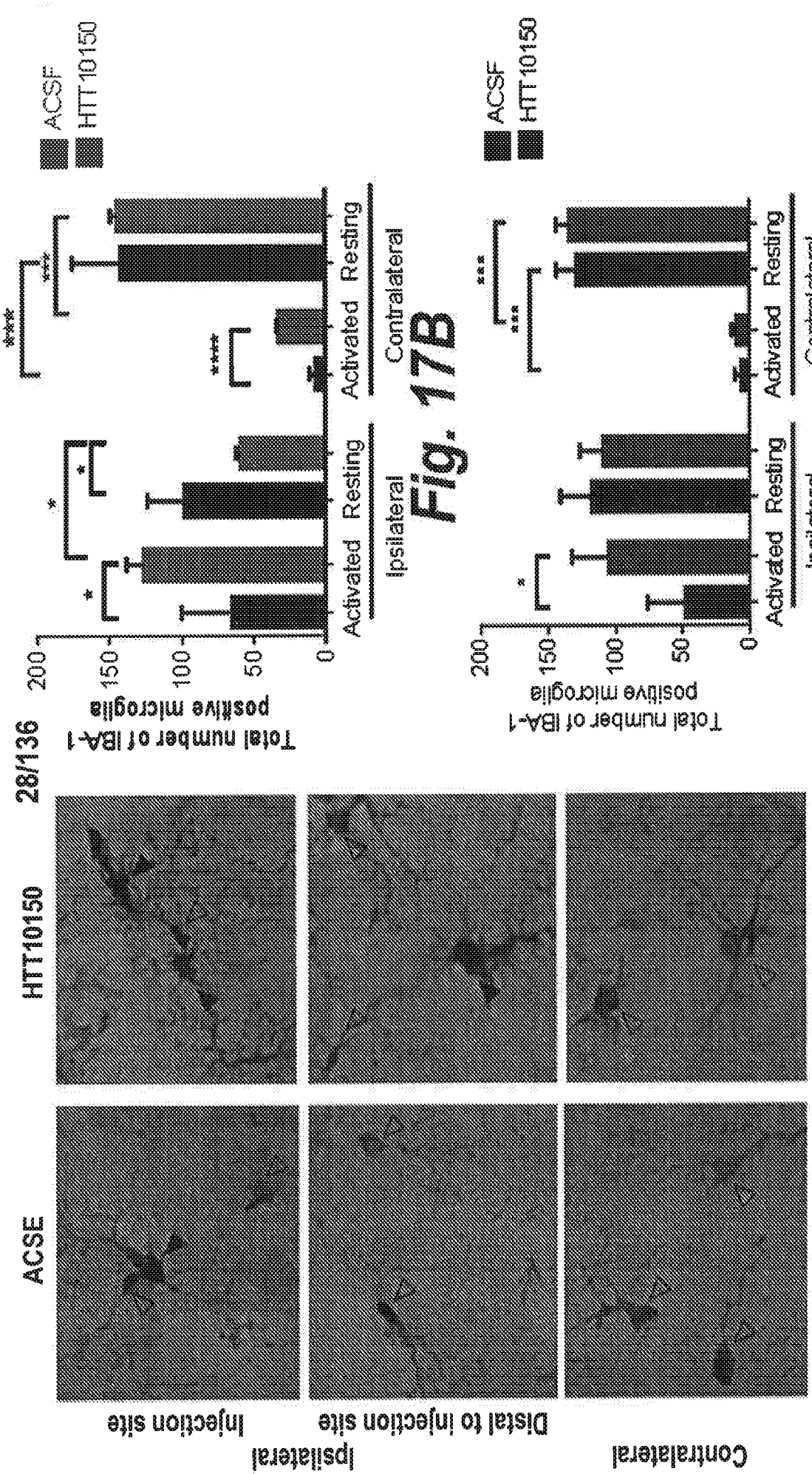

DARPP32 mRNA
Expression (% control)
150
100
50
0
*
*
Contralateral
Ipsilateral
ACSF
NTC
3.1
6.3
12.5
ACSF
NTC
3.1
6.3
12.5
HTT10150, (µg)
HTT10150, (µg)

Fig. 16


ACSE
HTT10150
28/136
Ipsilateral
Contralateral
Injection site
Distal to injection site
Fig. 17A
Total number of IBA-1 positive microglia
200
150
100
50
0
Activated Resting
Ipsilateral
Activated Resting
Contralateral
ACSF
HTT10150
Fig. 17B
Total number of IBA-1 positive microglia
200
150
100
50
0
Activated Resting
Ipsilateral
Activated Resting
Contralateral
ACSF
HTT10150
Fig. 17C

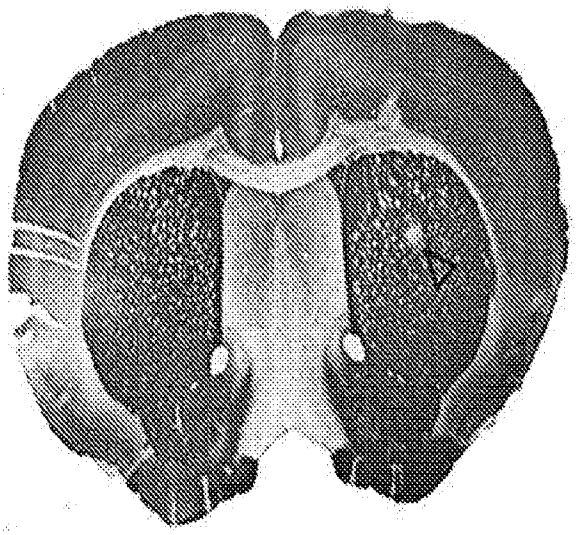


Fig. 18A

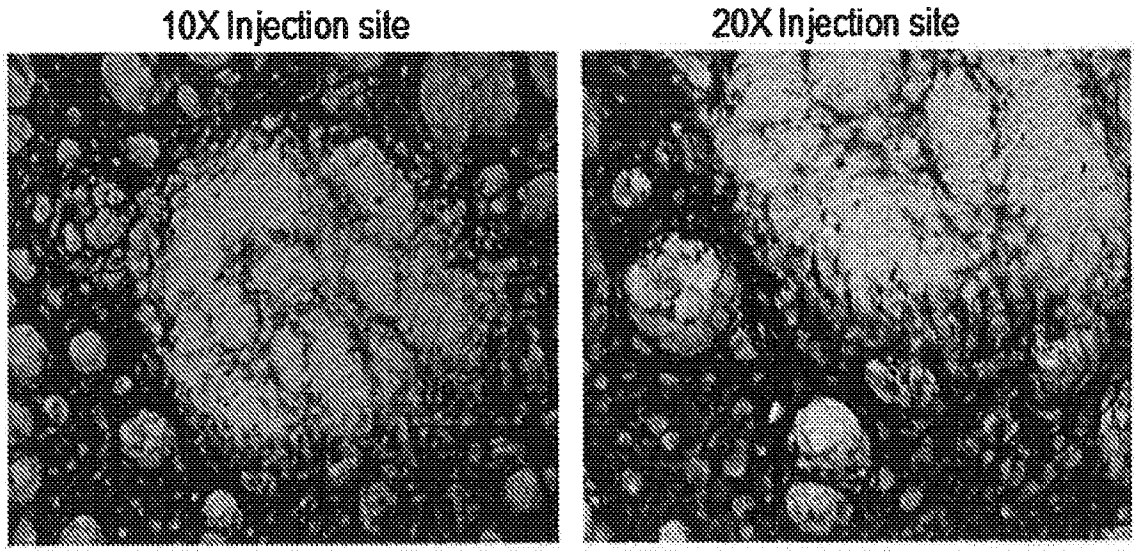


Fig. 18B

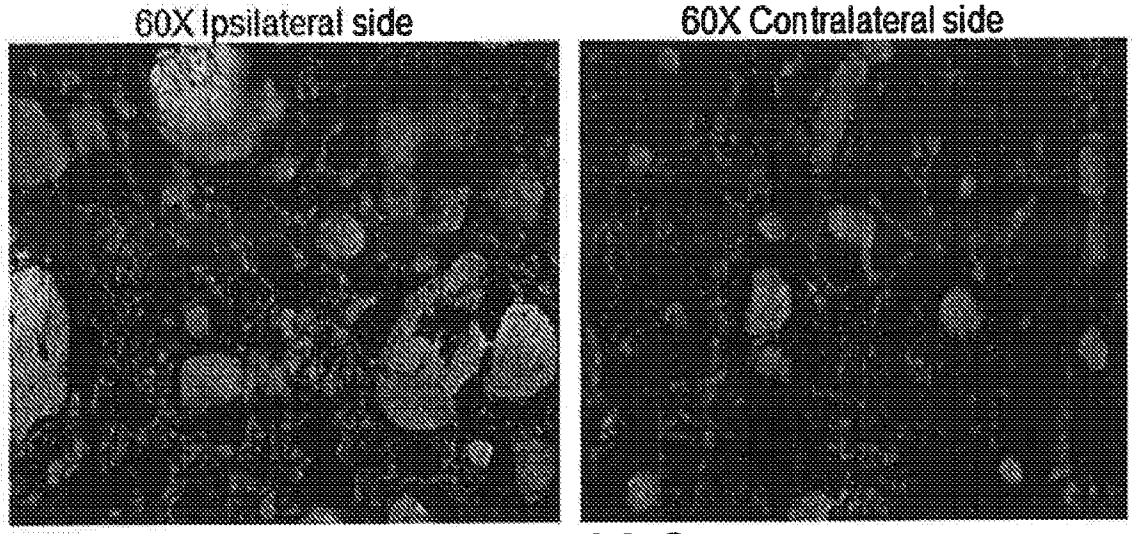


Fig. 18C

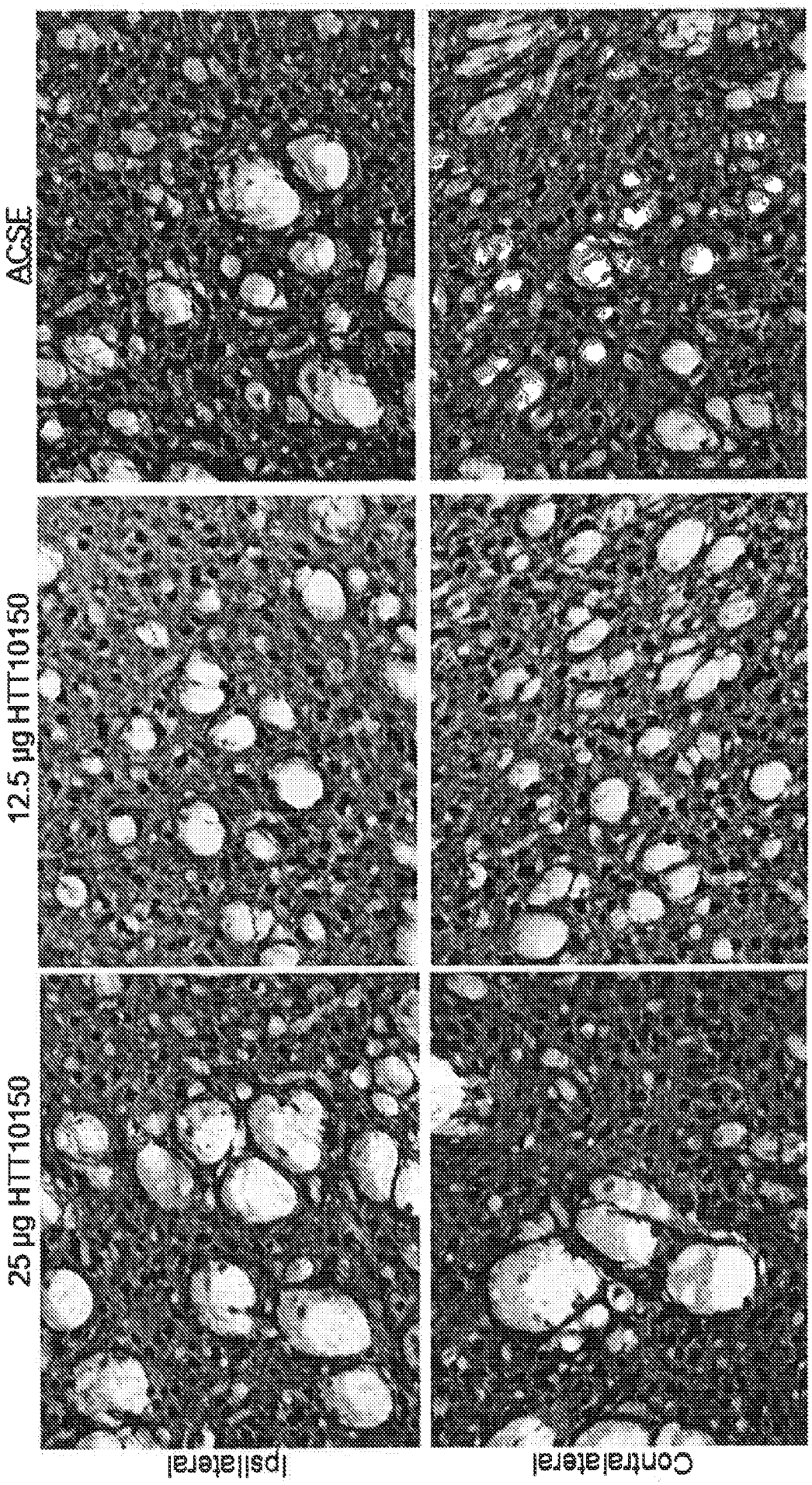

ACSF
30/136
12.5 µg HTT10150
25 µg HTT10150
Ipsilateral
Contralateral

Fig. 19

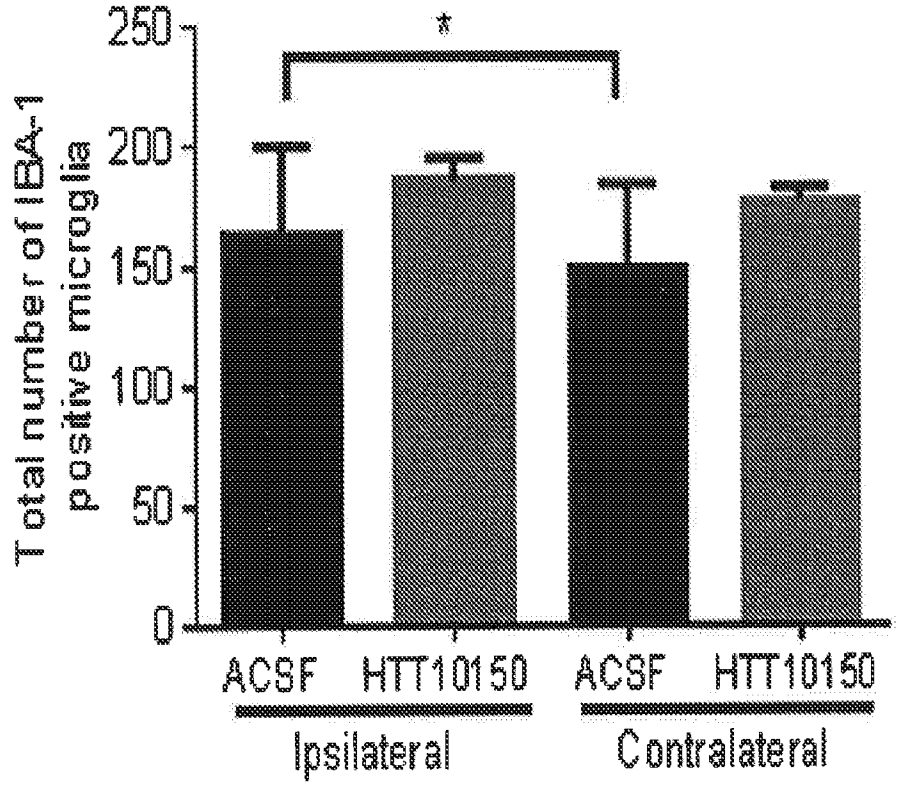


Fig. 20A

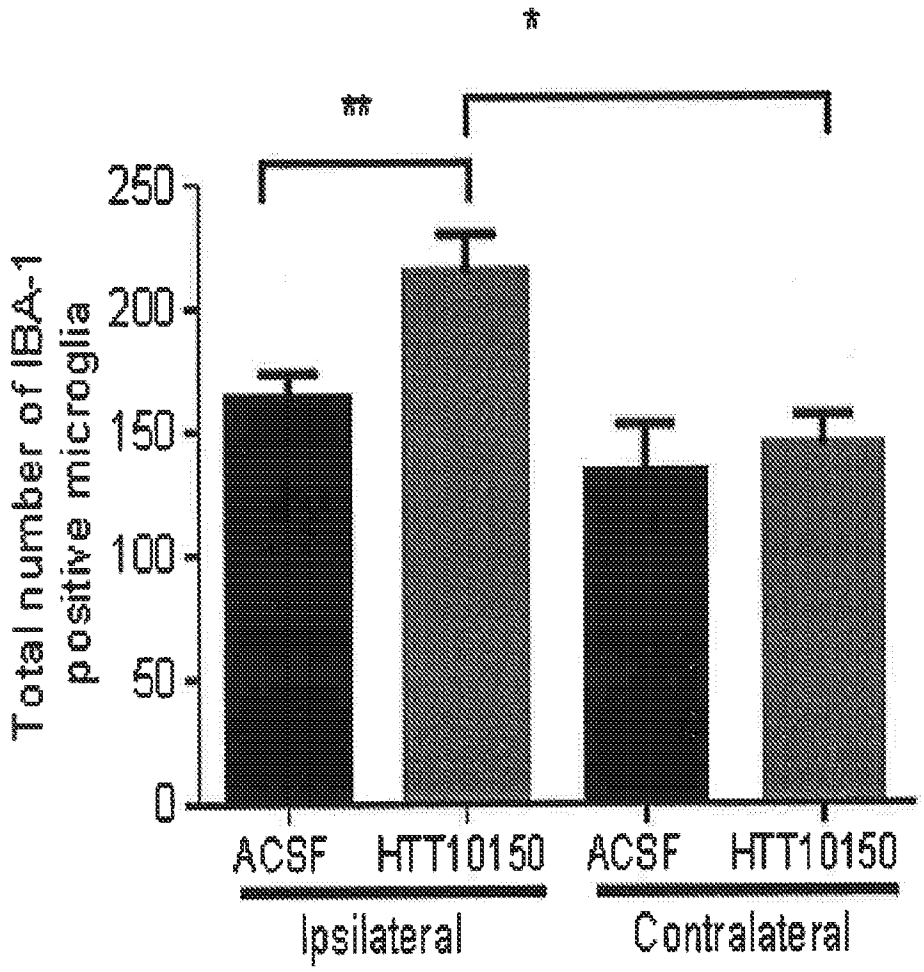


Fig. 20B

GCUGCCGGGA	Accession Number	Position	Targeting region (20 mer)	Targeting Region (30 mer)
HTT	NM_002111.6	1214	GUCCAGGUUUAUGAACUGAC	AGCUUGUCCAGGUUUAUGAACUGACGUUAC
HTT	NM_002111.6	1216	AGGUUUAUGAACUGACGUUA	UGUCCAGGUUUAUGAACUGACGUUACAUCA
HTT	NM_002111.6	1219	GGUUUAUGAACUGACGUUAC	GUCCAGGUUUAUGAACUGACGUUACAUCAU
HTT	NM_002111.6	1257	ACCACAAUGUUGUGACCGGA	CCAAGACCACAAUGUUGUGACCGGAGCCCU
HTT	NM_002111.6	1894	UGUGUUAGACGGUACCGACA	GAAAUUGUGUUAGACGGUACCGACAACCAG
HTT	NM_002111.6	1907	ACCGACAACCAGUAUUUGGG	ACGGUACCGACAACCAGUAUUUGGGCCUGC
HTT	NM_002111.6	2666	ACGAGUGCUCAAUAAUGUUG	CAAGAACGAGUGCUCAAUAAUGUUGUCAUC
HTT	NM_002111.6	4041	UGAAAUCCUGCUUUAGUCGA	AUACCUGAAAUCCUGCUUUAGUCGAGAACC
HTT	NM_002111.6	4049	UGCUUUAGUCGAGAACCAAU	AAUCCUGCUUUAGUCGAGAACCAAUGAUGG
HTT	NM_002111.6	5301	GGGACAGUACUUCAACGCUA	AGAUGGGGACAGUACUUCAACGCUAGAAGA
HTT	NM_002111.6	6016	GGCAAUUCAGUCUCGUUGUG	AUCCAGGCAAUUCAGUCUCGUUGUGAAAAC
HTT	NM_002111.6	6579	GCCUGCUAGCUCCAUGCUUA	CCUAAGCCUGCUAGCUCCAUGCUUAAGCCU
HTT	NM_002111.6	8603	GCCCACUGCGUGAACAUUCA	GGAUCGCCCACUGCGUGAACAUUCACAGCC
HTT	NM_002111.6	10125	UUCUUCUCAGGAUUUAAAAU	CUCUUUUCUUCUCAGGAUUUAAAAUUUAAU
HTT	NM_002111.6	10146	UAAUUAUAUCAGUAAAGAGA	AAAUUUAAUUAUAUCAGUAAAGAGAUUAAU
HTT	NM_002111.6	10150	UAUAUCAGUAAAGAGAUUAA	UUAAUUAUAUCAGUAAAGAGAUUAAUUUUA
HTT	NM_002111.6	424	ACUUUCAGCUACCAAGAAAG	AAAGAACUUUCAGCUACCAAGAAAGACCGU
HTT	NM_002111.6	456	AUUGUCUGACAAUAUGUGAA	GAAUCAUUGUCUGACAAUAUGUGAAAACAU
HTT	NM_002111.6	522	UUCUGGGCAUCGCUAUGGAA	
HTT	NM_002111.6	527	GGCAUCGCUAUGGAACUUUU	UUCUGGGCAUCGCUAUGGAACUUUUUCUGC
HTT	NM_002111.6	876	GCAAAUGACAAUGAAAUUAA	AUUUUGCAAAUGACAAUGAAAUUAAGGUUU
HTT	NM_002111.6	879	CAAAUGACAAUGAAAUUAAG	UUUUGCAAAUGACAAUGAAAUUAAGGUUUU
HTT	NM_002111.6	908	AAGGCCUUCAUAGCGAACCU	UGUUAAAGGCCUUCAUAGCGAACCUGAAGU
HTT	NM_002111.6	1024	ACUAAAUGUGCUCUUAGGCU	UGGCUACUAAAUGUGCUCUUAGGCUUACUC
HTT	NM_002111.6	1165	CGGAGUGACAAGGAAAGAAA	AGCUUCGGAGUGACAAGGAAAGAAAUGGAA
HTT	NM_002111.6	1207	GCAGCUUGUCCAGGUUUAUG	GCAGAGCAGCUUGUCCAGGUUUAUGAACUG
HTT	NM_002111.6	1212	UUGUCCAGGUUUAUGAACUG	GCAGCUUGUCCAGGUUUAUGAACUGACGUU
HTT	NM_002111.6	1217	CAGGUUUAUGAACUGACGUU	UUGUCCAGGUUUAUGAACUGACGUUACAUC
HTT	NM_002111.6	1220	GUUUAUGAACUGACGUUACA	UCCAGGUUUAUGAACUGACGUUACAUCAUA
HTT	NM_002111.6	1223	UAUGAACUGACGUUACAUCA	AGGUUUAUGAACUGACGUUACAUCAUACAC
HTT	NM_002111.6	1227	AACUGACGUUACAUCAUACA	UUAUGAACUGACGUUACAUCAUACACAGCA
HTT	NM_002111.6	1229	CUGACGUUACAUCAUACACA	AUGAACUGACGUUACAUCAUACACAGCACC
HTT	NM_002111.6	1260	ACAAUGUUGUGACCGGAGCC	AGACCACAAUGUUGUGACCGGAGCCCUGGA
HTT	NM_002111.6	1403	GGGAGUAUUGUGGAACUUAU	GUAGUGGGAGUAUUGUGGAACUUAUAGCUG
HTT	NM_002111.6	1470	AAGGCAAAGUGCUCUUAGGA	ACAAAAAGGCAAAGUGCUCUUAGGAGAAGA
HTT	NM_002111.6	1901	GACGGUACCGACAACCAGUA	UGUUAGACGGUACCGACAACCAGUAUUUGG
HTT	NM_002111.6	1903	CGGUACCGACAACCAGUAUU	UUAGACGGUACCGACAACCAGUAUUUGGGC
HTT	NM_002111.6	2411	UUGAACUACAUCGAUCAUGG	ACAUCUUGAACUACAUCGAUCAUGGAGACC
HTT	NM_002111.6	2412	UGAACUACAUCGAUCAUGGA	CAUCUUGAACUACAUCGAUCAUGGAGACCC
HTT	NM_002111.6	2865	AACGAGUGCUCAAUAAUGUU	GCAAGAACGAGUGCUCAAUAAUGUUGUCAU
HTT	NM_002111.6	3801	GUCCUGUUACAACAAGUAAA	CUCAGGUCCUGUUACAACAAGUAAAUCCUC
HTT	NM_002111.6	4040	CUGAAAUCCUGCUUUAGUCG	GAUACCUGAAAUCCUGCUUUAGUCGAGAAC
HTT	NM_002111.6	4048	CUGCUUUAGUCGAGAACCAA	AAAUCCUGCUUUAGUCGAGAACCAAUGAUG
HTT	NM_002111.6	4052	UUUAGUCGAGAACCAAUGAU	CCUGCUUUAGUCGAGAACCAAUGAUGGCAA
HTT	NM_002111.6	4055	AGUCGAGAACCAAUGAUGGC	GCUUUAGUCGAGAACCAAUGAUGGCAACUG
HTT	NM_002111.6	4083	GUGUUCAACAAUUGUUGAAG	UGUUUGUGUUCAACAAUUGUUGAAGACUCU
HTT	NM_002111.6	4275	UGAGGAACAUGGUGCAGGCG	CAGCCUGAGGAACAUGGUGCAGGCGGAGCA
HTT	NM_002111.6	4372	UGUCACAAAGAACCGUGCAG	ACGAGUGUCACAAAGAACCGUGCAGAUAAG
HTT	NM_002111.6	4374	UCACAAAGAACCGUGCAGAU	GAGUGUCACAAAGAACCGUGCAGAUAAGAA

Fig. 21

HTT	NM_002111.6	4376	ACAAAGAACCGUGCAGAUAA	GUGUCACAAAGAACCGUGCAGAUAAGAAUG
HTT	NM_002111.6	4425	UUGAACCUCUUGUUAUAAAA	UUUGUUUGAACCUCUUGUUAUAAAAGCUUU
HTT	NM_002111.6	4562	UUUAUUGGCUUUGUAUUGAA	AGGUGUUUAUUGGCUUUGUAUUGAAACAGU
HTT	NM_002111.6	4692	UCAUUGGAAUUCCUAAAAUC	ACAGAUCAUUGGAAUUCCUAAAUCAUUCA
HTT	NM_002111.6	4721	UGUGAUGGCAUCAUGGCCAG	AGCUCUGUGAUGGCAUCAUGGCCAGUGGAA
HTT	NM_002111.6	5200	GAUUUCCCAGUCAACUGAAG	GUUCUGAUUUCCCAGUCAACUGAAGAUAUU
HTT	NM_002111.6	5443	GAGUGAGCAGCAACAUACUU	GAAAUGAGUGAGCAGCAACAUACUUUCUAU
HTT	NM_002111.6	5515	GUCUGGAAUGUUCCGGAGAA	UUCAAGUCUGGAAUGUUCCGGAGAAUCACA
HTT	NM_002111.6	8609	UGCGUGAACAUUCACAGCCA	CCCACUGCGUGAACAUUCACAGCCAGCAGC
HTT	NM_002111.6	10130	CUCAGGAUUUAAAAUUUAAU	UUCUUCUCAGGAUUUAAAAUUUAAUUAUAU
HTT	NM_002111.6	10134	GGAUUUAAAAUUUAAUUAUA	UCUCAGGAUUUAAAAUUUAAUUAUAUCAGU
HTT	NM_002111.6	10142	AAUUUAAUUAUAUCAGUAAA	UUUAAAAUUUAAUUAUAUCAGUAAAGAGAU
HTT	NM_002111.6	10169	AUUUUAACGUAACUCUUUCU	GAUUAAUUUUAACGUAACUCUUUCUAUGCC
HTT	NM_002111.6	10182	UCUUUCUAUGCCCGUGUAAA	GUAACUCUUUCUAUGCCCGUGUAAAGUAUG
HTT	NM_002111.6	10186	UCUAUGCCCGUGUAAAGUAU	CUCUUUCUAUGCCCGUGUAAAGUAUGUGAA
HTT	NM_002111.6	10609	CUUUUAGUCAGGAGAGUGCA	GACCCCUUUUAGUCAGGAGAGUGCAGAUCU
HTT	NM_002111.6	11118	UGUUUUGGGUAUUGAAUGUG	GUCGAUGUUUUGGGUAUUGAAUGUGGUAAG
HTT	NM_002111.6	11129	GAAUGUGGUAAGUGGAGGAA	GUAUUGAAUGUGGUAAGUGGAGGAAAUGUU
HTT	NM_002111.6	11134	UGGUAAGUGGAGGAAAUGUU	GAAUGUGGUAAGUGGAGGAAAUGUUGGAAC
HTT	NM_002111.6	11147	AAAUGUUGGAACUCUGUGCA	GGAGGAAAUGUUGGAACUCUGUGCAGGUGC
HTT	NM_002111.6	11412	AUGUUUGAGGAGGCCCUUAA	GUCCGAUGUUUGAGGAGGCCCUUAAGGGAA
HTT	NM_002111.6	11426	CCUUAAGGGAAGCUACUGAA	GAGGCCCUUAAGGGAAGCUACUGAAUUAUA
HTT	NM_002111.6	11443	GAAUUAUAACACGUAAGAAA	CUACUGAAUUAUAACACGUAAGAAAAUCAC
HTT	NM_002111.6	11859	AUGUUUACAUUUGUAAGAAA	GCUAGAUGUUUUACAUUUGUAAGAAAUAACA
HTT	NM_002111.6	11866	CAUUUGUAAGAAAUAACACU	GUUUACAUUUGUAAGAAAUAACACUGUGAA
HTT	NM_002111.6	11877	AAUAACACUGUGAAUGUAAA	UAAGAAAUAACACUGUGAAUGUAAAACAGA
HTT	NM_002111.6	11883	AAUAUGAGCUCAUUAGUAAA	AGAUGAAUAUGAGCUCAUUAGUAAAAAUGA
HTT	NM_002111.6	11890	UCACCCACGCAUAUACAUAA	UGACUUCACCCACGCAUAUACAUAAAGUAU
HTT	NM_002111.6	11927	AUAUAGACACAUCUAUAAUU	UGUGCAUAUAGACACACUCUAUAAUUUUACA
HTT	NM_002111.6	11947	UUACACACACACCUCUCAAG	UAAUUUUACACACACCUCUCAAGACGGA
HTT	NM_002111.6	12163	GACUUUAUCAUGUUCCUAAA	AGGAAGACUUUAUCAUGUUCCUAAAAAUCU
HTT	NM_002111.6	12216	UUGUUGCAAAUGUGAUUAAU	AAAUUUUGUUGCAAAUGUGAUUAAUUUGGU
HTT	NM_002111.6	12223	GCAAAUGUGAUUAAUUUGGU	UUGUUGCAAAUGUGAUUAAUUUGGUUGUCA
HTT	NM_002111.6	12235	AAUUUGGUUGUCAAGUUUUG	UGAUUAAUUUGGUUGUCAAGUUUUCCGCGU
HTT	NM_002111.6	12279	UUUGUUUUCCUGCUGGUAAU	UUGCUUUUGUUUCCUGCUGGUAAUAUCGG
HTT	NM_002111.6	12282	GUUUUCCUGCUGGUAAUAUC	CUUUUGUUUUCCUGCUGGUAAUAUCGGGAA
HTT	NM_002111.6	12297	AUAUCGGGAAAGAUUUUAAU	UGGUAAUAUCGGGAAAGAUUUUAAUGAAAC
HTT	NM_002111.6	12309	AUUUUAAUGAAACCAGGGUA	GAAAGAUUUUAAUGAAACCAGGGUAGAAUU
HTT	NM_002111.6	12313	UAAUGAAACCAGGGUAGAAU	GAUUUUAAUGAAACCAGGGUAGAAUUGUUU
HTT	NM_002111.6	12331	AUUGUUUGGCAAUGCACUGA	GUAGAAUUGUUUGGCAAUGCACUGAAGCGU
HTT	NM_002111.6	13139	CCCCUCAGUUGUUUCUAAGA	GCCUUCCCCUCAGUUGUUUCUAAGAGCAGA
HTT	NM_002111.6	13398	GGACUGACGAGAGAUGUAUA	GGGAAGGACUGACGAGAGAUGUAUAUUUAA
HTT	NM_002111.6	13403	GACGAGAGAUGUAUAUUUAA	GGACUGACGAGAGAUGUAUAUUUAAUUUU
HTT	NM_002111.6	13423	UUUUUUAACUGCUGCAAACA	UUUAAUUUUUUAACUGCUGCAAACAUUGUA
HTT	NM_002111.6	13428	UAACUGCUGCAAACAUUGUA	UUUUUUAACUGCUGCAAACAUUGUACAUCC
HTT	NM_002111.6	152	ACCCUGGAAAAGCUGAUGAA	UGGCGACCCUGGAAAAGCUGAUGAAGGCCU
HTT	NM_002111.6	170	AAGGCCUUCGAGUCCCUCAA	UGAUGAAGGCCUUCGAGUCCCUCAAGUCCU
HTT	NM_002111.6	402	CGCUGCACGACGACCAAAGAAA	GGAGCCGCUGCACGACGACCAAAGAAAGAACU
HTT	NM_002111.6	420	AAGAACUUUCAGCUACCAAG	AAAGAAAGAACUUUCAGCUACCAAGAAAGA
HTT	NM_002111.6	430	AGCUACCAAGAAAGACCGUG	CUUUCAGCUACCAAGAAAGACCGUGUGAAU
HTT	NM_002111.6	446	CGUGUGAAAUCAUUGUCUGAC	AAGACCGUGUGAAAUCAUUGUCUGACAAUAU
HTT	NM_002111.6	454	UCAUUGUCUGACAAUAUGUG	GUGAAUCAUUGUCUGACAAUAUGUGAAAAC
HTT	NM_002111.6	462	UGACAAUAUGUGAAAACAUA	UUGUCUGACAAUAUGUGAAAACAUAGUGGC
HTT	NM_002111.6	467	AUAUGUGAAAACAUAGUGGC	UGACAAUAUGUGAAAACAUAGUGGCACAGU
HTT	NM_002111.6	211	GCAGCAGCAGCAGCAGCAGC	CAGCAGCAGCAGCAGCAGCAGCAGCAGCAG

Fig. 21 (Cont)

Sense Naked	Antisense Naked	Sense strand (P0)
GUCCAGGUUUAUGAACUGAC	GUCAGUUCAUAAACCUGGAC	PmU.fU.fC.A.G.fU.fU.fC.A.fU.A.A.mA.fC#fC#fU#G#G#mA#C
AGGUUUAUGAACUGACGUUA	UAACGUCAGUUCAUAAACCU	PmU.A.A.fC.G.fU.fC.A.G.fU.fU.fC.A.fU#A#A#mA#fC#fC#U
GGUUUAUGAACUGACGUUAC	GUAACGUCAGUUCAUAAACC	PmU.fU.A.A.fC.G.fU.fC.A.G.fU.fU.fC.A#fU#A#A#mA#fC#C
ACCACAAUGUUGUGACCGGA	UCCGGUCACAACAUUGUGGU	PmU.fC.fC.G.G.fU.fC.A.fC.A.A.fC.A.fU#fU#G#fU#G#G#U
UGUGUUAGACGGUACCGACA	UGUCGGUACCGUCUAACACA	PmU.G.fU.fC.G.G.fU.A.fC.fC.G.fU.fC.fU#A#A#fC#A#fC#A
ACCGACAACCAGUAUUUGGG	CCCAAAUACUGGUUGUCGGU	PmU.fC.fC.A.A.mA.fU.A.fC.fU.G.G.fU.fU#G#fU#fC#G#G#U
ACGAGUGCUCAAUAAUGUUG	CAACAUUAUUGAGCACUCGU	PmU.A.A.fC.A.fU.fU.A.fU.fU.G.A.mG.fC#A#fC#fU#fC#G#U
UGAAAUCCUGCUUUAGUCGA	UCGACUAAAGCAGGAUUUCA	PmU.fC.G.A.fC.fU.A.A.mA.G.fC.A.G.mG#A#fU#fU#fU#fC#A
UGCUUUAGUCGAGAACCAAU	AUUGGUUCUCGACUAAAGCA	PmU.fU.fU.G.G.fU.fU.fC.fU.fC.G.A.fC.fU#A#A#mA#G#fC#A
GGGACAGUACUUCAACGCUA	UAGCGUUGAAGUACUGUCCC	PmU.A.G.fC.G.fU.fU.G.A.mA.G.fU.A.fC#fU#G#fU#fC#C#C
GGCAAUUCAGUCUCGUUGUG	CACAACGAGACUGAAUUGCC	PmU.A.fC.A.A.fC.G.A.mG.A.fC.fU.G.A#mA#fU#fU#G#fC#C
GCCUGCUAGCUCCAUGCUUA	UAAGCAUGGAGCUAGCAGGC	PmU.A.A.mG.fC.A.fU.G.G.mA.G.fC.fU.A#G#fC#A#G#mG#C
GCCCACUGCGUGAACAUUCA	UGAAUGUUCACGCAGUGGGC	PmU.G.A.mA.fU.G.fU.fU.fC.A.fC.G.fC.A#G#fU#G#G#mG#C
UUCUUCUCAGGAUUUAAAAU	AUUUUAAAUCCUGAGAAGAA	PmU.fU.fU.fU.fU.A.A.mA.fU.fC.fC.fU.G.A#mG#A#A#mG#A#A
UAAUUAUAUCAGUAAAGAGA	UCUCUUUACUGAUAUAAUUA	PmU.fC.fU.fC.fU.fU.fU.A.fC.fU.G.A.fU.A#fU#A#A#fU#fU#A
UAUAUCAGUAAAGAGAUUAA	UUAAUCUCUUUACUGAUAUA	PmU.fU.A.A.fU.fC.fU.fC.fU.fU.fU.A.fC.fU#G#A#fU#A#fU#A
ACUUUCAGCUACCAAGAAAG	CUUUCUUGGUAGCUGAAAGU	PmU.fU.fU.fU.fC.fU.fU.G.G.fU.A.G.fC.fU#G#A#mA#A#G#U
AUUGUCUGACAAUAUGUGAA	UUCACAUAUUGUCAGACAAU	PmU.fU.fC.A.fC.A.fU.A.fU.fU.G.fU.fC.A#G#mA#fC#A#A#U
UUCUGGGCAUCGCUAUGGAA	UUCCAUAGCGAUGCCCAGAA	PmU.fU.fC.fC.A.fU.A.G.fC.G.A.fU.G.fC#fC#fC#A#G#mA#A
GGCAUCGCUAUGGAACUUUU	AAAAGUUCCAUAGCGAUGCC	PmU.A.A.mA.G.fU.fU.fC.fC.A.fU.A.G.fC#G#A#fU#G#fC#C
GCAAAUGACAAUGAAAUUAA	UUAAUUUCAUUGUCAUUUGC	PmU.fU.A.A.fU.fU.fU.fC.A.fU.fU.G.fU.fC#A#fU#fU#fU#G#C
CAAAUGACAAUGAAAUUAAG	CUUAAUUUCAUUGUCAUUUG	PmU.fU.fU.A.A.fU.fU.fU.fC.A.fU.fU.G.fU#fC#A#fU#fU#fU#G
AAGGCCUUCAUAGCGAACCU	AGGUUCGCUAUGAAGGCCUU	PmU.G.G.fU.fU.fC.G.fC.fU.A.fU.G.A.mA#G#G#fC#fC#fU#U
ACUAAAUGUGCUCUUAGGCU	AGCCUAAGAGCACAUUUAGU	PmU.G.fC.fC.fU.A.A.mG.A.G.fC.A.fC.A#fU#fU#fU#A#G#U
CGGAGUGACAAGGAAAGAAA	UUUCUUUCCUUGUCACUCCG	PmU.fU.fU.fC.fU.fU.fU.fC.fC.fU.fU.G.fU.fC#A#fC#fU#fC#fC#G
GCAGCUUGUCCAGGUUUAUG	CAUAAACCUGGACAAGCUGC	PmU.A.fU.A.A.mA.fC.fC.fU.G.G.mA.fC.A#A#mG#fC#fU#G#C
UUGUCCAGGUUUAUGAACUG	CAGUUCAUAAACCUGGACAA	PmU.A.G.fU.fU.fC.A.fU.A.A.mA.fC.fC.fU#G#G#mA#fC#A#A
CAGGUUUAUGAACUGACGUU	AACGUCAGUUCAUAAACCUG	PmU.A.fC.G.fU.fC.A.G.fU.fU.fC.A.fU.A#A#mA#fC#fC#fU#G
GUUUAUGAACUGACGUUACA	UGUAACGUCAGUUCAUAAAC	PmU.G.fU.A.A.fC.G.fU.fC.A.G.fU.fU.fC#A#fU#A#A#mA#C
UAUGAACUGACGUUACAUCA	UGAUGUAACGUCAGUUCAUA	PmU.G.A.fU.G.fU.A.A.fC.G.fU.fC.A.G#fU#fU#fC#A#fU#A
AACUGACGUUACAUCAUACA	UGUAUGAUGUAACGUCAGUU	PmU.G.fU.A.fU.G.A.fU.G.fU.A.A.fC.G#fU#fC#A#G#fU#U
CUGACGUUACAUCAUACACA	UGUGUAUGAUGUAACGUCAG	PmU.G.fU.G.fU.A.fU.G.A.fU.G.fU.A.A#fC#G#fU#fC#A#G
ACAAUGUUGUGACCGGAGCC	GGCUCCGGUCACAACAUUGU	PmU.G.fC.fU.fC.fC.G.G.fU.fC.A.fC.A.A#fC#A#fU#fU#G#U
GGGAGUAUUGUGGAACUUAU	AUAAGUUCCACAAUACUCCC	PmU.fU.A.A.mG.fU.fU.fC.fC.A.fC.A.A.fU#A#fC#fU#fC#fC#C
AAGGCAAAGUGCUCUUAGGA	UCCUAAGAGCACUUUGCCUU	PmU.fC.fC.fU.A.A.mG.A.G.fC.A.fC.fU.fU#fU#G#fC#fC#fU#U
GACGGUACCGACAACCAGUA	UACUGGUUGUCGGUACCGUC	PmU.A.fC.fU.G.G.fU.fU.G.fU.fC.G.G.fU#A#fC#fC#G#fU#C
CGGUACCGACAACCAGUAUU	AAUACUGGUUGUCGGUACCG	PmU.A.fU.A.fC.fU.G.G.fU.fU.G.fU.fC.G#G#fU#A#fC#fC#G
UUGAACUACAUCGAUCAUGG	CCAUGAUCGAUGUAGUUCAA	PmU.fC.A.fU.G.A.fU.fC.G.A.fU.G.fU.A#G#fU#fU#fC#A#A
UGAACUACAUCGAUCAUGGA	UCCAUGAUCGAUGUAGUUCA	PmU.fC.fC.A.fU.G.A.fU.fC.G.A.fU.G.fU#A#G#fU#fU#fC#A
AACGAGUGCUCAAUAAUGUU	AACAUUAUUGAGCACUCGUU	PmU.A.fC.A.fU.fU.A.fU.fU.G.A.mG.fC.A#fC#fU#fC#G#fU#U
GUCCUGUUACAACAAGUAAA	UUUACUUGUUGUAACAGGAC	PmU.fU.fU.A.fC.fU.fU.G.fU.fU.G.fU.A.A#fC#A#G#mG#A#C
CUGAAAUCCUGCUUUAGUCG	CGACUAAAGCAGGAUUUCAG	PmU.G.A.fC.fU.A.A.mA.G.fC.A.G.mG.A#fU#fU#fU#fC#A#G
CUGCUUUAGUCGAGAACCAA	UUGGUUCUCGACUAAAGCAG	PmU.fU.G.G.fU.fU.fC.fU.fC.G.A.fC.fU.A#A#mA#G#fC#A#G
UUUAGUCGAGAACCAAUGAU	AUCAUUGGUUCUCGACUAAA	PmU.fU.fC.A.fU.fU.G.G.fU.fU.fC.fU.fC.G#A#fC#fU#A#A#A
AGUCGAGAACCAAUGAUGGC	GCCAUCAUUGGUUCUCGACU	PmU.fC.fC.A.fU.fC.A.fU.fU.G.G.fU.fU.fC#fU#fC#G#A#fC#U
GUGUUCAACAAUUGUUGAAG	CUUCAACAAUUGUUGAACAC	PmU.fU.fU.fC.A.A.fC.A.A.fU.fU.G.fU.fU#G#A#mA#fC#A#C
UGAGGAACAUGGUGCAGGCG	CGCCUGCACCAUGUUCCUCA	PmU.G.fC.fC.fU.G.fC.A.fC.fC.A.fU.G.fU#fU#fC#fC#fU#fC#A
UGUCACAAAGAACCGUGCAG	CUGCACGGUUCUUUGUGACA	PmU.fU.G.fC.A.fC.G.G.fU.fU.fC.fU.fU.fU#G#fU#G#A#fC#A
UCACAAAGAACCGUGCAGAU	AUCUGCACGGUUCUUUGUGA	PmU.fU.fC.fU.G.fC.A.fC.G.G.fU.fU.fC.fU#fU#fU#G#fU#G#A

Continued on sheet 35/136

Continued on sheet 37/136

Fig. 21 (Cont)

Continued from sheet 34/136

Antisense Strand (P0)	Sense strand (P1)
PmU.fU.fC.A.G.fU.fU.fC.A.fU.A.A.mA.fC#fC#fU#G#G#mA#C	fGmUfCmCfAmGfGmUfUmUfAmUfG#mA#fA-linkerX
PmU.A.A.fC.G.fU.fC.A.G.fU.fU.fC.A.fU#A#A#mA#fC#fC#U	fAmGfGmUfUmUfAmUfGmAfAmCfU#mG#fA-linkerX
PmU.fU.A.A.fC.G.fU.fC.A.G.fU.fU.fC.A#fU#A#A#mA#fC#C	fGmGfUmUfUmAfUmGfAmAfCmUfG#mA#fA-linkerX
PmU.fC.fC.G.G.fU.fC.A.fC.A.A.fC.A.fU#fU#G#fU#G#G#U	fAmCfCmAfCmAfAmUfGmUfUmGfU#mG#fA-linkerX
PmU.G.fU.fC.G.G.fU.A.fC.fC.G.fU.fC.fU#A#A#fC#A#fC#A	fUmGfUmGfUmUfAmGfAmCfGmGfU#mA#fA-linkerX
PmU.fC.fC.A.A.mA.fU.A.fC.fU.G.G.fU.fU#G#fU#fC#G#G#U	fAmCfCmGfAmCfAmAfCmCfAmGfU#mA#fA-linkerX
PmU.A.A.fC.A.fU.fU.A.fU.fU.G.A.mG.fC#A#fC#fU#fC#G#U	fAmCfGmAfGmUfGmCfUmCfAmAfU#mA#fA-linkerX
PmU.fC.G.A.fC.fU.A.A.mA.G.fC.A.G.mG#A#fU#fU#fU#fC#A	fUmGfAmAfAmUfCmCfUmGfCmUfU#mU#fA-linkerX
PmU.fU.fU.G.G.fU.fU.fC.fU.fC.G.A.fC.fU#A#A#mA#G#fC#A	fUmGfCmUfUmUfAmGfUmCfGmAfG#mA#fA-linkerX
PmU.A.G.fC.G.fU.fU.G.A.mA.G.fU.A.fC#fU#G#fU#fC#fC#C	fGmGfGmAfCmAfGmUfAmCfUmUfC#mA#fA-linkerX
PmU.A.fC.A.A.fC.G.A.mG.A.fC.fU.G.A#mA#fU#fU#G#fC#C	fGmGfCmAfAmUfUmCfAmGfUmCfU#mC#fA-linkerX
PmU.A.A.mG.fC.A.fU.G.G.mA.G.fC.fU.A#G#fC#A#G#mG#C	fGmCfCmUfGmCfUmAfGmCfUmCfC#mA#fA-linkerX
PmU.G.A.mA.fU.G.fU.fU.fC.A.fC.G.fC.A#G#fU#G#G#mG#C	fGmCfCmCfAmCfUmGfCmGfUmGfA#mA#fA-linkerX
PmU.fU.fU.fU.fU.A.A.mA.fU.fC.fC.fU.G.A#mG#A#A#mG#A#A	fUmUfCmUfUmCfUmCfAmGfGmAfU#mU#fA-linkerX
PmU.fC.fU.fC.fU.fU.fU.A.fC.fU.G.A.fU.A#fU#A#A#fU#fU#A	fUmAfAmUfUmAfUmAfUmCfAmGfU#mA#fA-linkerX
PmU.fU.A.A.fU.fC.fU.fC.fU.fU.fU.A.fC.fU#G#A#fU#A#fU#A	fUmAfUmAfUmCfAmGfUmAfAmAfG#mA#fA-linkerX
PmU.fU.fU.fU.fC.fU.fU.G.G.fU.A.G.fC.fU#G#A#mA#A#G#U	fAmCfUmUfUmCfAmGfCmUfAmCfC#mA#fA-linkerX
PmU.fU.fC.A.fC.A.fU.A.fU.fU.G.fU.fC.A#G#mA#fC#A#A#U	fAmUfUmGfUmCfUmGfAmCfAmAfU#mA#fA-linkerX
PmU.fU.fC.fC.A.fU.A.G.fC.G.A.fU.G.fC#fC#fC#A#G#mA#A	fUmUfCmUfGmGfGmCfAmUfCmGfC#mU#fA-linkerX
PmU.A.A.mA.G.fU.fU.fC.fC.A.fU.A.G.fC#G#A#fU#G#fC#C	fGmGfCmAfUmCfGmCfUmAfUmGfG#mA#fA-linkerX
PmU.fU.A.A.fU.fU.fU.fC.A.fU.fU.G.fU.fC#A#fU#fU#fU#G#C	fGmCfAmAfAmUfGmAfCmAfAmUfG#mA#fA-linkerX
PmU.fU.fU.A.A.fU.fU.fU.fC.A.fU.fU.G.fU#fC#A#fU#fU#fU#G	fCmAfAmAfUmGfAmCfAmAfUmGfA#mA#fA-linkerX
PmU.G.G.fU.fU.fC.G.fC.fU.A.fU.G.A.mA#G#G#fC#fC#fU#U	fAmAfGmGfCmCfUmUfCmAfUmAfG#mC#fA-linkerX
PmU.G.fC.fC.fU.A.A.mG.A.G.fC.A.fC.A#fU#fU#fU#A#G#U	fAmCfUmAfAmAfUmGfUmGfCmUfC#mU#fA-linkerX
PmU.fU.fU.fC.fU.fU.fU.fC.fC.fU.fU.G.fU.fC#A#fC#fU#fC#fC#G	fCmGfGmAfGmUfGmAfCmAfAmGfG#mA#fA-linkerX
PmU.A.fU.A.A.mA.fC.fC.fU.G.G.mA.fC.A#A#mG#fC#fU#G#C	fGmCfAmGfCmUfUmGfUmCfCmAfG#mG#fA-linkerX
PmU.A.G.fU.fU.fC.A.fU.A.A.mA.fC.fC.fU#G#G#mA#fC#A#A	fUmUfGmUfCmCfAmGfGmUfUmUfA#mU#fA-linkerX
PmU.A.fC.G.fU.fC.A.G.fU.fU.fC.A.fU.A#A#mA#fC#fC#fU#G	fCmAfGmGfUmUfUmAfUmGfAmAfC#mU#fA-linkerX
PmU.G.fU.A.A.fC.G.fU.fC.A.G.fU.fU.fC#A#fU#A#A#mA#C	fGmUfUmUfAmUfGmAfAmCfUmGfA#mC#fA-linkerX
PmU.G.A.fU.G.fU.A.A.fC.G.fU.fC.A.G#fU#fU#fC#A#fU#A	fUmAfUmGfAmAfCmUfGmAfCmGfU#mU#fA-linkerX
PmU.G.fU.A.fU.G.A.fU.G.fU.A.A.fC.G#fU#fC#A#G#fU#U	fAmAfCmUfGmAfCmGfUmUfAmCfA#mU#fA-linkerX
PmU.G.fU.G.fU.A.fU.G.A.fU.G.fU.A.A#fC#G#fU#fC#A#G	fCmUfGmAfCmGfUmUfAmCfAmUfC#mA#fA-linkerX
PmU.G.fC.fU.fC.fC.G.G.fU.fC.A.fC.A.A#fC#A#fU#fU#G#U	fAmCfAmAfUmGfUmUfGmUfGmAfC#mC#fA-linkerX
PmU.fU.A.A.mG.fU.fU.fC.fC.A.fC.A.A.fU#A#fC#fU#fC#fC#C	fGmGfGmAfGmUfAmUfUmGfUmGfG#mA#fA-linkerX
PmU.fC.fC.fU.A.A.mG.A.G.fC.A.fC.fU.fU#fU#G#fC#fC#fU#U	fAmAfGmGfCmAfAmAfGmUfGmCfU#mC#fA-linkerX
PmU.A.fC.fU.G.G.fU.fU.G.fU.fC.G.G.fU#A#fC#fC#G#fU#C	fGmAfCmGfGmUfAmCfCmGfAmCfA#mA#fA-linkerX
PmU.A.fU.A.fC.fU.G.G.fU.fU.G.fU.fC.G#G#fU#A#fC#fC#G	fCmGfGmUfAmCfCmGfAmCfAmAfC#mC#fA-linkerX
PmU.fC.A.fU.G.A.fU.fC.G.A.fU.G.fU.A#G#fU#fU#fU#fC#A#A	fUmUfGmAfAmCfUmAfCmAfUmCfG#mA#fA-linkerX
PmU.fC.fC.A.fU.G.A.fU.fC.G.A.fU.G.fU#A#G#fU#fU#fC#A	fUmGfAmAfCmUfAmCfAmUfCmGfA#mU#fA-linkerX
PmU.A.fC.A.fU.fU.A.fU.fU.G.A.mG.fC.A#fC#fU#fC#G#fU#U	fAmAfCmGfAmGfUmGfCmUfCmAfA#mU#fA-linkerX
PmU.fU.fU.A.fC.fU.fU.G.fU.fU.G.fU.A.A#fC#A#G#mG#A#C	fGmUfCmCfUmGfUmUfAmCfAmAfC#mA#fA-linkerX
PmU.G.A.fC.fU.A.A.mA.G.fC.A.G.mG.A#fU#fU#fU#fC#A#G	fCmUfGmAfAmAfUmCfCmUfGmCfU#mU#fA-linkerX
PmU.fU.G.G.fU.fU.fC.fU.fC.G.A.fC.fU.A#A#mA#G#fC#A#G	fCmUfGmCfUmUfUmAfGmUfCmGfA#mG#fA-linkerX
PmU.fU.fC.A.fU.fU.G.G.fU.fU.fC.fU.fC.G#A#fC#fU#A#A#A	fUmUfUmAfGmUfCmGfAmGfAmAfC#mC#fA-linkerX
PmU.fC.fC.A.fU.fC.A.fU.fU.G.G.fU.fU.fC#fU#fC#G#A#fC#U	fAmGfUmCfGmAfGmAfAmCfCmAfA#mU#fA-linkerX
PmU.fU.fU.fC.A.A.fC.A.A.fU.fU.G.fU.fU#G#A#mA#fC#A#C	fGmUfGmUfUmCfAmAfCmAfAmUfU#mG#fA-linkerX
PmU.G.fC.fC.fU.G.fC.A.fC.fC.A.fU.G.fU#fU#fC#fC#fU#fC#A	fUmGfAmGfGmAfAmCfAmUfGmGfU#mG#fA-linkerX
PmU.fU.G.fC.A.fC.G.G.fU.fU.fC.fU.fU.fU#G#fU#G#A#fC#A	fUmGfUmCfAmCfAmAfAmGfAmAfC#mC#fA-linkerX
PmU.fU.fC.fU.G.fC.A.fC.G.G.fU.fU.fC.fU#fU#fU#G#fU#G#A	fUmCfAmCfAmAfAmGfAmAfCmCfG#mU#fA-linkerX

Continued on sheet 36/136

Continued on sheet 38/136

Fig. 21 (Cont)

Continued from sheet 35/136

Antisense Strand (P1)
P'-mUfUmCfAmGfUmUfCmAfUmAfAmAfC#mC#fU#mG#fG#mA#fC
P'-mUfAmAfCmGfUmCfAmGfUmUfCmAfU#mA#fA#mA#fC#mC#fU
P'-mUfUmAfAmCfGmUfCmAfGmUfUmCfA#mU#fA#mA#fA#mC#fC
P'-mUfCmCfGmGfUmCfAmCfAmAfCmAfU#mU#fG#mU#fG#mG#fU
P'-mUfGmUfCmGfGmUfAmCfCmGfUmCfU#mA#fA#mC#fA#mC#fA
P'-mUfCmCfAmAfAmUfAmCfUmGfGmUfU#mG#fU#mC#fG#mG#fU
P'-mUfAmAfCmAfUmUfAmUfUmGfAmGfC#mA#fC#mU#fC#mG#fU
P'-mUfCmGfAmCfUmAfAmAfGmCfAmGfG#mA#fU#mU#fU#mC#fA
P'-mUfUmUfGmGfUmUfCmUfCmGfAmCfU#mA#fA#mA#fG#mC#fA
P'-mUfAmGfCmGfUmUfGmAfAmGfUmAfC#mU#fG#mU#fC#mC#fC
P'-mUfAmCfAmAfCmGfAmGfAmCfUmGfA#mA#fU#mU#fG#mC#fC
P'-mUfAmAfGmCfAmUfGmGfAmGfCmUfA#mG#fC#mA#fG#mG#fC
P'-mUfGmAfAmUfGmUfUmCfAmCfGmCfA#mG#fU#mG#fG#mG#fC
P'-mUfUmUfUmUfAmAfAmUfCmCfUmGfA#mG#fA#mA#fG#mA#fA
P'-mUfCmUfCmUfUmUfAmCfUmGfAmUfA#mU#fA#mA#fU#mU#fA
P'-mUfUmAfAmUfCmUfCmUfUmUfAmCfU#mG#fA#mU#fA#mU#fA
P'-mUfUmUfUmCfUmUfGmGfUmAfGmCfU#mG#fA#mA#fA#mG#fU
P'-mUfUmCfAmCfAmUfAmUfUmGfUmCfA#mG#fA#mC#fA#mA#fU
P'-mUfUmCfCmAfUmAfGmCfGmAfUmGfC#mC#fC#mA#fG#mA#fA
P'-mUfAmAfAmGfUmUfCmCfAmUfAmGfC#mG#fA#mU#fG#mC#fC
P'-mUfUmAfAmUfUmUfCmAfUmUfGmUfC#mA#fU#mU#fU#mG#fC
P'-mUfUmUfAmAfUmUfUmCfAmUfUmGfU#mC#fA#mU#fU#mU#fG
P'-mUfGmGfUmUfCmGfCmUfAmUfGmAfA#mG#fG#mC#fC#mU#fU
P'-mUfGmCfCmUfAmAfGmAfGmCfAmCfA#mU#fU#mU#fA#mG#fU
P'-mUfUmUfCmUfUmUfCmCfUmUfGmUfC#mA#fC#mU#fC#mC#fG
P'-mUfAmUfAmAfAmCfCmUfGmGfAmCfA#mA#fG#mC#fU#mG#fC
P'-mUfAmGfUmUfCmAfUmAfAmAfCmCfU#mG#fG#mA#fC#mA#fA
P'-mUfAmCfGmUfCmAfGmUfUmCfAmUfA#mA#fA#mC#fC#mU#fG
P'-mUfGmUfAmAfCmGfUmCfAmGfUmUfC#mA#fU#mA#fA#mA#fC
P'-mUfGmAfUmGfUmAfAmCfGmUfCmAfG#mU#fU#mC#fA#mU#fA
P'-mUfGmUfAmUfGmAfUmGfUmAfAmCfG#mU#fC#mA#fG#mU#fU
P'-mUfGmUfGmUfAmUfGmAfUmGfUmAfA#mC#fG#mU#fC#mA#fG
P'-mUfGmCfUmCfCmGfGmUfCmAfCmAfA#mC#fA#mU#fU#mG#fU
P'-mUfUmAfAmGfUmUfCmCfAmCfAmAfU#mA#fC#mU#fC#mC#fC
P'-mUfCmCfUmAfAmGfAmGfCmAfCmUfU#mU#fG#mC#fC#mU#fU
P'-mUfAmCfUmGfGmUfUmGfUmCfGmGfU#mA#fC#mC#fG#mU#fC
P'-mUfAmUfAmCfUmGfGmUfUmGfUmCfG#mG#fU#mA#fC#mC#fG
P'-mUfCmAfUmGfAmUfCmGfAmUfGmUfA#mG#fU#mU#fC#mA#fA
P'-mUfCmCfAmUfGmAfUmCfGmAfUmGfU#mA#fG#mU#fU#mC#fA
P'-mUfAmCfAmUfUmAfUmUfGmAfGmCfA#mC#fU#mC#fG#mU#fU
P'-mUfUmUfAmCfUmUfGmUfUmGfUmAfA#mC#fA#mG#fG#mA#fC
P'-mUfGmAfCmUfAmAfAmGfCmAfGmGfA#mU#fU#mU#fC#mA#fG
P'-mUfUmGfGmUfUmCfUmCfGmAfCmUfA#mA#fA#mG#fC#mA#fG
P'-mUfUmCfAmUfUmGfGmUfUmCfUmCfG#mA#fC#mU#fA#mA#fA
P'-mUfCmCfAmUfCmAfUmUfGmGfUmUfC#mU#fC#mG#fA#mC#fU
P'-mUfUmUfCmAfAmCfAmAfUmUfGmUfU#mG#fA#mA#fC#mA#fC
P'-mUfGmCfCmUfGmCfAmCfCmAfUmGfU#mU#fC#mC#fU#mC#fA
P'-mUfUmGfCmAfCmGfGmUfUmCfUmUfU#mG#fU#mG#fA#mC#fA
P'-mUfUmCfUmGfCmAfCmGfGmUfUmCfU#mU#fU#mG#fU#mG#fA

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Fig. 21 (Cont)

Continued from sheet 34/136

Sense Naked	Antisense Naked	Sense strand (P0)
ACAAAGAACCGUGCAGAUAA	UUAUCUGCACGGUUCUUUGU	mG.mA.A.mC.mC.G.mU.G.mC.A.G.A.mU#mA#mA.tegChol
UUGAACCUCUUGUUAUAAAA	UUUUAUAACAAGAGGUUCAA	mC.mC.mU.mC.mU.mU.G.mU.mU.A.mU.A.A#mA#mA.tegChol
UUUAUUGGCUUUGUAUUGAA	UUCAAUACAAAGCCAAUAAA	mU.mG.G.mC.mU.mU.mU.G.mU.A.mU.mU.G#mA#mA.tegChol
UCAUUGGAAUUCCUAAAAUC	GAUUUUAGGAAUUCCAAUGA	mG.mG.A.A.mU.mU.mC.mC.mU.A.A.A.A#mU#mA.tegChol
UGUGAUGGCAUCAUGGCCAG	CUGGCCAUGAUGCCAUCACA	mU.mG.G.mC.A.mU.mC.A.mU.G.G.mC.mC#mA#mA.tegChol
GAUUUCCCAGUCAACUGAAG	CUUCAGUUGACUGGGAAAUC	mC.mC.mC.A.G.mU.mC.A.A.mC.mU.G.A#mA#mA.tegChol
GAGUGAGCAGCAACAUACUU	AAGUAUGUUGCUGCUCACUC	mA.mG.mC.A.G.mC.A.A.mC.A.mU.A.mC#mU#mA.tegChol
GUCUGGAAUGUUCCGGAGAA	UUCUCCGGAACAUUCCAGAC	mG.mA.A.mU.G.mU.mU.mC.mC.G.G.A.G#mA#mA.tegChol
UGCGUGAACAUUCACAGCCA	UGGCUGUGAAUGUUCACGCA	mG.mA.A.mC.A.mU.mU.mC.A.mC.A.G.mC#mC#mA.tegChol
CUCAGGAUUUAAAAUUUAAU	AUUAAAUUUUAAAUCCUGAG	mG.mA.mU.mU.mU.A.A.A.A.mU.mU.mU.A#mA#mA.tegChol
GGAUUUAAAAUUUAAUUAUA	UAUAAUUAAAUUUUAAAUCC	mU.mA.A.A.A.mU.mU.mU.A.A.mU.mU.A#mU#mA.tegChol
AAUUUAAUUAUAUCAGUAAA	UUUACUGAUAUAAUUAAAUU	mA.mA.mU.mU.A.mU.A.mU.mC.A.G.mU.A#mA#mA.tegChol
AUUUUAACGUAACUCUUUCU	AGAAAGAGUUACGUUAAAAU	mA.mA.mC.G.mU.A.A.mC.mU.mC.mU.mU.mU.mU#mC#mA.tegChol
UCUUUCUAUGCCCGUGUAAA	UUUACACGGGCAUAGAAAGA	mC.mU.A.mU.G.mC.mC.mC.G.mU.G.mU.A#mA#mA.tegChol
UCUAUGCCCGUGUAAAGUAU	AUACUUUACACGGGCAUAGA	mG.mC.mC.mC.G.mU.G.mU.A.A.A.G.mU#mA#mA.tegChol
CUUUUAGUCAGGAGAGUGCA	UGCACUCUCCUGACUAAAAG	mA.mG.mU.mC.A.G.G.A.G.A.G.mU.G#mC#mA.tegChol
UGUUUUGGGUAUUGAAUGUG	CACAUUCAAUACCCAAAACA	mU.mG.G.G.mU.A.mU.mU.G.A.A.mU.G#mU#mA.tegChol
GAAUGUGGUAAGUGGAGGAA	UUCCUCCACUUACCACAUUC	mU.mG.G.mU.A.A.G.mU.G.G.A.G.G#mA#mA.tegChol
UGGUAAGUGGAGGAAAUGUU	AACAUUUCCUCCACUUACCA	mA.mG.mU.G.G.A.G.G.A.A.A.mU.G#mU#mA.tegChol
AAAUGUUGGAACUCUGUGCA	UGCACAGAGUUCCAACAUUU	mU.mU.G.G.A.A.mC.mU.mC.mU.G.mU.G#mC#mA.tegChol
AUGUUUGAGGAGGCCCUUAA	UUAAGGGCCUCCUCAAACAU	mU.mG.A.G.G.mA.G.G.mC.mC.mC.mU.mU#mA#mA.tegChol
CCUUAAGGGAAGCUACUGAA	UUCAGUAGCUUCCCUUAAGG	mA.mG.G.G.A.A.G.mC.mU.A.mC.mU.G#mA#mA.tegChol
GAAUUAUAACACGUAAGAAA	UUUCUUACGUGUUAUAAUUC	mA.mU.A.A.mC.A.mC.G.mU.A.A.G.A#mA#mA.tegChol
AUGUUUACAUUUGUAAGAAA	UUUCUUACAAAUGUAAACAU	mU.mA.mC.A.mU.mU.mU.G.mU.A.A.G.A#mA#mA.tegChol
CAUUUGUAAGAAAUAACACU	AGUGUUAUUUCUUACAAAUG	mG.mU.A.A.G.mA.A.A.mU.A.A.mC.A#mC#mA.tegChol
AAUAACAGUGUGAAUGUAAA	UUUACAUUCACAGUGUUAUU	mC.mA.mC.mU.G.mU.G.A.A.mU.G.mU.A#mA#mA.tegChol
AAUAUGAGCUCAUUAGUAAA	UUUACUAAUGAGCUCAUAUU	mG.mA.G.mC.mU.mC.A.mU.mU.A.G.mU.A#mA#mA.tegChol
UCACCCACGCAUAUACAUAA	UUAUGUAUAUGCGUGGGUGA	mC.mA.mC.G.mC.A.mU.A.mU.A.mC.A.mU#mA#mA.tegChol
AUAUAGACACAUCUAUAAUU	AAUUAUAGAUGUGUCUAUAU	mG.mA.mC.A.mC.A.mU.mC.mU.A.mU.A.A#mU#mA.tegChol
UUACACACACACCUCUCAAG	CUUGAGAGGUGUGUGUGUAA	mC.mA.mC.A.mC.A.mC.mC.mU.mC.mU.mC.A#mA#mA.tegChol
GACUUUAUCAUGUUCCUAAA	UUUAGGAACAUGAUAAAGUC	mU.mA.mU.mC.A.mU.G.mU.mU.mC.mC.mU.A#mA#mA.tegChol
UUGUUGCAAAUGUGAUUAAU	AUUAAUCACAUUUGCAACAA	mG.mC.A.A.A.mU.G.mU.G.A.mU.mU.A#mA#mA.tegChol
GCAAAUGUGAUUAAUUUGGU	ACCAAAUUAAUCACAUUUGC	mU.mG.mU.G.A.mU.mU.A.A.mU.mU.mU.G#mG#mA.tegChol
AAUUUGGUUGUCAAGUUUUG	CAAAACUUGACAACCAAAUU	mG.mG.mU.mU.G.mU.mC.A.A.G.mU.mU.mU#mU#mA.tegChol
UUUGUUUUCCUGCUGGUAAU	AUUACCAGCAGGAAAACAAA	mU.mU.mU.mC.mC.mU.G.mC.mU.G.G.mU.A#mA#mA.tegChol
GUUUUCCUGCUGGUAAUAUC	GAUAUUACCAGCAGGAAAAC	mC.mC.mU.G.mC.mU.G.G.mU.A.A.mU.A#mU#mA.tegChol
AUAUCGGGAAAGAUUUUAAU	AUUAAAAUCUUUCCCGAUAU	mG.mG.G.A.A.A.G.A.mU.mU.mU.mU.A#mA#mA.tegChol
AUUUUAAUGAAACCAGGGUA	UACCCUGGUUUCAUUAAAAU	mA.mA.mU.G.A.A.A.mC.mC.A.G.G.G#mU#mA.tegChol
UAAUGAAACCAGGGUAGAAU	AUUCUACCCUGGUUUCAUUA	mA.mA.A.mC.mC.A.G.G.G.mU.A.G.A#mA#mA.tegChol
AUUGUUUGGCAAUGCACUGA	UCAGUGCAUUGCCAAACAAU	mU.mU.G.G.mC.A.A.mU.G.mC.A.mC.mU#mG#mA.tegChol
CCCCUCAGUUGUUUCUAAGA	UCUUAGAAACAACUGAGGGG	mC.mA.G.mU.mU.G.mU.mU.mU.mC.mU.A.A#mG#mA.tegChol
GGACUGACGAGAGAUGUAUA	UAUACAUCUCUCGUCAGUCC	mG.mA.mC.G.A.G.A.G.A.mU.G.mU.A#mU#mA.tegChol
GACGAGAGAUGUAUAUUUAA	UUAAAUAUACAUCUCUCGUC	mG.mA.G.A.mU.G.mU.A.mU.A.mU.mU.mU#mA#mA.tegChol
UUUUUUAACUGCUGCAAACA	UGUUUGCAGCAGUUAAAAAA	mU.mA.A.mC.mU.G.mC.mU.G.mC.A.A.A#mC#mA.tegChol
UAACUGCUGCAAACAUUGUA	UACAAUGUUUGCAGCAGUUA	mG.mC.mU.G.mC.A.A.A.mC.A.mU.mU.G#mU#mA.tegChol
ACCCUGGAAAAGCUGAUGAA	UUCAUCAGCUUUUCCAGGGU	mG.mG.A.A.A.A.G.mC.mU.G.A.mU.G#mA#mAtegChol
AAGGCCUUCGAGUCCCUCAA	UUGAGGGACUCGAAGGCCUU	mC.mU.mU.mC.G.A.G.mU.mC.mC.mC.mU.mC#mA#mAtegChol
CGCUGCACCGACCAAAGAAA	UUUCUUUGGUCGGUGCAGCG	mC.mA.mC.mC.G.A.mC.mC.A.A.A.G.A#mA#mAtegChol
AAGAACUUUCAGCUACCAAG	CUUGGUAGCUGAAAGUUCUU	mC.mU.mU.mU.mC.A.G.mC.mU.A.mC.mC.A#mA#mAtegChol
AGCUACCAAGAAAGACCGUG	CACGGUCUUUCUUGGUAGCU	mC.mC.A.A.G.A.A.A.G.A.mC.mC.G#mU#mAtegChol
CGUGUGAAUCAUUGUCUGAC	GUCAGACAAUGAUUCACACG	mG.mA.A.mU.mC.A.mU.mU.G.mU.mC.mU.G#mA#mAtegChol
UCAUUGUCUGACAAUAUGUG	CACAUAUUGUCAGACAAUGA	mG.mU.mC.mU.G.A.mC.A.A.mU.A.mU.G#mU#mAtegChol
UGACAAUAUGUGAAAACAUA	UAUGUUUUCACAUAUUGUCA	mA.mU.A.mU.G.mU.G.A.A.A.A.mC.A#mU#mAtegChol
AUAUGUGAAAACAUAGUGGC	GCCACUAUGUUUUCACAUAU	mU.mG.A.A.A.A.mC.A.mU.A.G.mU.G#mG#mAtegChol
GCAGCAGCAGCAGCAGCAGC	GCUGCUGCUGCUGCUGCUGC	mA.mG.mC.A.G.mC.A.G.mC.A.G.mC.A.mG#mGmAtegChol

Continued on sheet 38/136

Fig. 21 (Cont)

Continued from sheet 35/136

Continued from sheet 37/136

Antisense Strand (P0)	Sense strand (P1)
PmU.fU.A.fU.fC.fU.G.fC.A.fC.G.G.fU.fU#fC#fU#fU#fU#G#U	fAmCfAmAfAmGfAmAfCmCfGmUfG#mC#fA-linkerX
PmU.fU.fU.fU.A.fU.A.A.fC.A.A.mG.A.G#mG#fU#fU#fC#A#A	fUmUfGmAfAmCfCmUfCmUfUmGfU#mU#fA-linkerX
PmU.fU.fC.A.A.fU.A.fC.A.A.mA.G.fC.fC#A#A#fU#A#A#A	fUmUfUmAfUmUfGmGfCmUfUmUfG#mU#fA-linkerX
PmU.A.fU.fU.fU.fU.A.G.mG.A.A.fU.fU.fC#fC#A#A#fU#G#A	fUmCfAmUfUmGfGmAfAmUfUmCfC#mU#fA-linkerX
PmU.fU.G.G.fC.fC.A.fU.G.A.fU.G.fC.fC#A#fU#fC#A#fC#A	fUmGfUmGfAmUfGmGfCmAfUmCfA#mU#fA-linkerX
PmU.fU.fU.fC.A.G.fU.fU.G.A.fC.fU.G.G#mG#A#A#mA#fU#C	fGmAfUmUfUmCfCmCfAmGfUmCfA#mA#fA-linkerX
PmU.A.G.fU.A.fU.G.fU.fU.G.fC.fU.G.fC#fU#fC#A#fC#fU#C	fGmAfGmUfGmAfGmCfAmGfCmAfA#mC#fA-linkerX
PmU.fU.fC.fU.fC.fC.G.G.mA.A.fC.A.fU.fU#fC#fC#A#G#mA#C	fGmUfCmUfGmGfAmAfUmGfUmUfC#mC#fA-linkerX
PmU.G.G.fC.fU.G.fU.G.A.mA.fU.G.fU.fU#fC#A#fC#G#fC#A	fUmGfCmGfUmGfAmAfCmAfUmUfC#mA#fA-linkerX
PmU.fU.fU.A.A.mA.fU.fU.fU.fU.A.A.mA.fU#fC#fC#fU#G#A#G	fCmUfCmAfGmGfAmUfUmUfAmAfA#mA#fA-linkerX
PmU.A.fU.A.A.fU.fU.A.A.mA.fU.fU.fU.fU#A#A#mA#fU#fC#C	fGmGfAmUfUmUfAmAfAmAfUmUfU#mA#fA-linkerX
PmU.fU.fU.A.fC.fU.G.A.fU.A.fU.A.A.fU#fU#A#A#mA#fU#U	fAmAfUmUfUmAfAmUfUmAfUmAfU#mC#fA-linkerX
PmU.G.A.mA.A.G.mA.G.fU.fU.A.fC.G.fU#fU#A#A#mA#A#U	fAmUfUmUfUmAfAmCfGmUfAmAfC#mU#fA-linkerX
PmU.fU.fU.A.fC.A.fC.G.G.mG.fC.A.fU.A#G#mA#A#A#mG#A	fUmCfUmUfUmCfUmAfUmGfCmCfC#mG#fA-linkerX
PmU.fU.A.fC.fU.fU.fU.A.fC.A.fC.G.G.mG#fC#A#fU#A#G#A	fUmCfUmAfUmGfCmCfCmGfUmGfU#mA#fA-linkerX
PmU.G.fC.A.fC.fU.fC.fU.fC.fC.fU.G.A.fC#fU#A#A#mA#A#G	fCmUfUmUfUmAfGmUfCmAfGmGfA#mG#fA-linkerX
PmU.A.fC.A.fU.fU.fC.A.A.fU.A.fC.fC.fC#A#A#mA#A#fC#A	fUmGfUmUfUmUfGmGfGmUfAmUfU#mG#fA-linkerX
PmU.fU.fC.fC.fU.fC.fC.A.fC.fU.fU.A.fC.fC#A#fC#A#fU#fU#C	fGmAfAmUfGmUfGmGfUmAfAmGfU#mG#fA-linkerX
PmU.A.fC.A.fU.fU.fU.fC.fC.fU.fC.fC.A.fC#fU#fU#A#fC#fC#A	fUmGfGmUfAmAfGmUfGmGfAmGfG#mA#fA-linkerX
PmU.G.fC.A.fC.A.G.mA.G.fU.fU.fC.fC.A#A#fC#A#fU#fU#U	fAmAfAmUfGmUfUmGfGmAfAmCfU#mC#fA-linkerX
PmU.fU.A.A.mG.G.G.fC.fC.fU.fC.fC.fU.fC#A#A#mA#fC#A#U	fAmUfGmUfUmUfGmAfGmGfAmGfG#mC#fA-linkerX
PmU.fU.fC.A.G.fU.A.G.fC.fU.fU.fC.fC.fC#fU#fU#A#A#mG#G	fCmCfUmUfAmAfGmGfGmAfAmGfC#mU#fA-linkerX
PmU.fU.fU.fC.fU.fU.A.fC.G.fU.G.fU.fU.A#fU#A#A#fU#fU#C	fGmAfAmUfUmAfUmAfAmCfAmCfG#mU#fA-linkerX
PmU.fU.fU.fC.fU.fU.A.fC.A.A.mA.fU.G.fU#A#A#mA#fC#A#U	fAmUfGmUfUmUfAmCfAmUfUmUfG#mU#fA-linkerX
PmU.G.fU.G.fU.fU.A.fU.fU.fU.fC.fU.fU.A#fC#A#A#mA#fU#G	fCmAfUmUfUmGfUmAfAmGfAmAfA#mU#fA-linkerX
PmU.fU.fU.A.fC.A.fU.fU.fC.A.fC.A.G.fU#G#fU#fU#A#fU#U	fAmAfUmAfAmCfAmCfUmGfUmGfA#mA#fA-linkerX
PmU.fU.fU.A.fC.fU.A.A.fU.G.A.mG.fC.fU#fC#A#fU#A#fU#U	fAmAfUmAfUmGfAmGfCmUfCmAfU#mU#fA-linkerX
PmU.fU.A.fU.G.fU.A.fU.A.fU.G.fC.G.fU#G#G#mG#fU#G#A	fUmCfAmCfCmCfAmCfGmCfAmUfA#mU#fA-linkerX
PmU.A.fU.fU.A.fU.A.G.mA.fU.G.fU.G.fU#fC#fU#A#fU#A#U	fAmUfAmUfAmGfAmCfAmCfAmUfC#mU#fA-linkerX
PmU.fU.fU.G.A.mG.A.G.mG.fU.G.fU.G.fU#G#fU#G#fU#A#A	fUmUfAmCfAmCfAmCfAmCfAmCfC#mU#fA-linkerX
PmU.fU.fU.A.G.mG.A.A.fC.A.fU.G.A.fU#A#A#mA#G#fU#C	fGmAfCmUfUmUfAmUfCmAfUmGfU#mU#fA-linkerX
PmU.fU.fU.A.A.fU.fC.A.fC.A.fU.fU.fU.G#fC#A#A#fC#A#A	fUmUfGmUfUmGfCmAfAmAfUmGfU#mG#fA-linkerX
PmU.fC.fC.A.A.mA.fU.fU.A.A.fU.fC.A.fC#A#fU#fU#fU#G#C	fGmCfAmAfAmUfGmUfGmAfUmUfA#mA#fA-linkerX
PmU.A.A.mA.A.fC.fU.fU.G.A.fC.A.A.fC#fC#A#A#mA#fU#U	fAmAfUmUfUmGfGmUfUmGfUmCfA#mA#fA-linkerX
PmU.fU.fU.A.fC.fC.A.G.fC.A.G.mG.A.A#mA#A#fC#A#A#A	fUmUfUmGfUmUfUmUfCmCfUmGfC#mU#fA-linkerX
PmU.A.fU.A.fU.fU.A.fC.fC.A.G.fC.A.G#mG#A#A#mA#A#C	fGmUfUmUfUmCfCmUfGmCfUmGfG#mU#fA-linkerX
PmU.fU.fU.A.A.mA.A.fU.fC.fU.fU.fU.fC.fC#fC#G#A#fU#A#U	fAmUfAmUfCmGfGmGfAmAfAmGfA#mU#fA-linkerX
PmU.A.fC.fC.fC.fU.G.G.fU.fU.fU.fC.A.fU#fU#A#A#mA#A#U	fAmUfUmUfUmAfAmUfGmAfAmAfC#mC#fA-linkerX
PmU.fU.fU.fC.fU.A.fC.fC.fC.fU.G.G.fU.fU#fU#fC#A#fU#fU#A	fUmAfAmUfGmAfAmAfCmCfAmGfG#mG#fA-linkerX
PmU.fC.A.G.fU.G.fC.A.fU.fU.G.fC.fC.A#A#mA#fC#A#A#U	fAmUfUmGfUmUfUmGfGmCfAmAfU#mG#fA-linkerX
PmU.fC.fU.fU.A.G.mA.A.A.fC.A.A.fC.fU#G#A#mG#G#G#G	fCmCfCmCfUmCfAmGfUmUfGmUfU#mU#fA-linkerX
PmU.A.fU.A.fC.A.fU.fC.fU.fC.fU.fC.G.fU#fC#A#G#fU#fC#C	fGmGfAmCfUmGfAmCfGmAfGmAfG#mA#fA-linkerX
PmU.fU.A.A.mA.fU.A.fU.A.fC.A.fU.fC.fU#fC#fU#fC#G#fU#C	fGmAfCmGfAmGfAmGfAmUfGmUfA#mU#fA-linkerX
PmU.G.fU.fU.fU.G.fC.A.G.fC.A.G.fU.fU#A#A#mA#A#A#A	fUmUfUmUfUmUfAmAfCmUfGmCfU#mG#fA-linkerX
PmU.A.fC.A.A.fU.G.fU.fU.fU.G.fC.A.G#fC#A#G#fU#fU#A	fUmAfAmCfUmGfCmUfGmCfAmAfA#mC#fA-linkerX
PmU.fU.fC.A.fU.fC.A.G.fC.fU.fU.fU.fU.fC#fC#A#G#mG#G#U	fAmCfCmCfUmGfGmAfAmAfAmGfC#mU#fA-linkerX
PmU.fU.G.A.mG.G.G.mA.fC.fU.fC.G.A.mA#G#G#fC#fC#fU#U	fAmAfGmGfCmCfUmUfCmGfAmGfU#mC#fA-linkerX
PmU.fU.fU.fC.fU.fU.fU.G.G.fU.fC.G.G.fU#G#fC#A#G#fC#G	fCmGfCmUfGmCfAmCfCmGfAmCfC#mA#fA-linkerX
PmU.fU.fU.G.G.fU.A.G.fC.fU.G.A.mA.A#G#fU#fU#fC#fU#U	fAmAfGmAfAmCfUmUfUmCfAmGfC#mU#fA-linkerX
PmU.A.fC.G.G.fU.fC.fU.fU.fU.fC.fU.fU.G#G#fU#A#G#fC#U	fAmGfCmUfAmCfCmAfAmGfAmAfA#mG#fA-linkerX
PmU.fU.fC.A.G.mA.fC.A.A.fU.G.A.fU.fU#fC#A#fC#A#fC#G	fCmGfUmGfUmGfAmAfUmCfAmUfU#mG#fA-linkerX
PmU.A.fC.A.fU.A.fU.fU.G.fU.fC.A.G.mA#fC#A#A#fU#G#A	fUmCfAmUfUmGfUmCfUmGfAmCfA#mA#fA-linkerX
PmU.A.fU.G.fU.fU.fU.fU.fC.A.fC.A.fU.A#fU#fU#G#fU#fC#A	fUmGfAmCfAmAfUmAfUmGfUmGfA#mA#fA-linkerX
PmU.fC.fC.A.fC.fU.A.fU.G.fU.fU.fU.fC#A#fC#A#fU#A#U	fAmUfAmUfGmUfGmAfAmAfAmCfA#mU#fA-linkerX
PmU.fC.fU.G.fC.fU.G.fC.fU.G.fC.fU.G.fC#fU#G#fC#fU#G#C	fGmCfAmGfCmAfGmCfAmGfCmAfG#mC#fA-linkerX

Continued on sheet 39/136

Fig. 21 (Cont)

Continued from sheet 36/136

Antisense Strand (P1)
P'-mUfUmAfUmCfUmGfCmAfCmGfGmUfU#mC#fU#mU#fU#mG#fU
P'-mUfUmUfUmAfUmAfAmCfAmAfGmAfG#mG#fU#mU#fC#mA#fA
P'-mUfUmCfAmAfUmAfCmAfAmAfGmCfC#mA#fA#mU#fA#mA#fA
P'-mUfAmUfUmUfUmAfGmGfAmAfUmUfC#mC#fA#mA#fU#mG#fA
P'-mUfUmGfGmCfCmAfUmGfAmUfGmCfC#mA#fU#mC#fA#mC#fA
P'-mUfUmUfCmAfGmUfUmGfAmCfUmGfG#mG#fA#mA#fA#mU#fC
P'-mUfAmGfUmAfUmGfUmUfGmCfUmGfC#mU#fC#mA#fC#mU#fC
P'-mUfUmCfUmCfCmGfGmAfAmCfAmUfU#mC#fC#mA#fG#mA#fC
P'-mUfGmGfCmUfGmUfGmAfAmUfGmUfU#mC#fA#mC#fG#mC#fA
P'-mUfUmUfAmAfAmUfUmUfUmAfAmAfU#mC#fC#mU#fG#mA#fG
P'-mUfAmUfAmAfUmUfAmAfAmUfUmUfU#mA#fA#mA#fU#mC#fC
P'-mUfUmUfAmCfUmGfAmUfAmUfAmAfU#mU#fA#mA#fA#mU#fU
P'-mUfGmAfAmAfGmAfGmUfUmAfCmGfU#mU#fA#mA#fA#mA#fU
P'-mUfUmUfAmCfAmCfGmGfGmCfAmUfA#mG#fA#mA#fA#mG#fA
P'-mUfUmAfCmUfUmUfAmCfAmCfGmGfG#mC#fA#mU#fA#mG#fA
P'-mUfGmCfAmCfUmCfUmCfCmUfGmAfC#mU#fA#mA#fA#mA#fG
P'-mUfAmCfAmUfUmCfAmAfUmAfCmCfC#mA#fA#mA#fA#mC#fA
P'-mUfUmCfCmUfCmCfAmCfUmUfAmCfC#mA#fC#mA#fU#mU#fC
P'-mUfAmCfAmUfUmUfCmCfUmCfCmAfC#mU#fU#mA#fC#mC#fA
P'-mUfGmCfAmCfAmGfAmGfUmUfCmCfA#mA#fC#mA#fU#mU#fU
P'-mUfUmAfAmGfGmGfCmCfUmCfCmUfC#mA#fA#mA#fC#mA#fU
P'-mUfUmCfAmGfUmAfGmCfUmUfCmCfC#mU#fU#mA#fA#mG#fG
P'-mUfUmUfCmUfUmAfCmGfUmGfUmUfA#mU#fA#mA#fU#mU#fC
P'-mUfUmUfCmUfUmAfCmAfAmAfUmGfU#mA#fA#mA#fC#mA#fU
P'-mUfGmUfGmUfUmAfUmUfUmCfUmUfA#mC#fA#mA#fA#mU#fG
P'-mUfUmUfAmCfAmUfUmCfAmCfAmGfU#mG#fU#mU#fA#mU#fU
P'-mUfUmUfAmCfUmAfAmUfGmAfGmCfU#mC#fA#mU#fA#mU#fU
P'-mUfUmAfUmGfUmAfUmAfUmGfCmGfU#mG#fG#mG#fU#mG#fA
P'-mUfAmUfUmAfUmAfGmAfUmGfUmGfU#mC#fU#mA#fU#mA#fU
P'-mUfUmUfGmAfGmAfGmGfUmGfUmGfU#mG#fU#mG#fU#mA#fA
P'-mUfUmUfAmGfGmAfAmCfAmUfGmAfU#mA#fA#mA#fG#mU#fC
P'-mUfUmUfAmAfUmCfAmCfAmUfUmUfG#mC#fA#mA#fC#mA#fA
P'-mUfCmCfAmAfAmUfUmAfAmUfCmAfC#mA#fU#mU#fU#mG#fC
P'-mUfAmAfAmAfCmUfUmGfAmCfAmAfC#mC#fA#mA#fA#mU#fU
P'-mUfUmUfAmCfCmAfGmCfAmGfGmAfA#mA#fA#mC#fA#mA#fA
P'-mUfAmUfAmUfUmAfCmCfAmGfCmAfG#mG#fA#mA#fA#mA#fC
P'-mUfUmUfAmAfAmAfUmCfUmUfUmCfC#mC#fG#mA#fU#mA#fU
P'-mUfAmCfCmCfUmGfGmUfUmUfCmAfU#mU#fA#mA#fA#mA#fU
P'-mUfUmUfCmUfAmCfCmCfUmGfGmUfU#mU#fC#mA#fU#mU#fA
P'-mUfCmAfGmUfGmCfAmUfUmGfCmCfA#mA#fA#mC#fA#mA#fU
P'-mUfCmUfUmAfGmAfAmAfCmAfAmCfU#mG#fA#mG#fG#mG#fG
P'-mUfAmUfAmCfAmUfCmUfCmUfCmGfU#mC#fA#mG#fU#mC#fC
P'-mUfUmAfAmAfUmAfUmAfCmAfUmCfU#mC#fU#mC#fG#mU#fC
P'-mUfGmUfUmUfGmCfAmGfCmAfGmUfU#mA#fA#mA#fA#mA#fA
P'-mUfAmCfAmAfUmGfUmUfUmGfCmAfG#mC#fA#mG#fU#mU#fA
P'-mUfUmCfAmUfCmAfGmCfUmUfUmUfC#mC#fA#mG#fG#mG#fU
P'-mUfUmGfAmGfGmGfAmCfUmCfGmAfA#mG#fG#mC#fC#mU#fU
P'-mUfUmUfCmUfUmUfGmGfUmCfGmGfU#mG#fC#mA#fG#mC#fG
P'-mUfUmUfGmGfUmAfGmCfUmGfAmAfA#mG#fU#mU#fC#mU#fU
P'-mUfAmCfGmGfUmCfUmUfUmCfUmUfG#mG#fU#mA#fG#mC#fU
P'-mUfUmCfAmGfAmCfAmAfUmGfAmUfU#mC#fA#mC#fA#mC#fG
P'-mUfAmCfAmUfAmUfUmGfUmCfAmGfA#mC#fA#mA#fU#mG#fA
P'-mUfAmUfGmUfUmUfUmCfAmCfAmUfA#mU#fU#mG#fU#mC#fA
P'-mUfCmCfAmCfUmAfUmGfUmUfUmUfC#mA#fC#mA#fU#mA#fU
P'-mUfCmUfGmCfUmGfCmUfGmCfUmGfC#mU#fG#mC#fU#mG#fC

Continued from sheet 38/136

Fig. 21 (Cont)

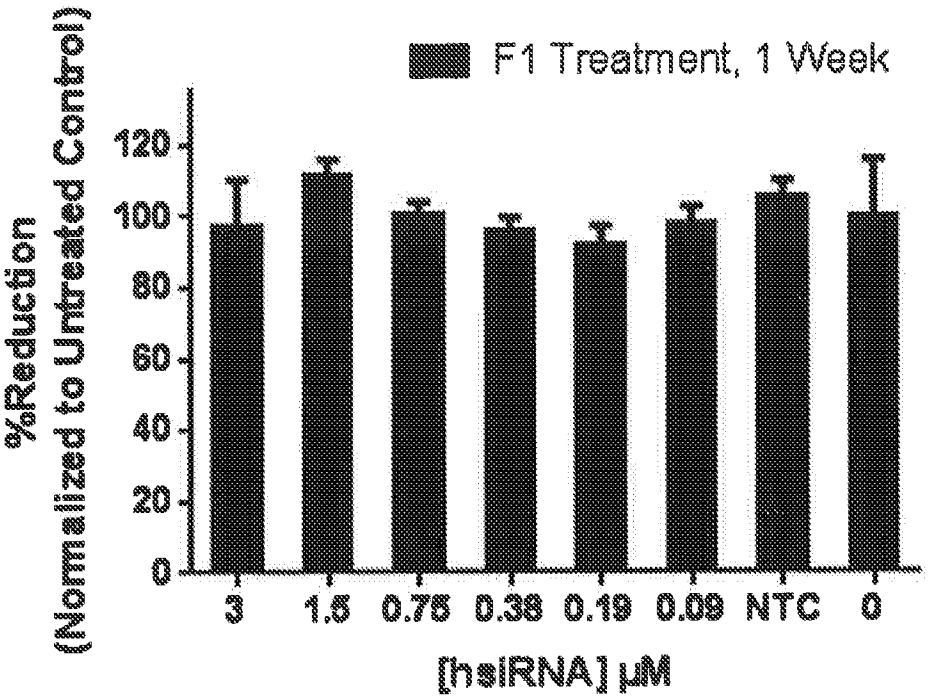

F1 Treatment, 1 Week
%Reduction
(Normalized to Untreated Control)
120
100
80
60
40
20
0
3
1.5
0.75
0.38
0.19
0.09
NTC
0
[hsiRNA] µM

Fig. 22

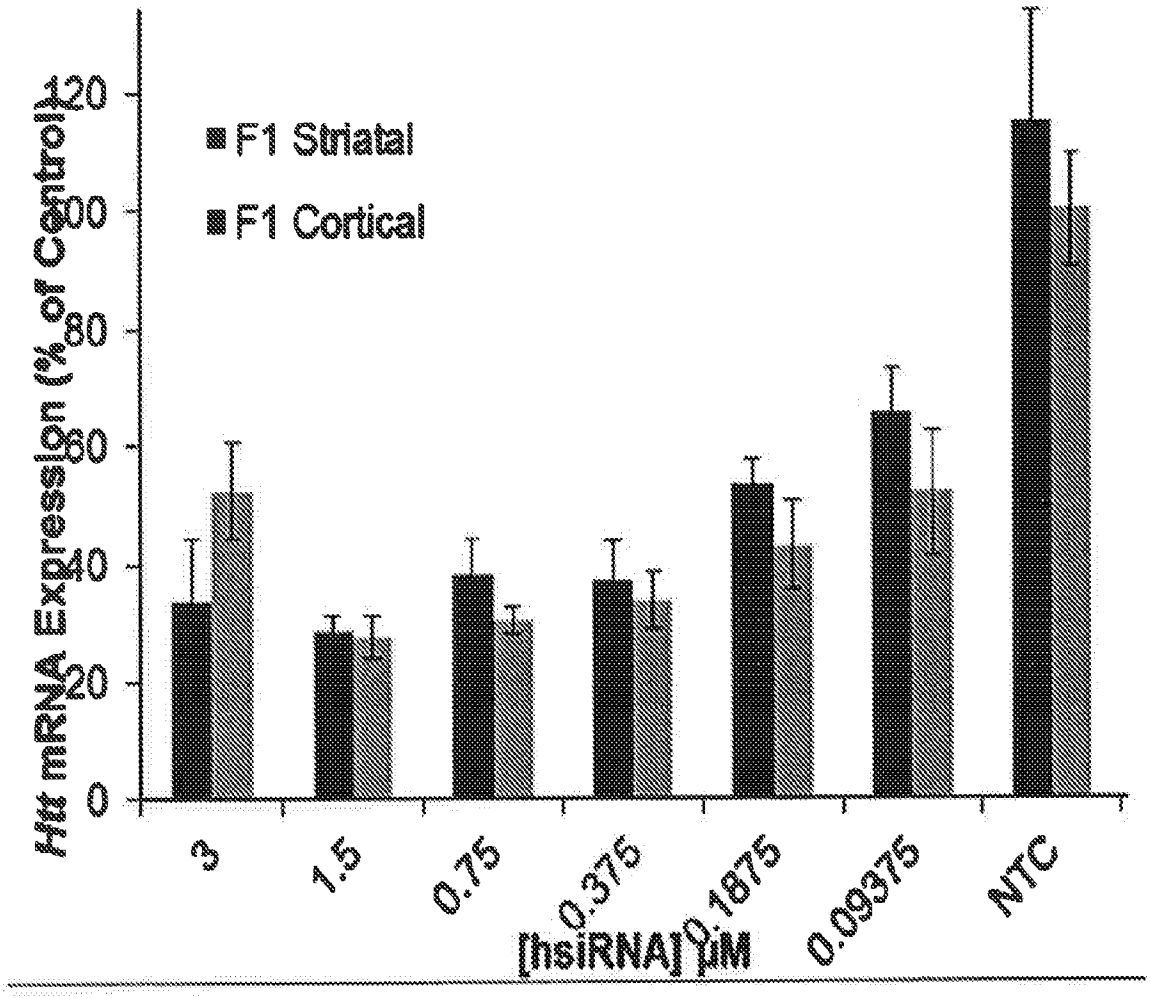

F1 Striatal
F1 Cortical
Htt mRNA Expression (% of Control)
120
100
80
60
40
20
0
3
1.5
0.75
0.375
0.1875
0.09375
NTC
[hsiRNA] µM

Fig. 23

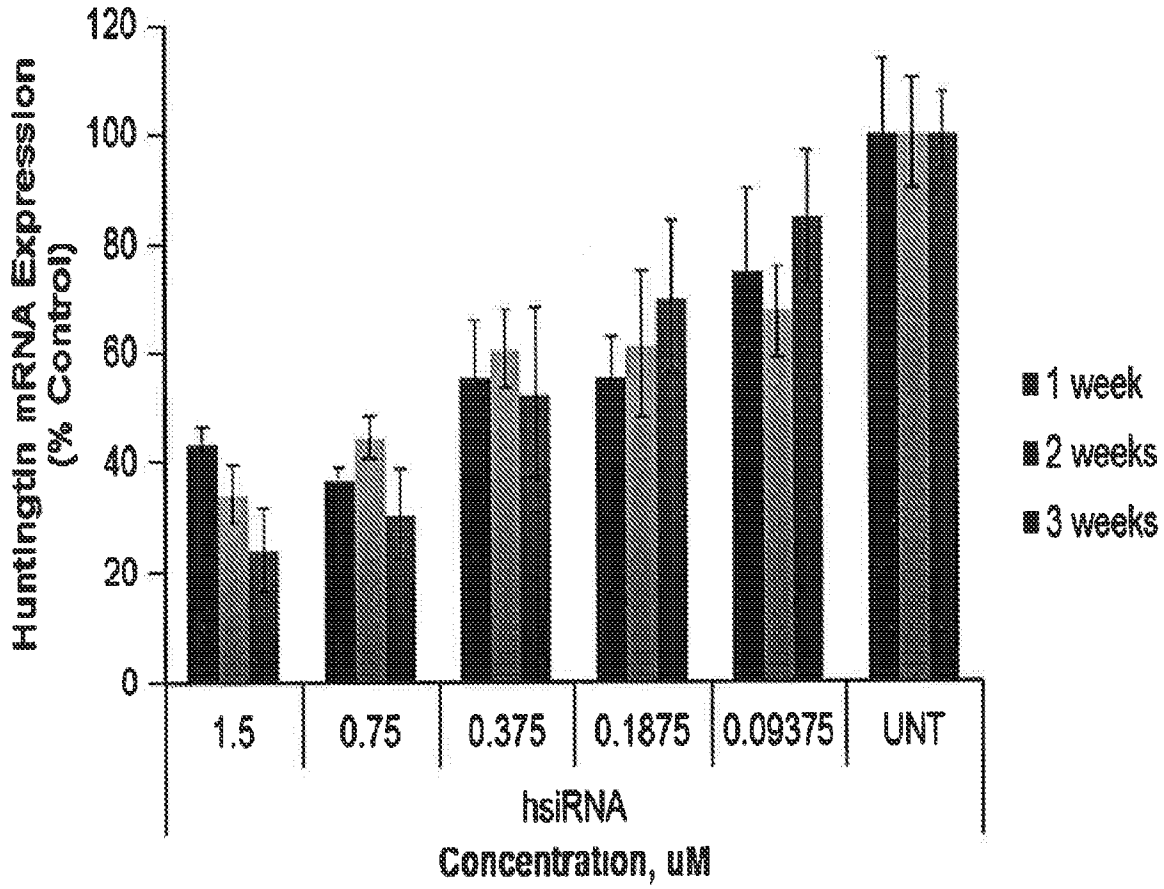

Huntingtin mRNA Expression
(% Control)
120
100
80
60
40
20
0
1.5
0.75
0.375
0.1875
0.09375
UNT
hsiRNA
Concentration, uM
1 week
2 weeks
3 weeks

Fig. 24

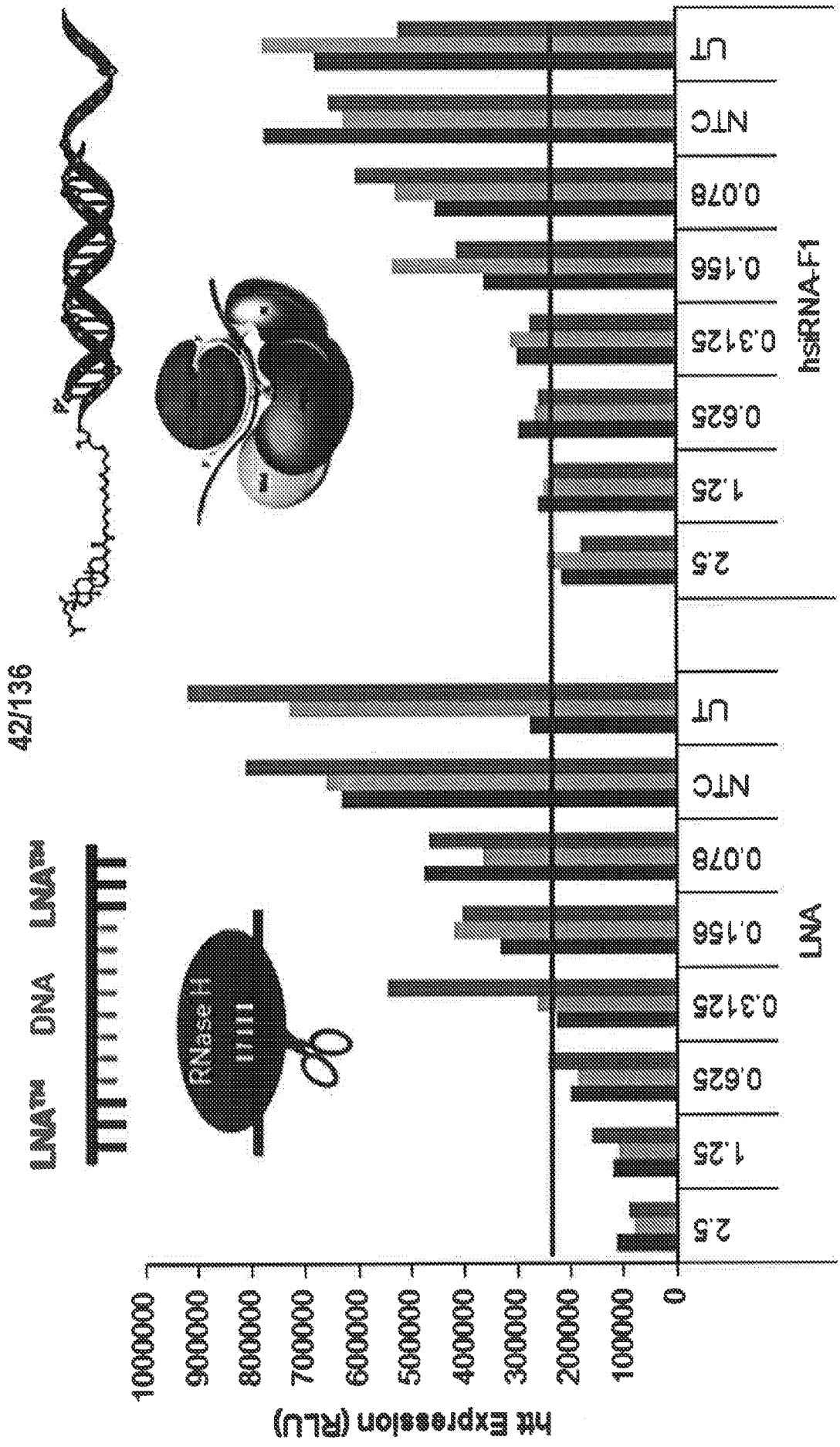

42/136
LNA™ DNA LNA™
RNase H
htt expression (RLU)
1000000
900000
800000
700000
600000
500000
400000
300000
200000
100000
0
2.5
1.25
0.625
0.3125
0.156
0.078
NTC
UT
LNA
2.5
1.25
0.625
0.3125
0.156
0.078
NTC
UT
hsiRNA-F1

Fig. 25

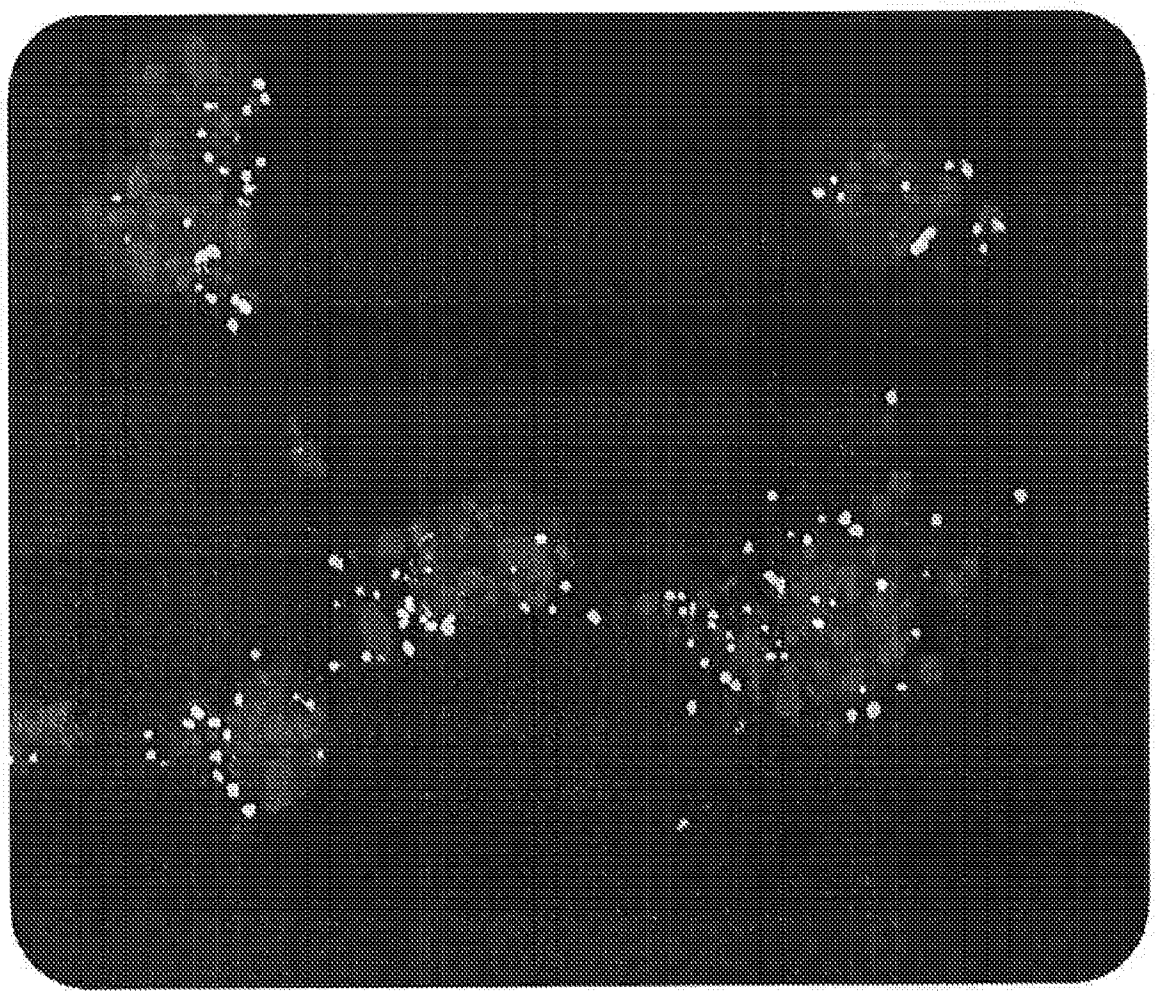


Fig. 26

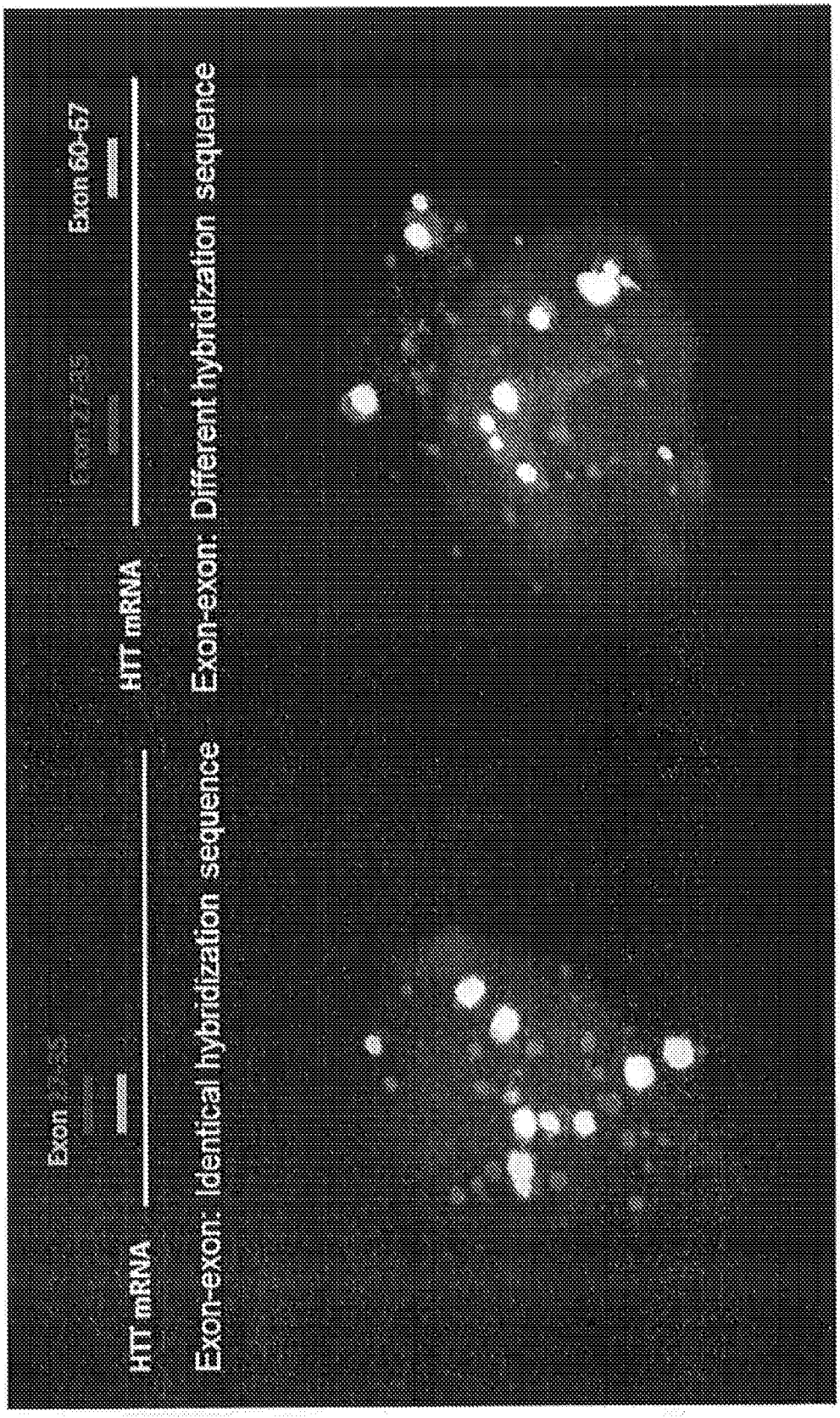

Exon 60-67
HTT mRNA
HTT mRNA
Exon-exon: Identical hybridization sequence
Exon-exon: Different hybridization sequence

Fig. 27

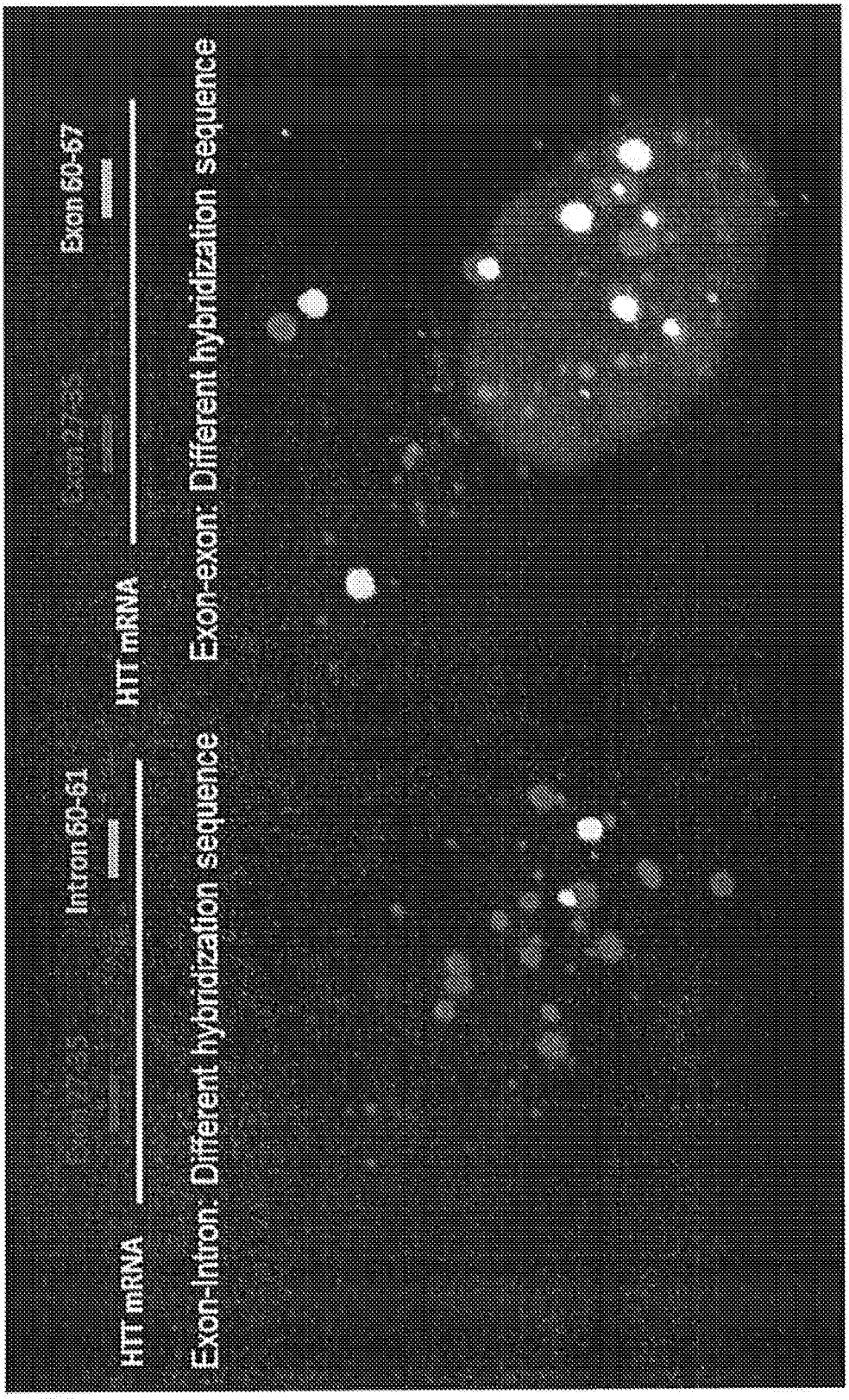

Exon-Intron: Different hybridization sequence
HTT mRNA
Intron 60-61
Exon-exon: Different hybridization sequence
HTT mRNA
Exon 60-67

Fig. 28

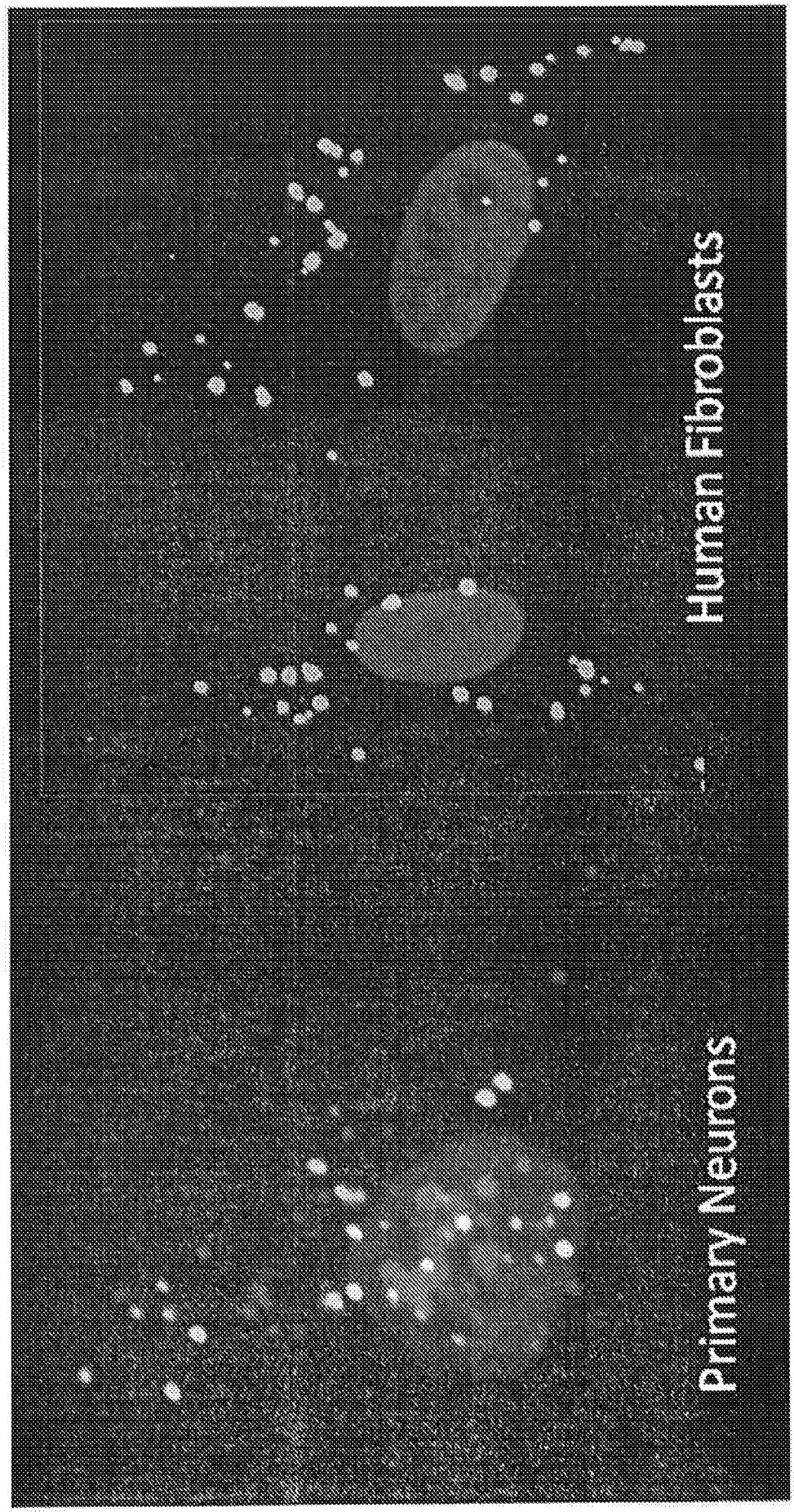


Fig. 29

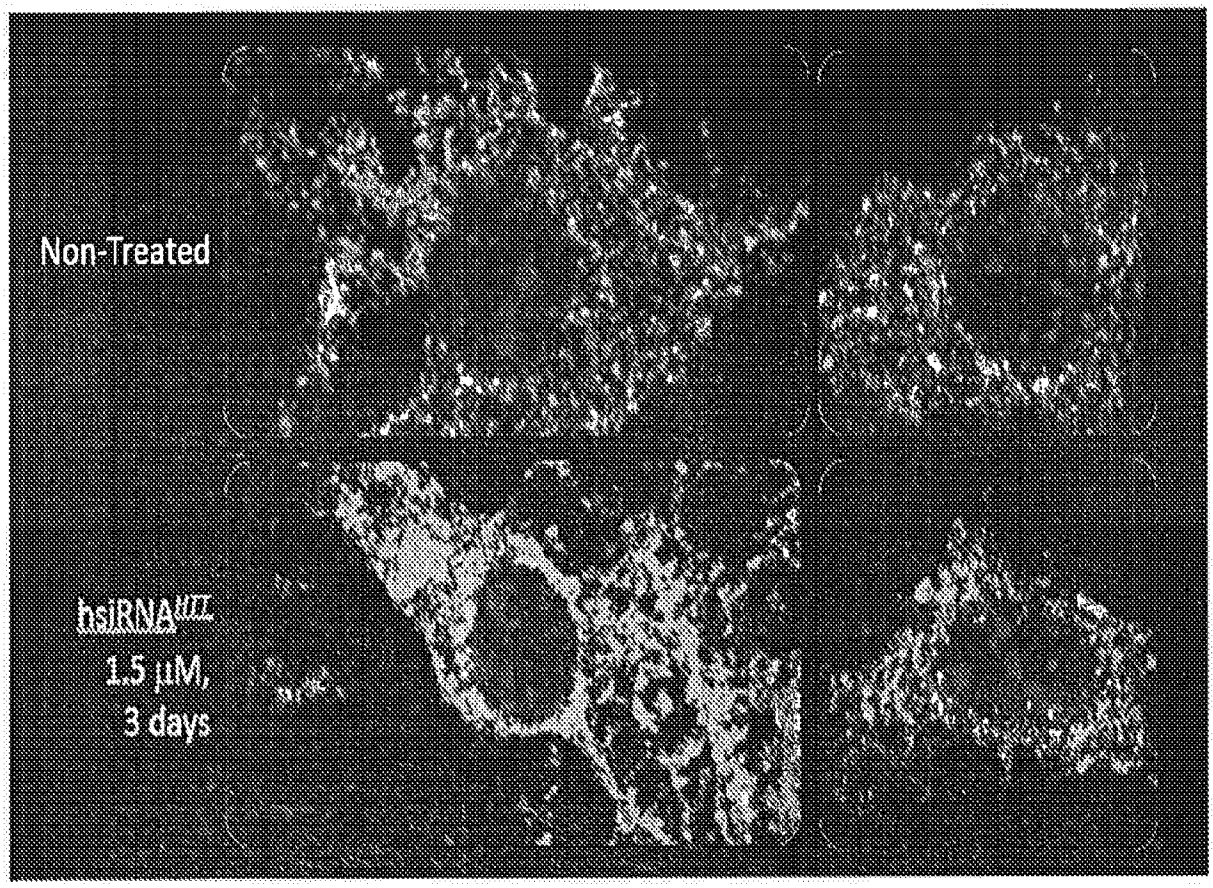


Fig. 30

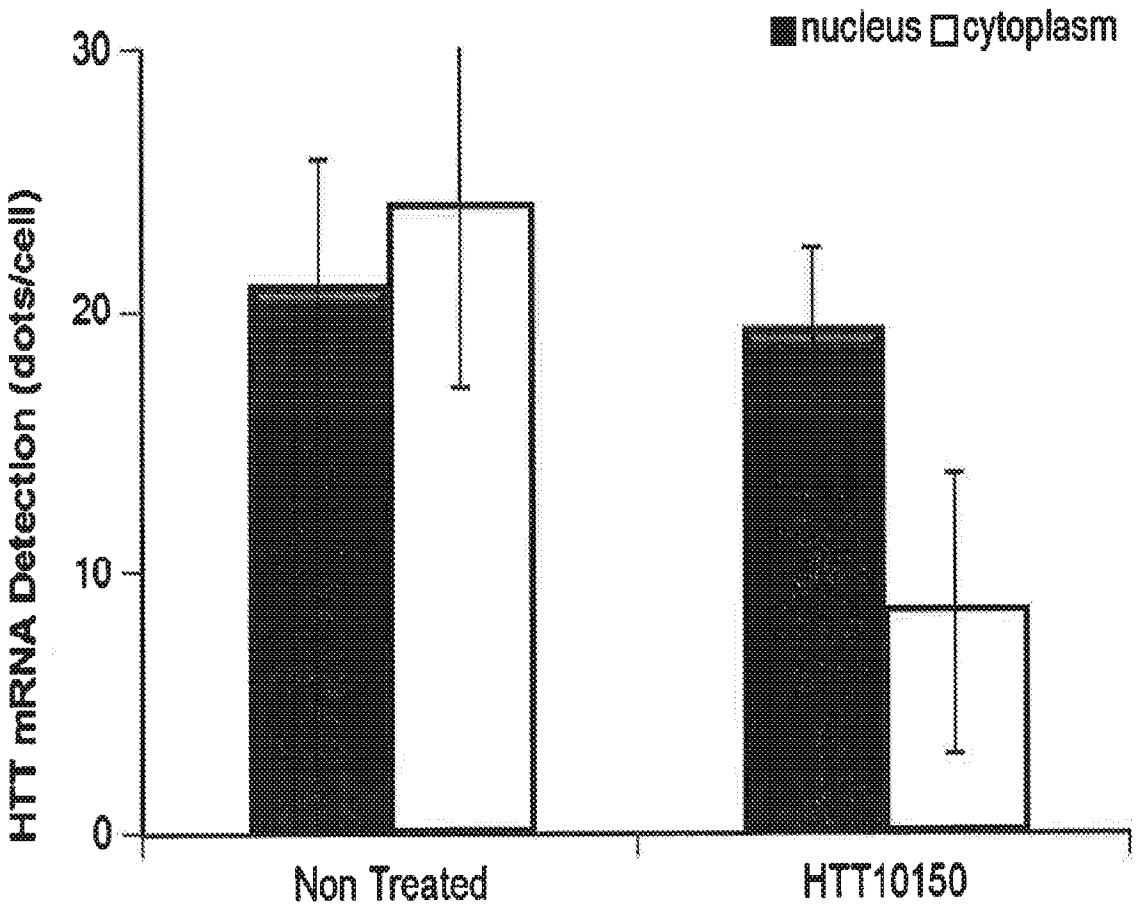

nucleus
cytoplasm
HTT mRNA Detection (dots/cell)
30
20
10
0
Non Treated
HTT10150

Fig. 31

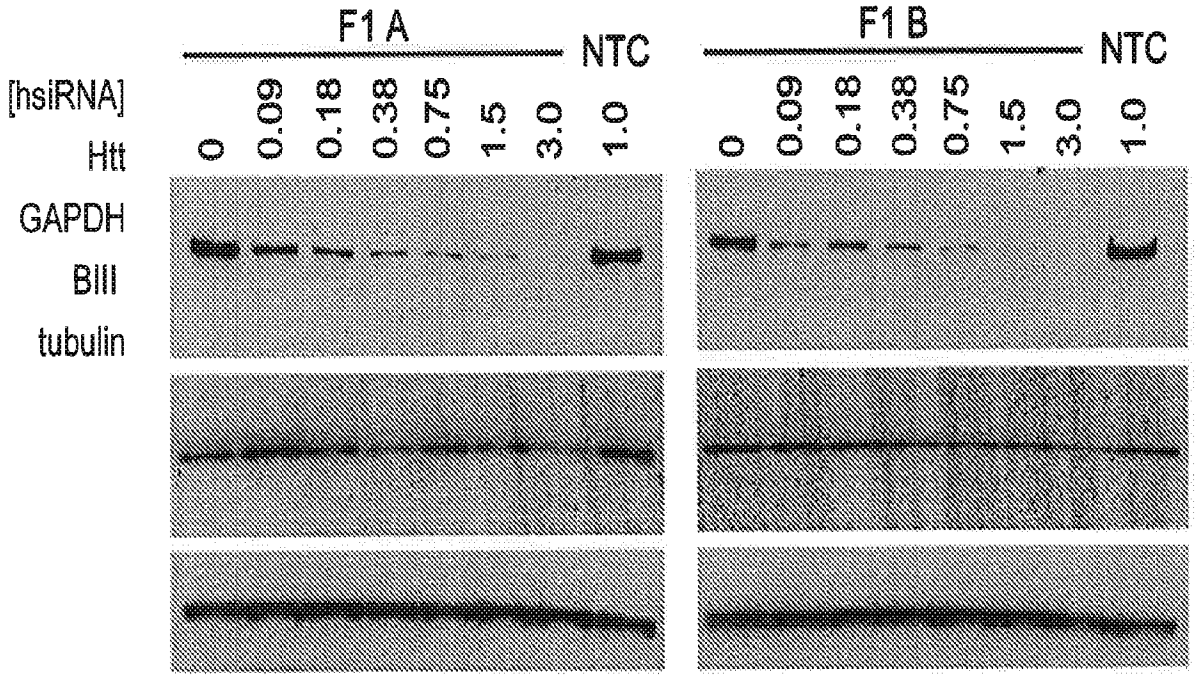

F1 A
NTC
F1 B
NTC
[hsiRNA]
Htt
GAPDH
BIII
tubulin
0
0.09
0.18
0.38
0.75
1.5
3.0
1.0
0
0.09
0.18
0.38
0.75
1.5
3.0
1.0

Fig. 32

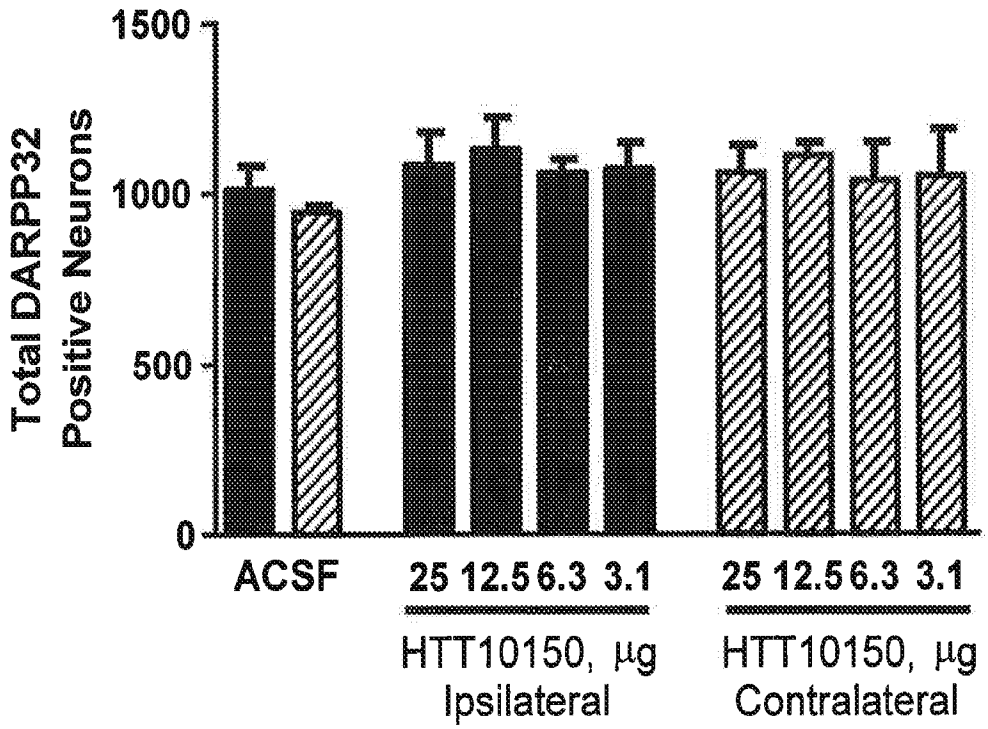

Total DARPP32 Positive Neurons
1500
1000
500
0
ACSF
25 12.5 6.3 3.1
HTT10150, µg
Ipsilateral
25 12.5 6.3 3.1
HTT10150, µg
Contralateral

Fig. 33

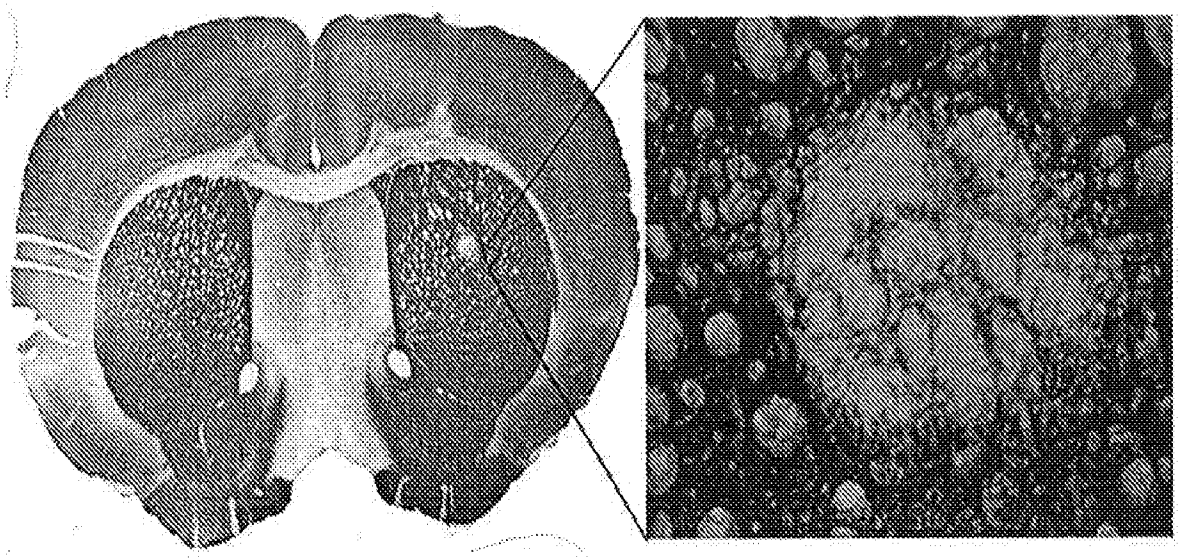


Fig. 34

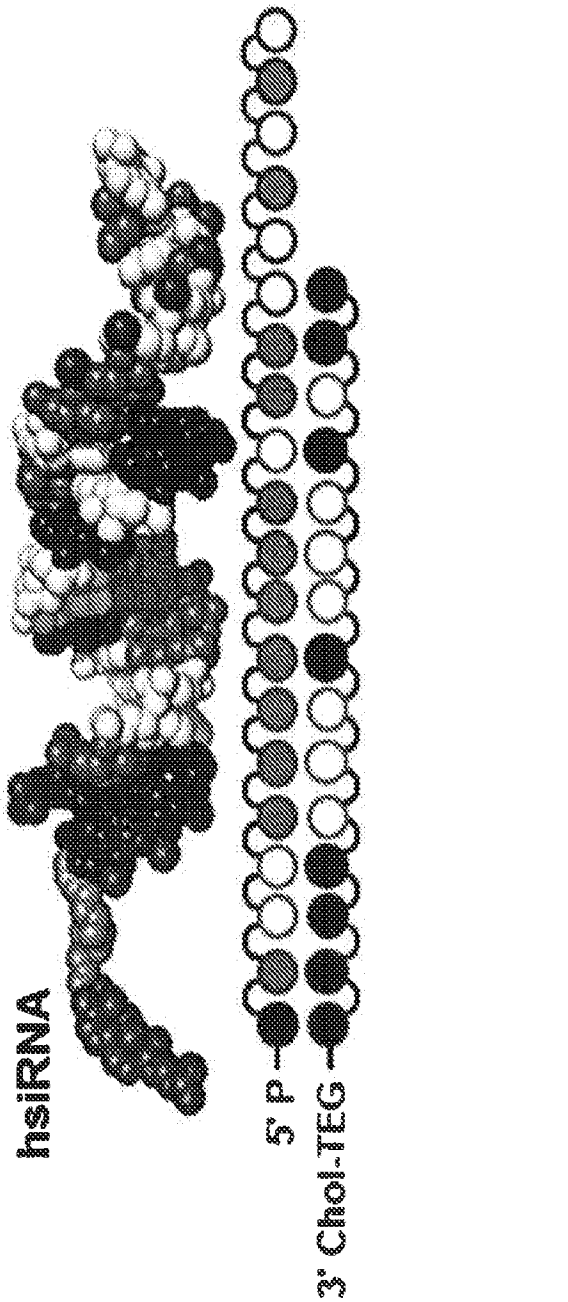

hsiRNA
5' P
3' Chol-TEG
2'-O-Methyl RNA
2'-Fluoro RNA
2'-Hydroxyl RNA
Phosphorothioate
5'-Phosphate/ vinylphosphonate
Cholesterol
TEG linker

Fig. 35A

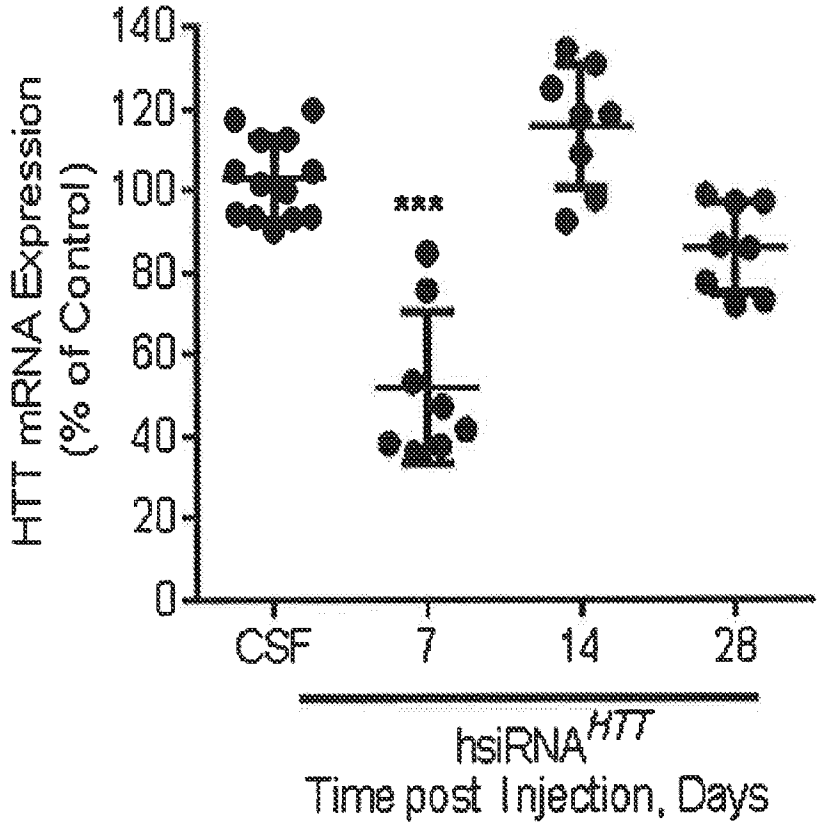


Fig. 35B

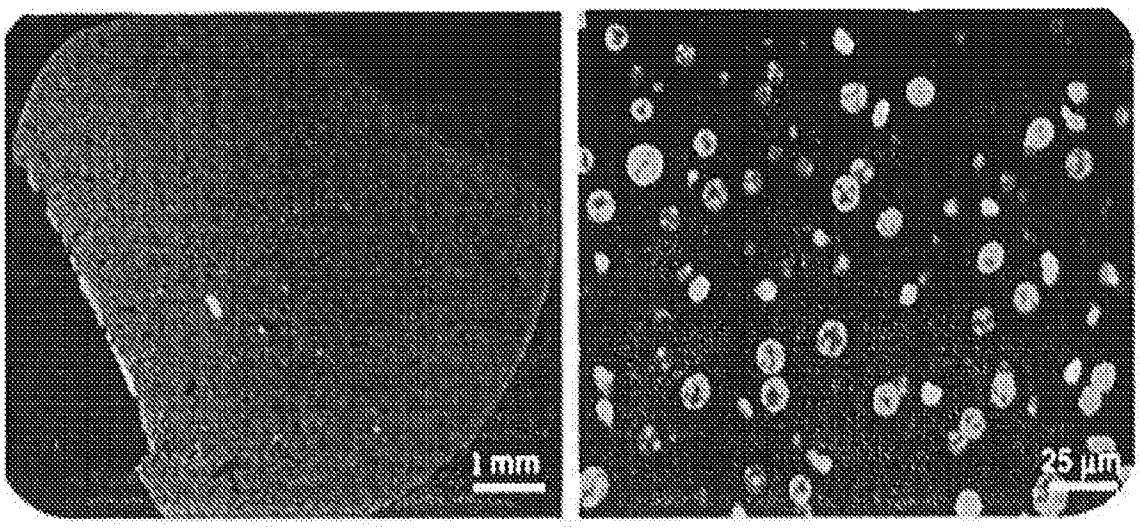
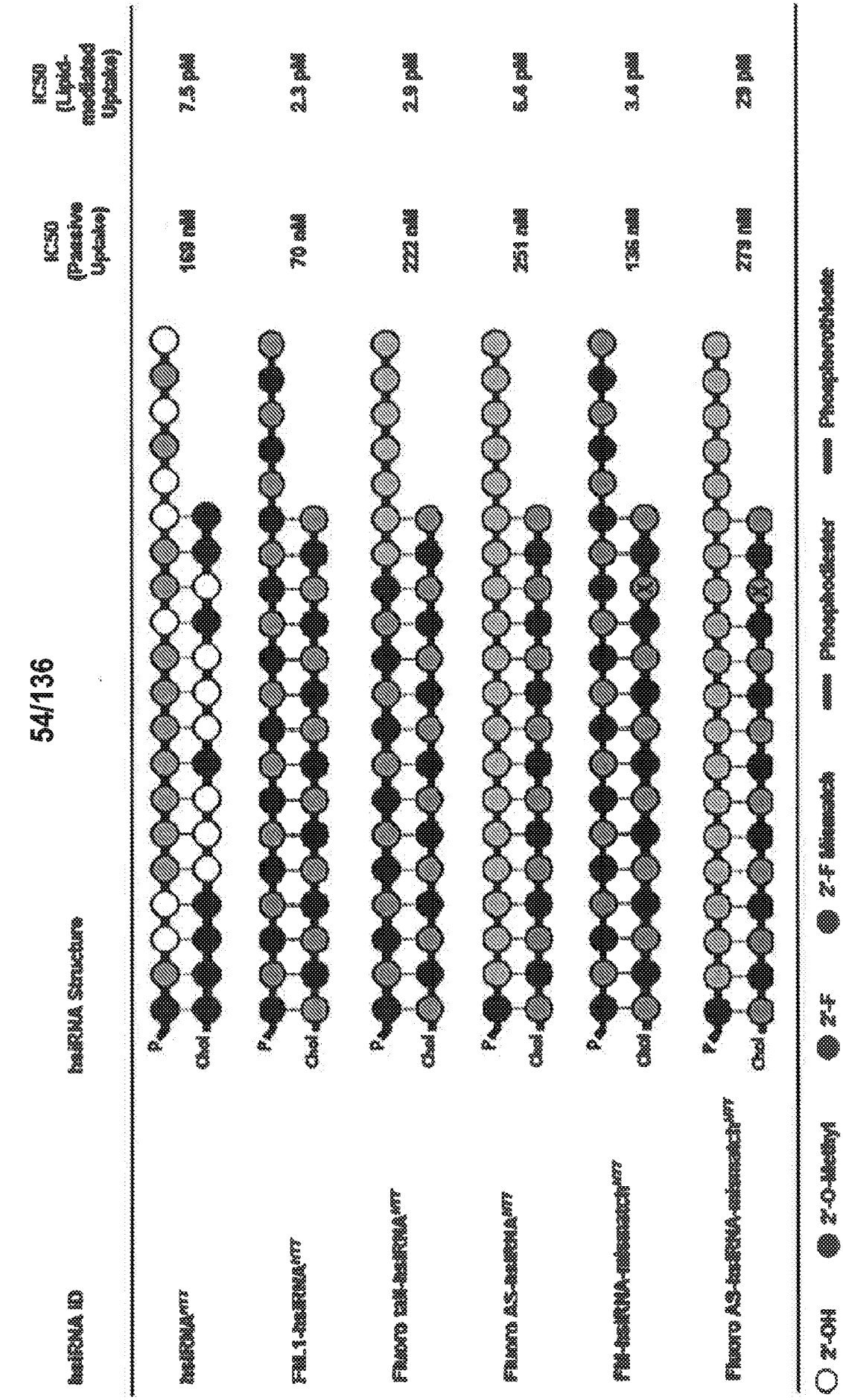


Fig. 35C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IC50 (Passive Uptake)
IC50 (Lipid-mediated Uptake)
2'-OH
2'-O-Methyl
2'-F
2'-F Mismatch
Phosphorothioate
7.5 pM
2.3 pM
2.9 pM
5.4 pM
3.4 pM
29 pM

Fig. 36A

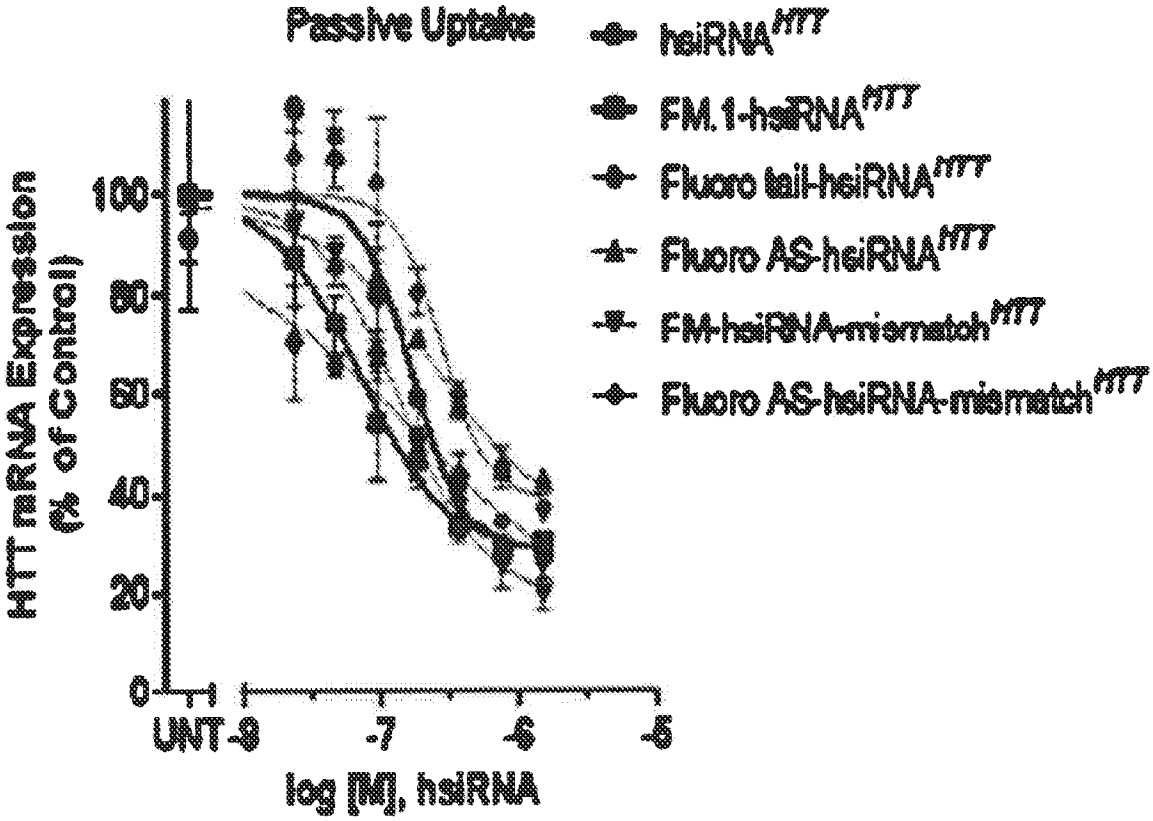


Fig. 36B

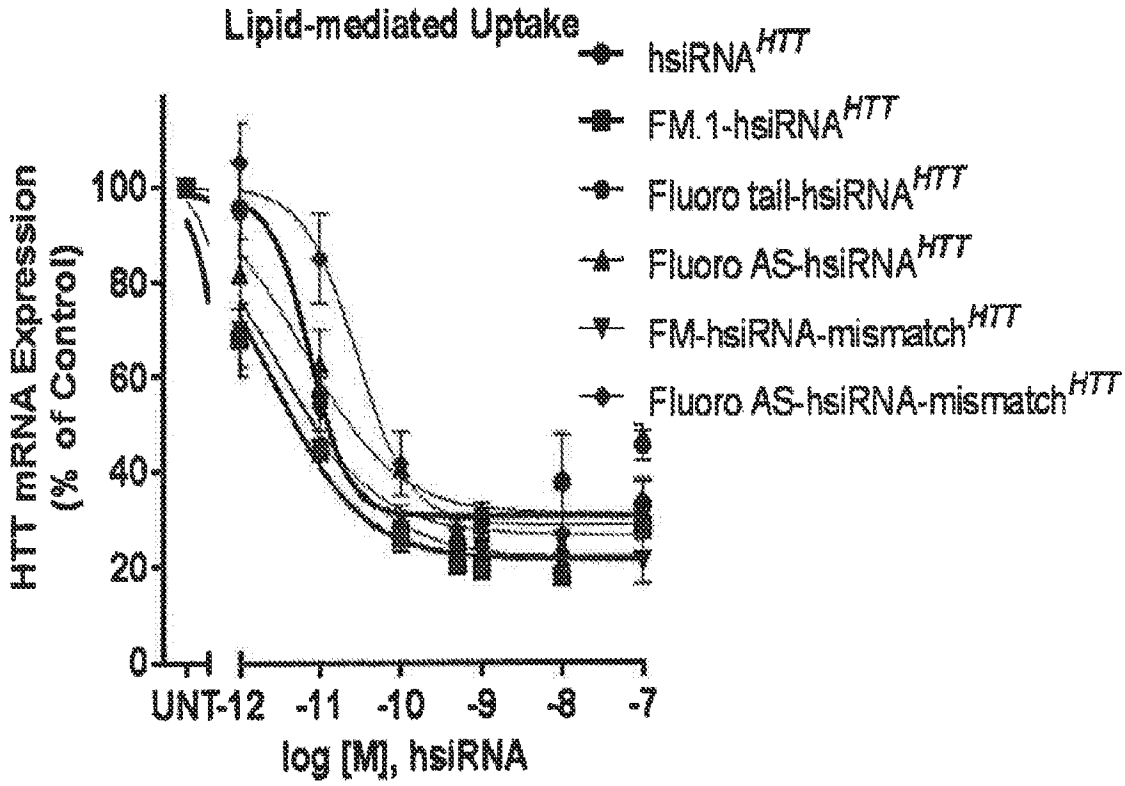


Fig. 36C

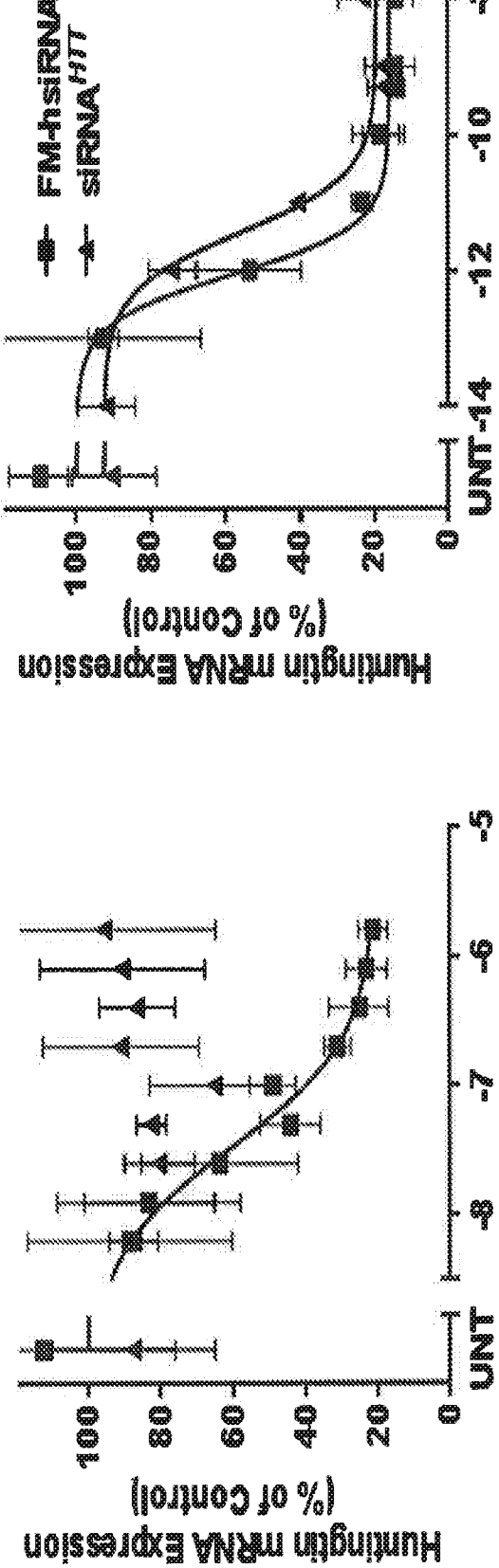
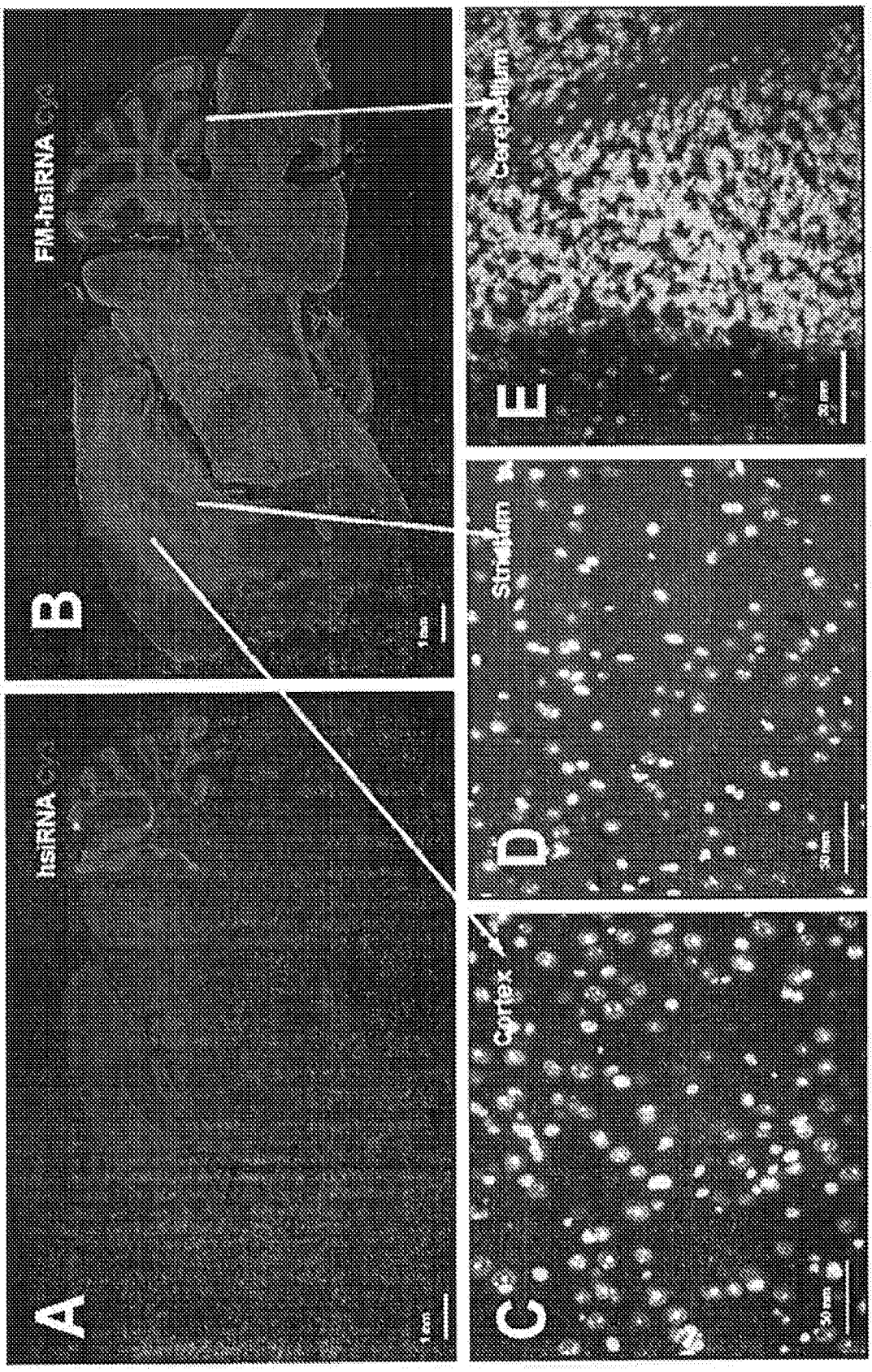


Fig. 37A

Fig. 37B

	siRNA^{HTT}	FM-hsiRNA^{HTT}
IC50 (Passive Uptake)	N/A	33.5 nM
IC50 (Lipid Mediated Uptake)	3.5 pM	0.9 pM

Fig. 37C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A
hsiRNA
1 cm
B
FIM-hsiRNA
1 cm
C
Cortex
50 μm
D
Striatum
50 μm
E
Cerebellum
50 μm

Fig. 38A-E

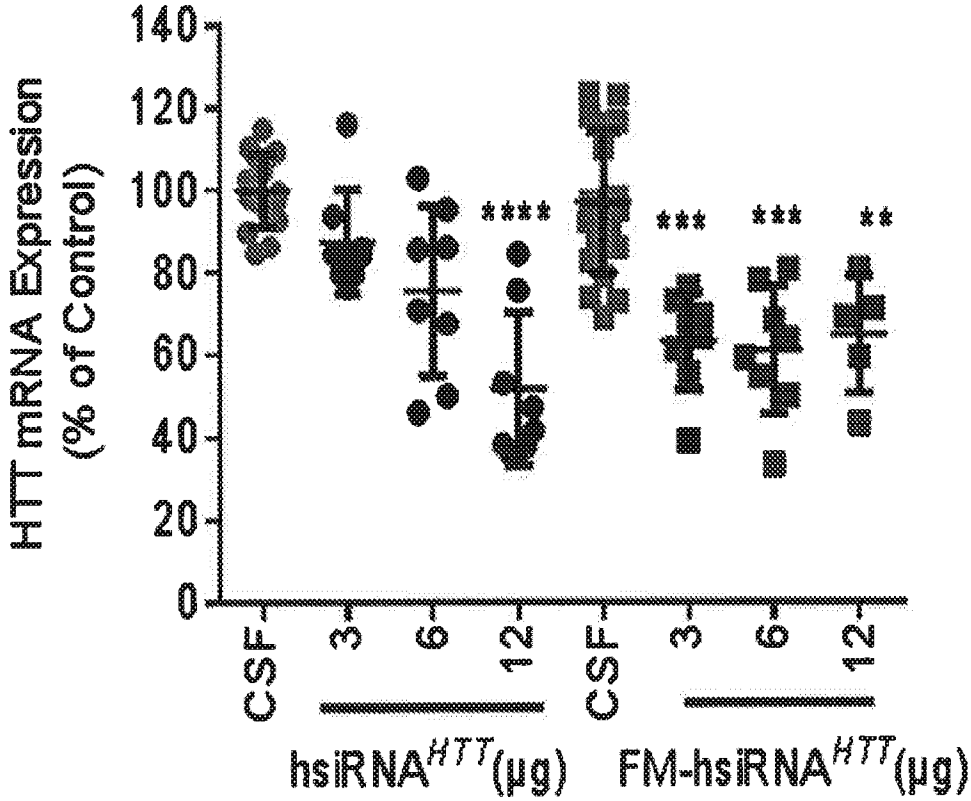


Fig. 39A

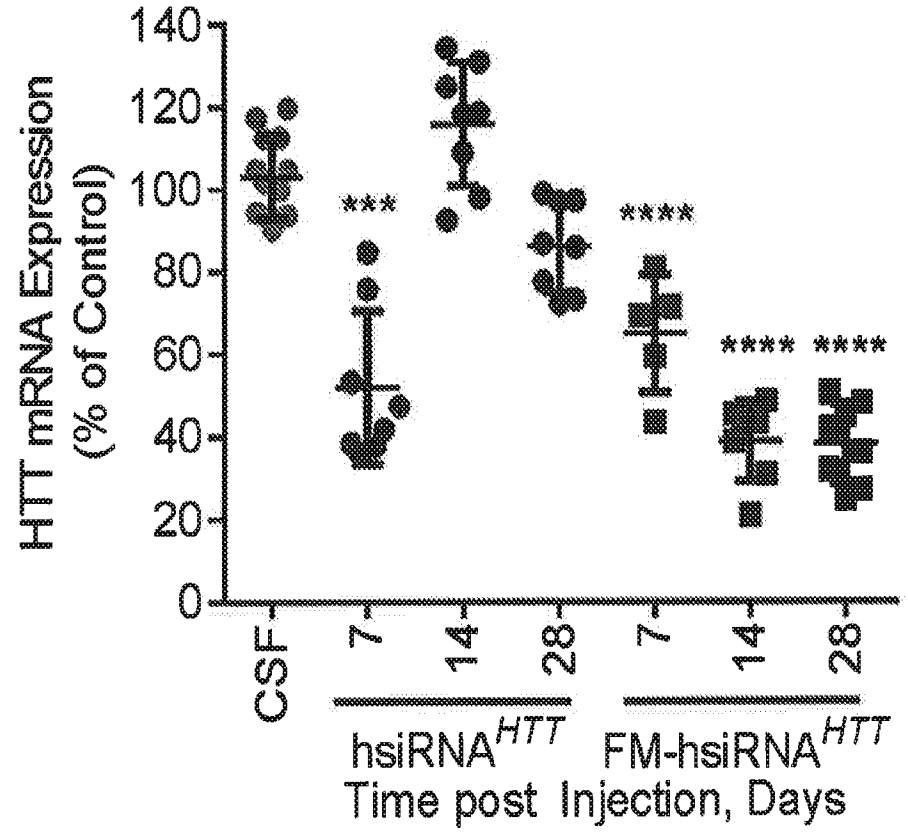


Fig. 39B

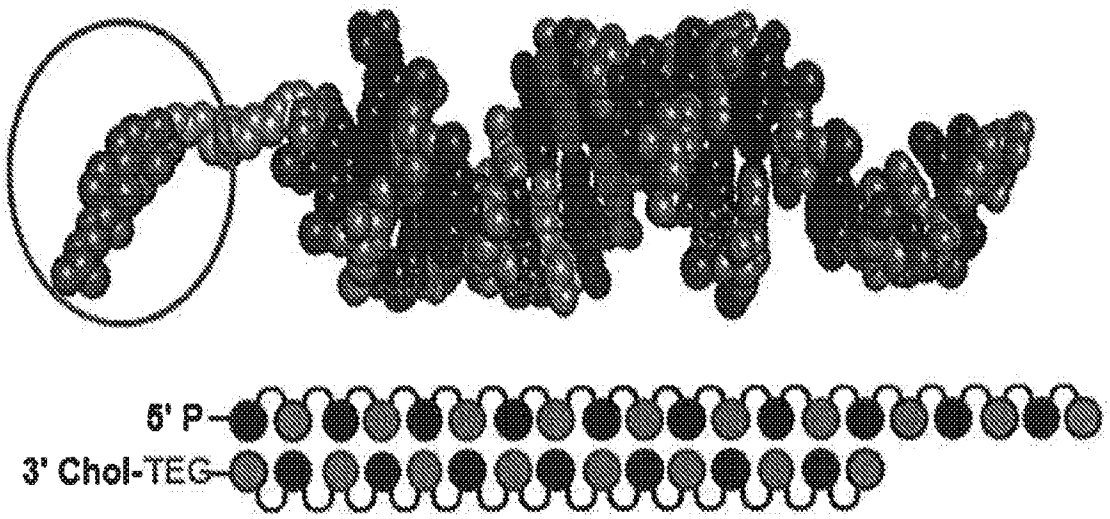


Fig. 40A

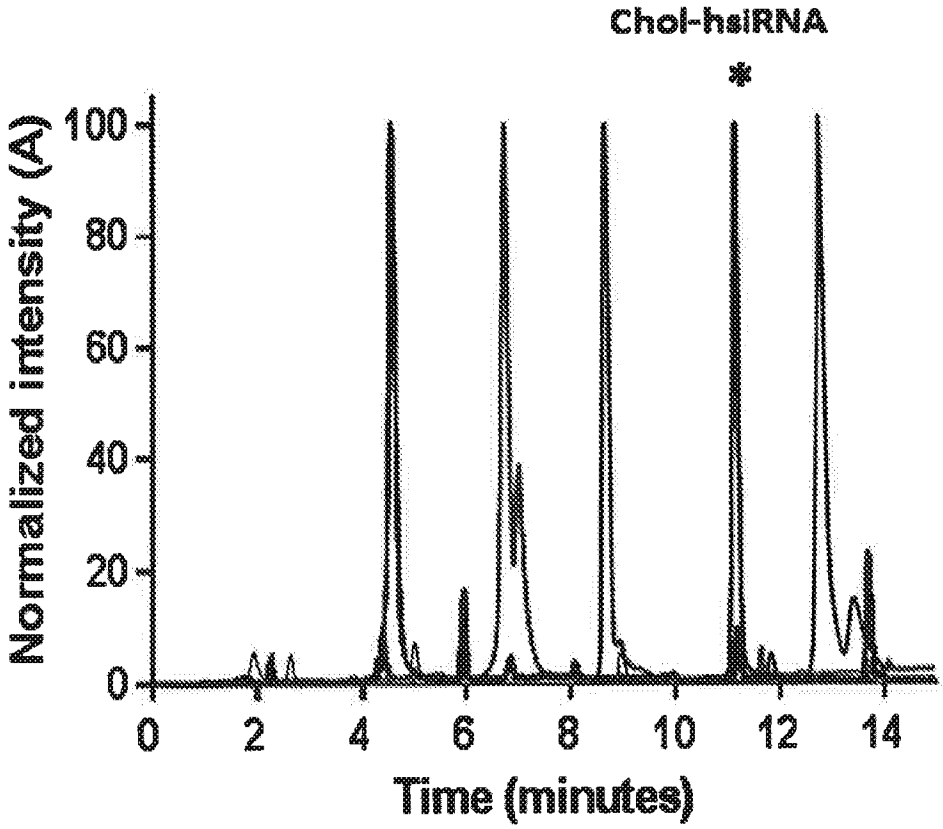


Fig. 40B

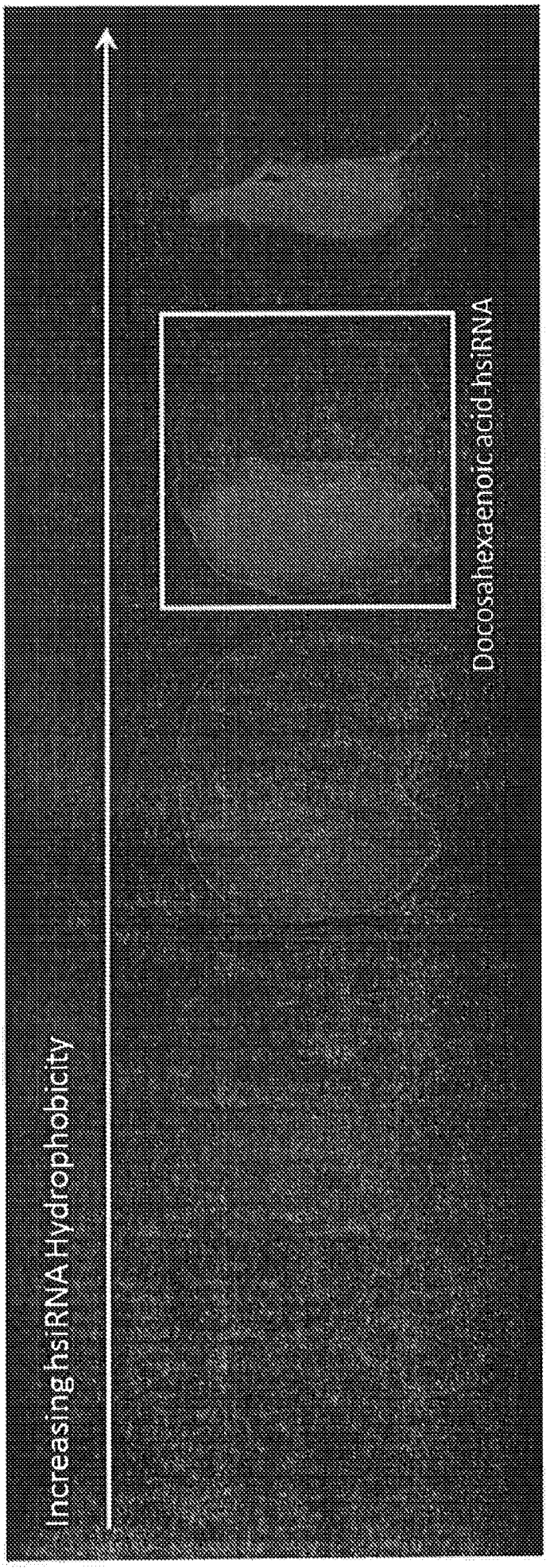
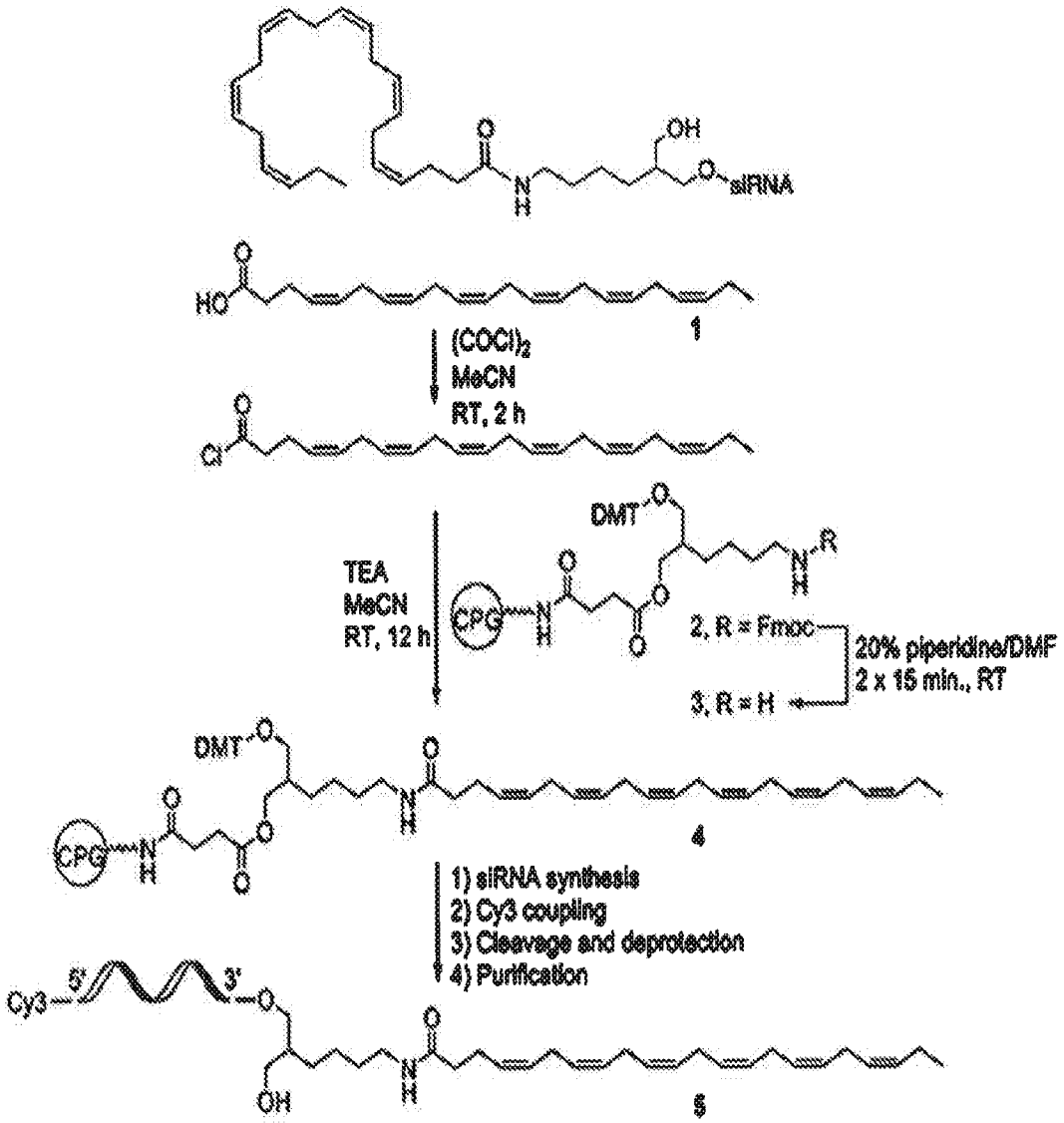

Increasing hsiRNA Hydrophobicity
Docosahexaenoic acid hsiRNA

Fig. 41


OH
siRNA
HO
1
(COCl)2
MeCN
RT, 2 h
Cl
DMT
R
TEA
MeCN
RT, 12 h
CPG
2, R = Fmoc
20% piperidine/DMF
2 x 15 min., RT
3, R = H
4
1) siRNA synthesis
2) Cy3 coupling
3) Cleavage and deprotection
4) Purification
Cy3
5'
3'
5

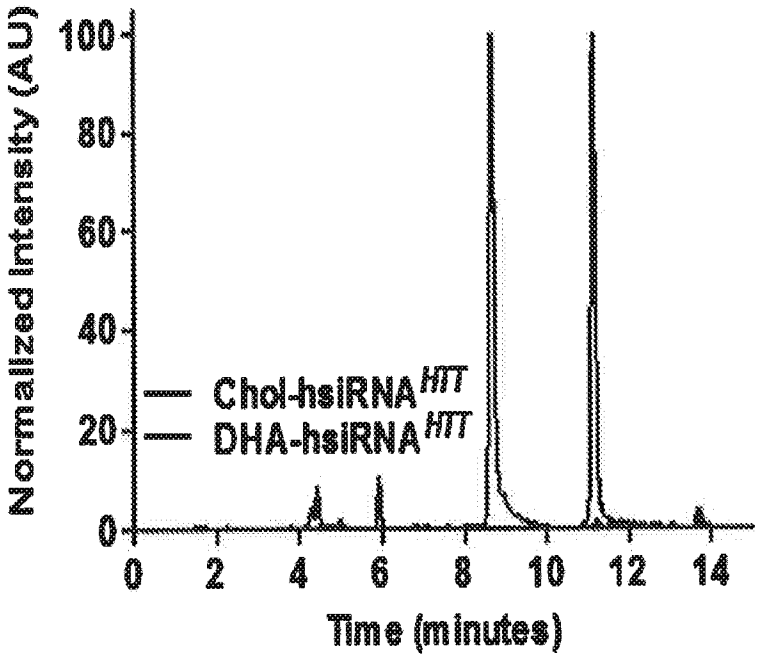
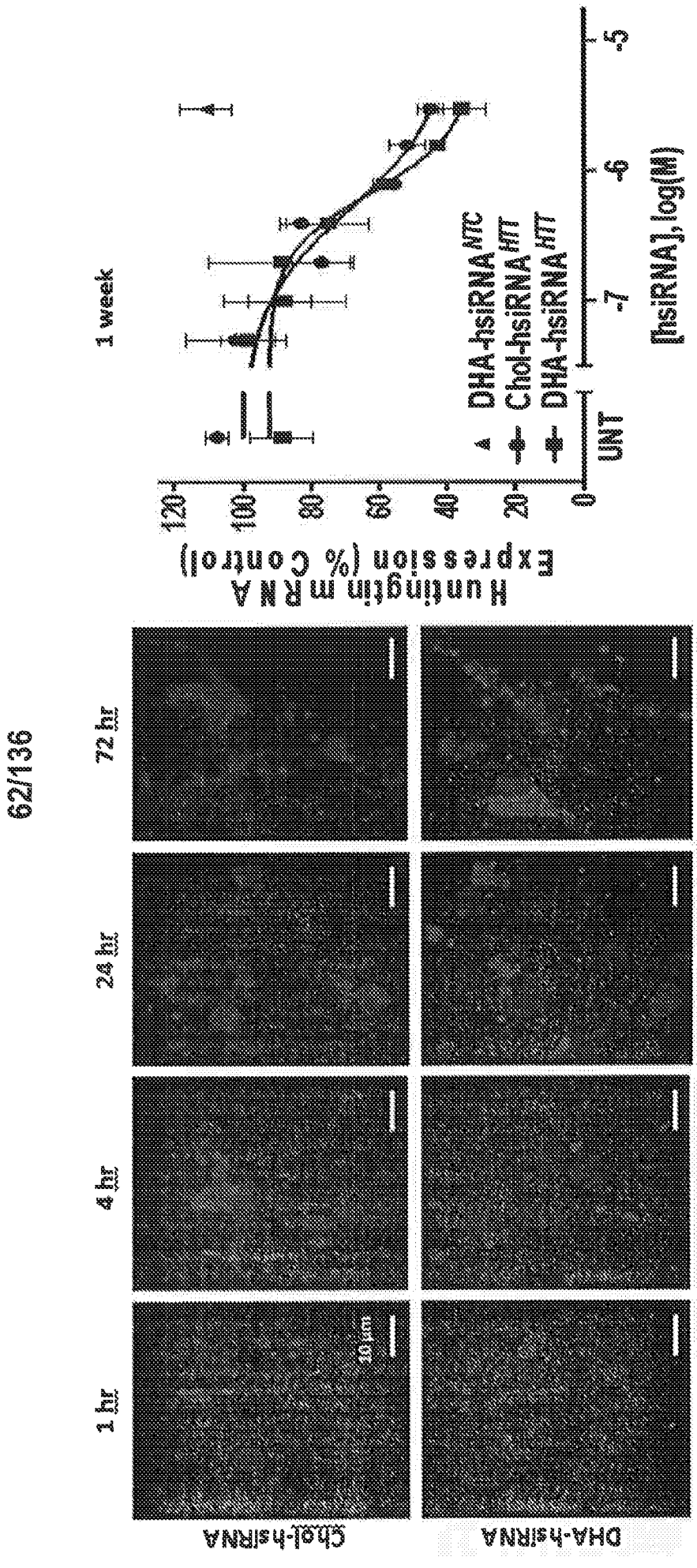

Normalized Intensity (AU)
100
80
60
40
20
0
Chol-hsiRNA HIT
DHA-hsiRNA HIT
0
2
4
6
8
10
12
14
Time (minutes)

Fig. 42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1 hr
4 hr
24 hr
72 hr
10 μm
Chol-hsiRNA
DHA-hsiRNA
1 week
Huntingtin mRNA Expression (% Control)
120
100
80
60
40
20
0
UNT
-7
-6
-5
[hsiRNA], log(M)
DHA-hsiRNA^NTC
Chol-hsiRNA^HTT
DHA-hsiRNA^HTT

Fig. 43

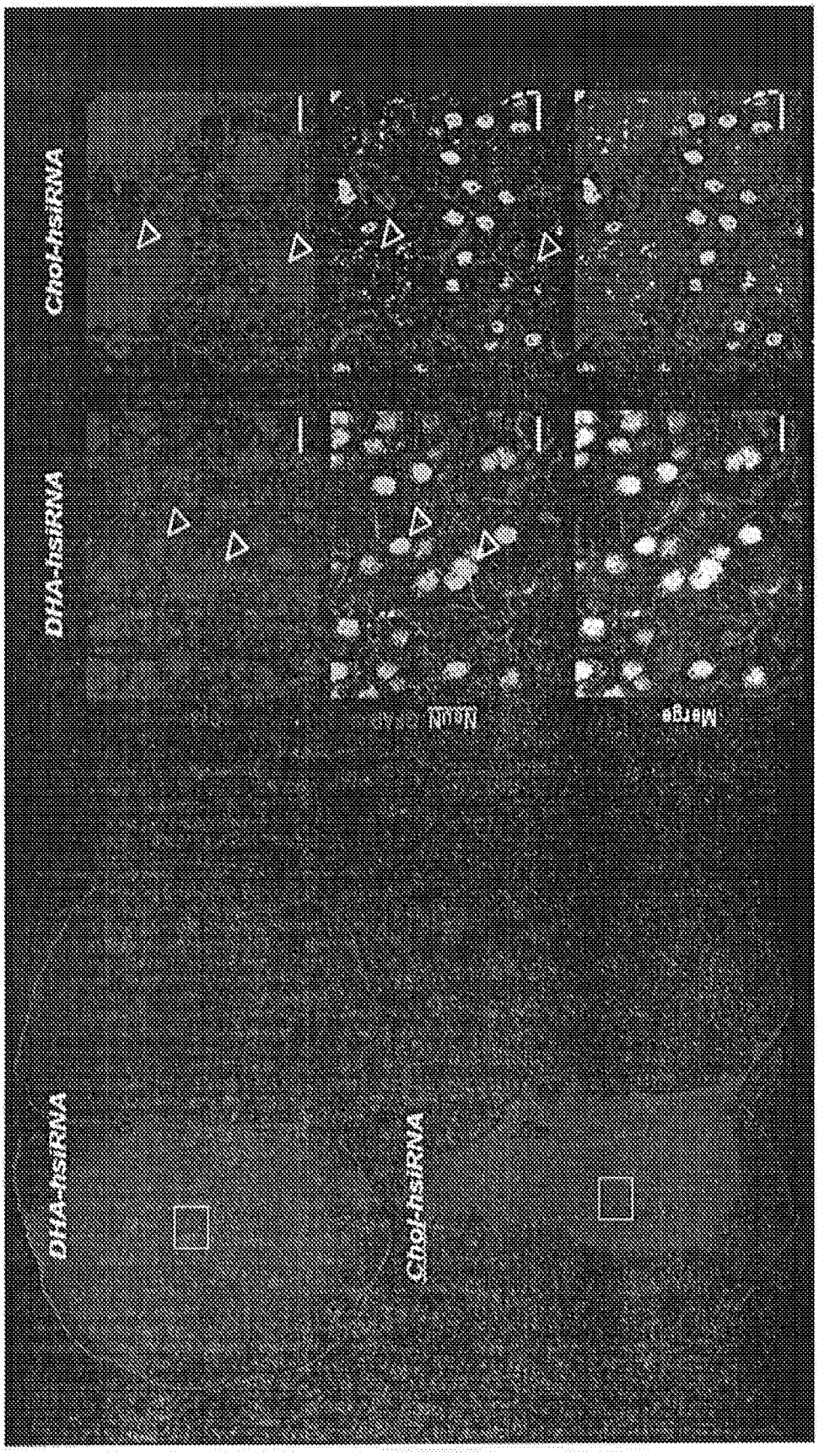

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Fig. 44

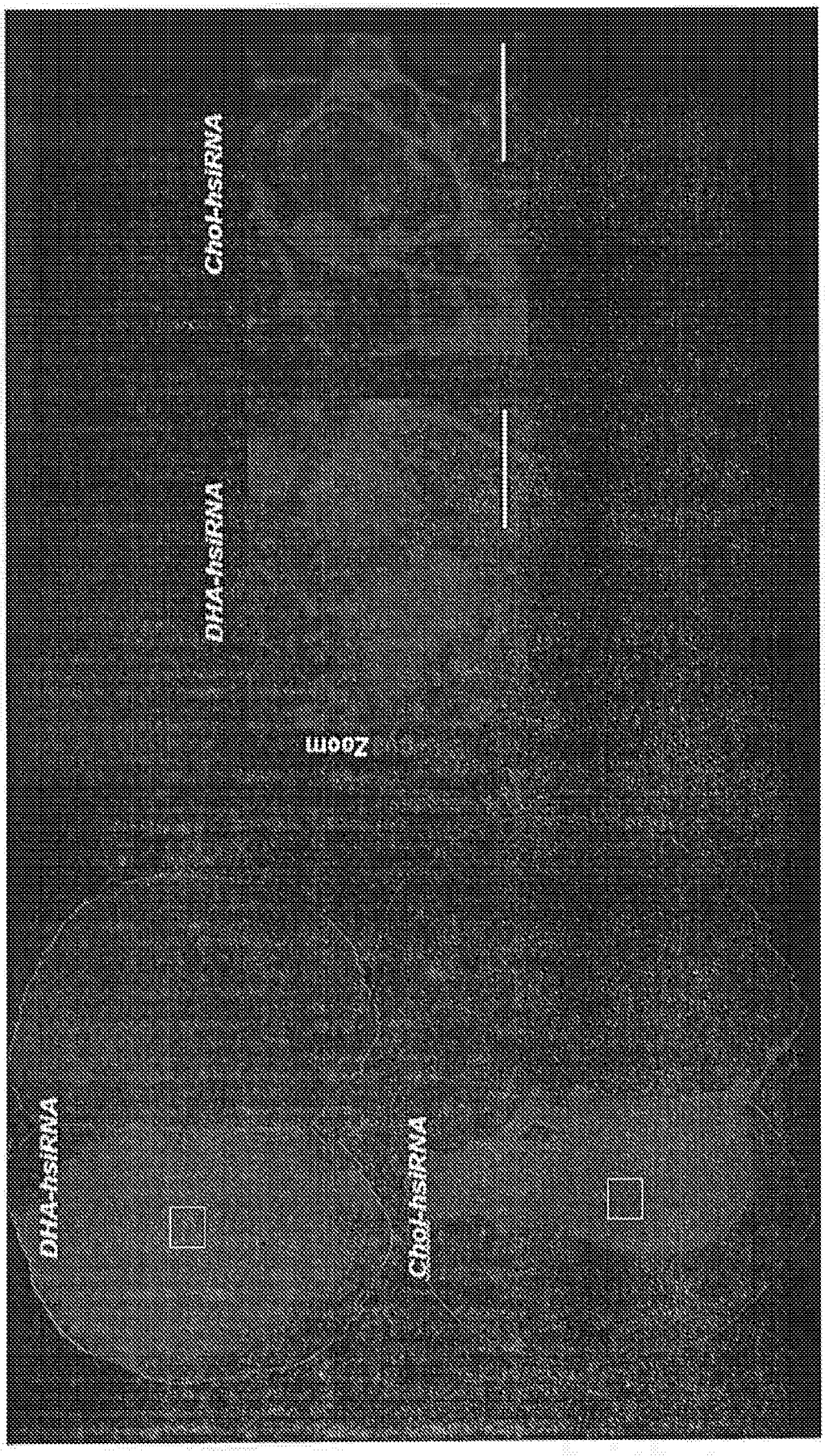

Fig. 45

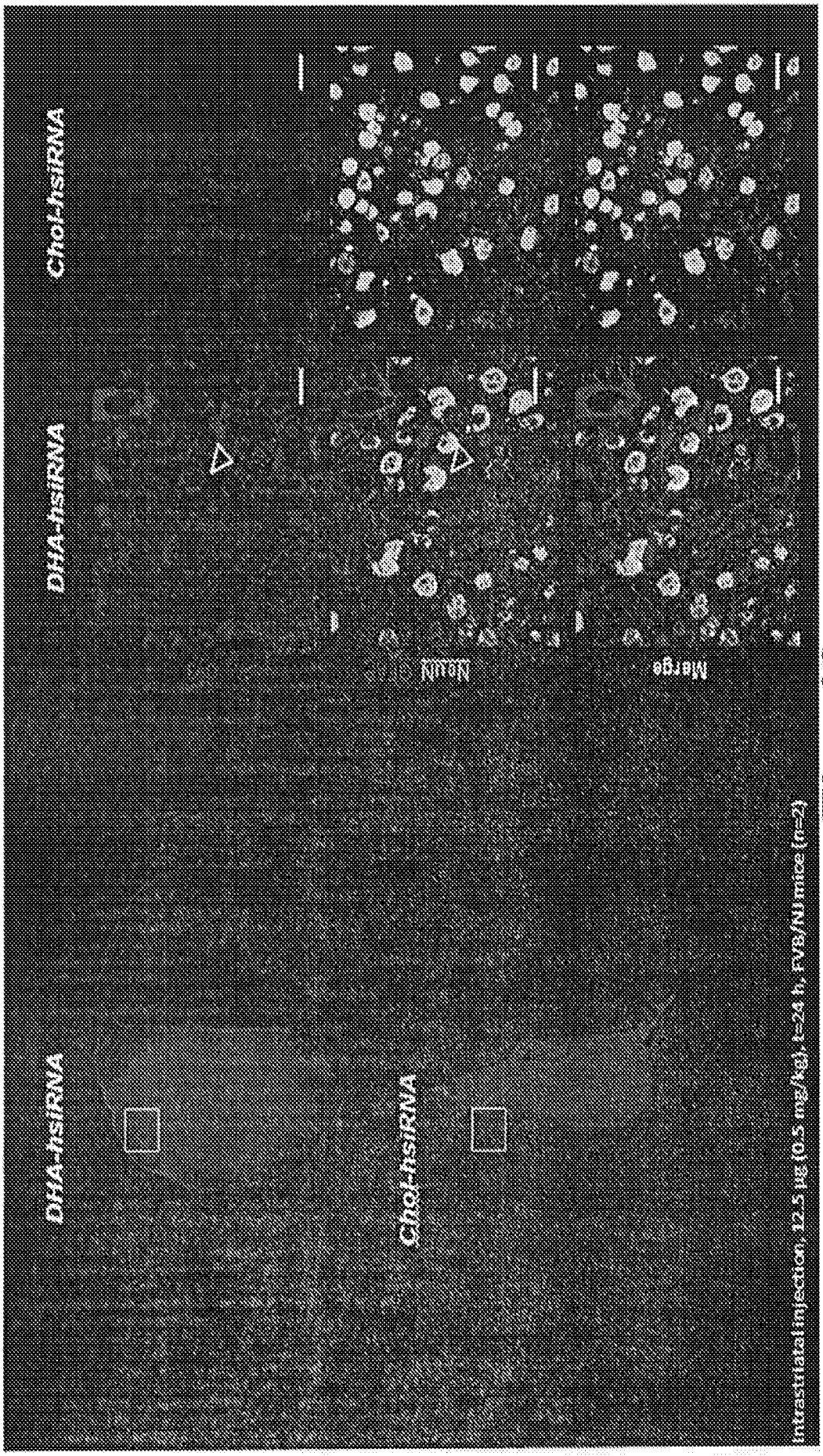
DHA-hsiRNA
Chol-hsiRNA
DHA-hsiRNA
Chol-hsiRNA
Nolin
Merge
intrastriatal injection, 12.5 μg (0.5 mg/kg), t=24 h, FVB/NJ mice (n=2)

Fig. 46

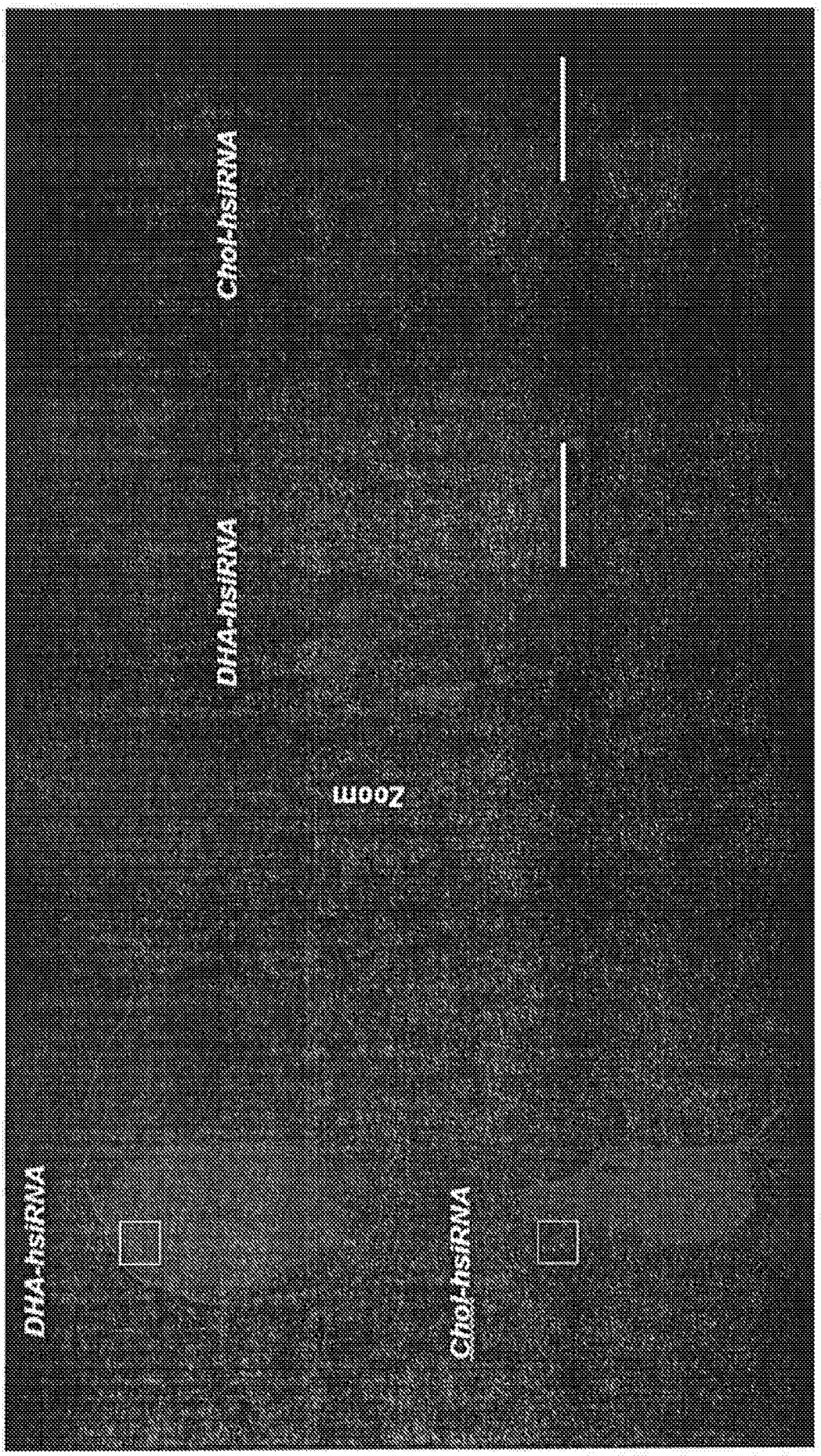

Fig. 47

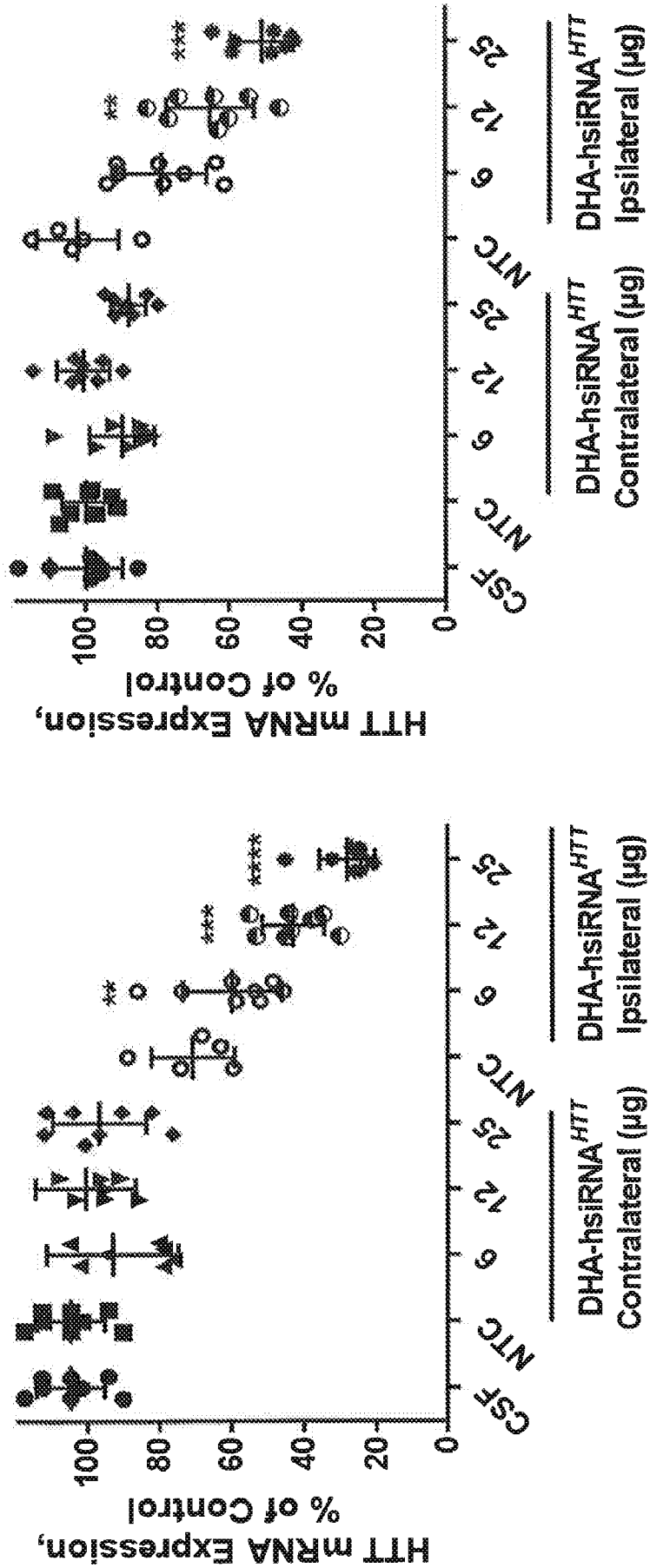
HTT mRNA Expression, % of Control
100
80
60
40
20
0
CSF
NTC
6
12
25
DHA-hsiRNA^HTT Contralateral (µg)
DHA-hsiRNA^HTT Ipsilateral (µg)
HTT mRNA Expression, % of Control
100
80
60
40
20
0
CSF
NTC
6
12
25
DHA-hsiRNA^HTT Contralateral (µg)
DHA-hsiRNA^HTT Ipsilateral (µg)

Fig. 48

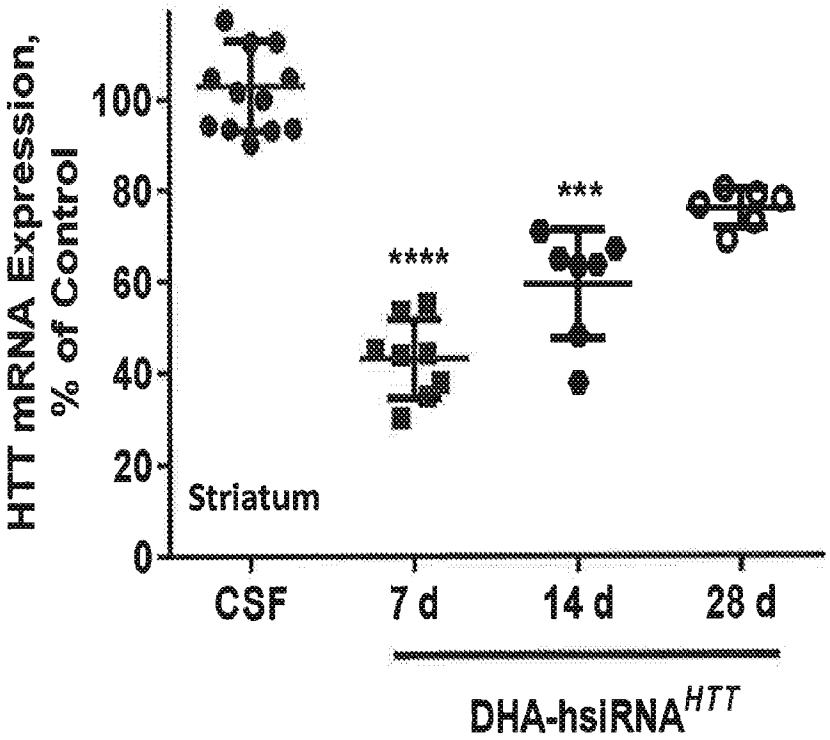
HTT mRNA Expression, % of Control
100
80
60
40
20
0
Striatum
****
***
CSF
7 d
14 d
28 d
DHA-hsiRNA^HTT

Fig. 49

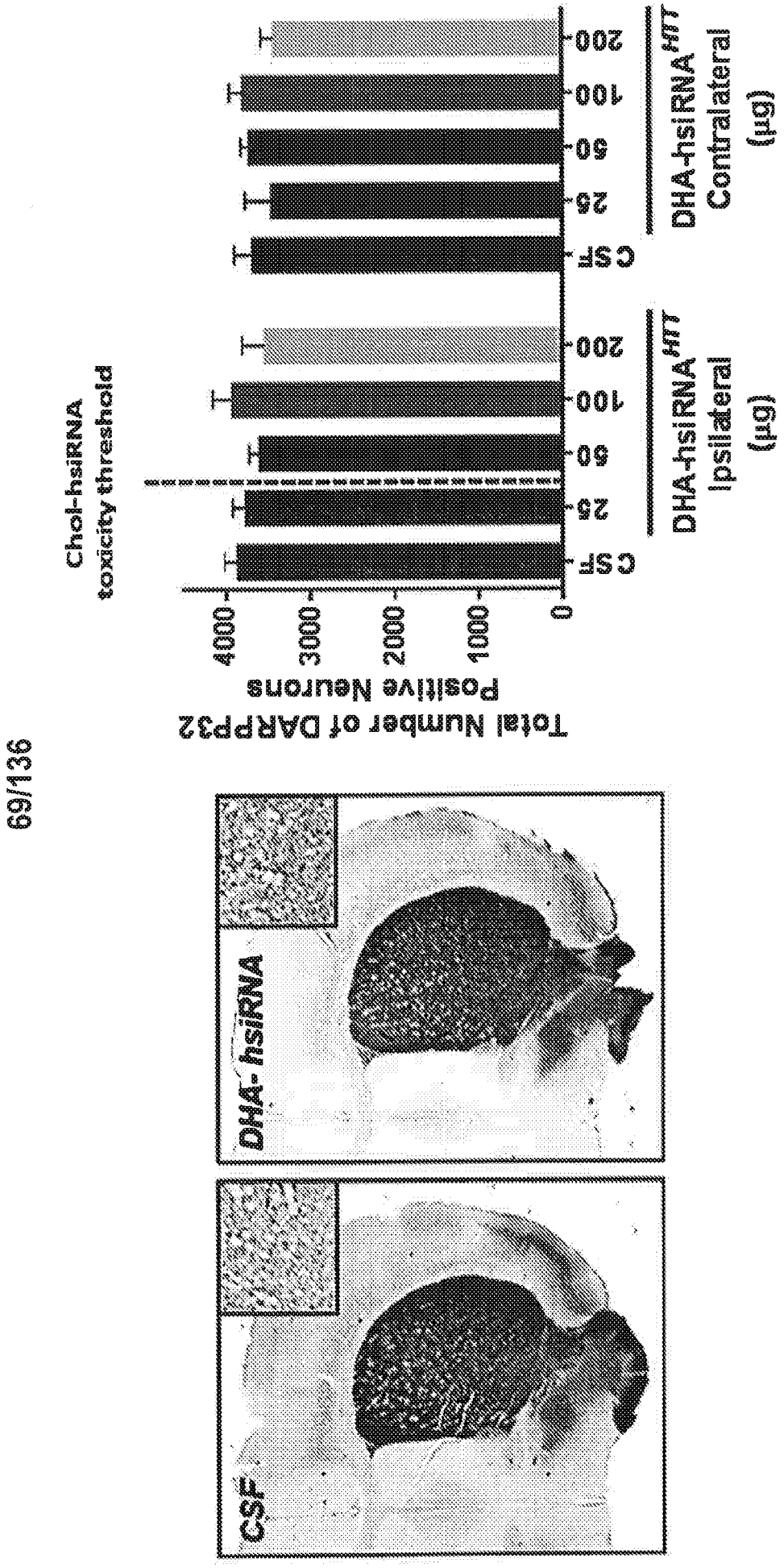
CSF
DHA- hsiRNA
Total Number of DARPP32 Positive Neurons
4000
3000
2000
1000
0
CSF
25
50
100
200
DHA-hsiRNA^HTT Ipsilateral (µg)
CSF
25
50
100
200
DHA-hsiRNA^HTT Contralateral (µg)
Chol-hsiRNA toxicity threshold

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Fig. 50

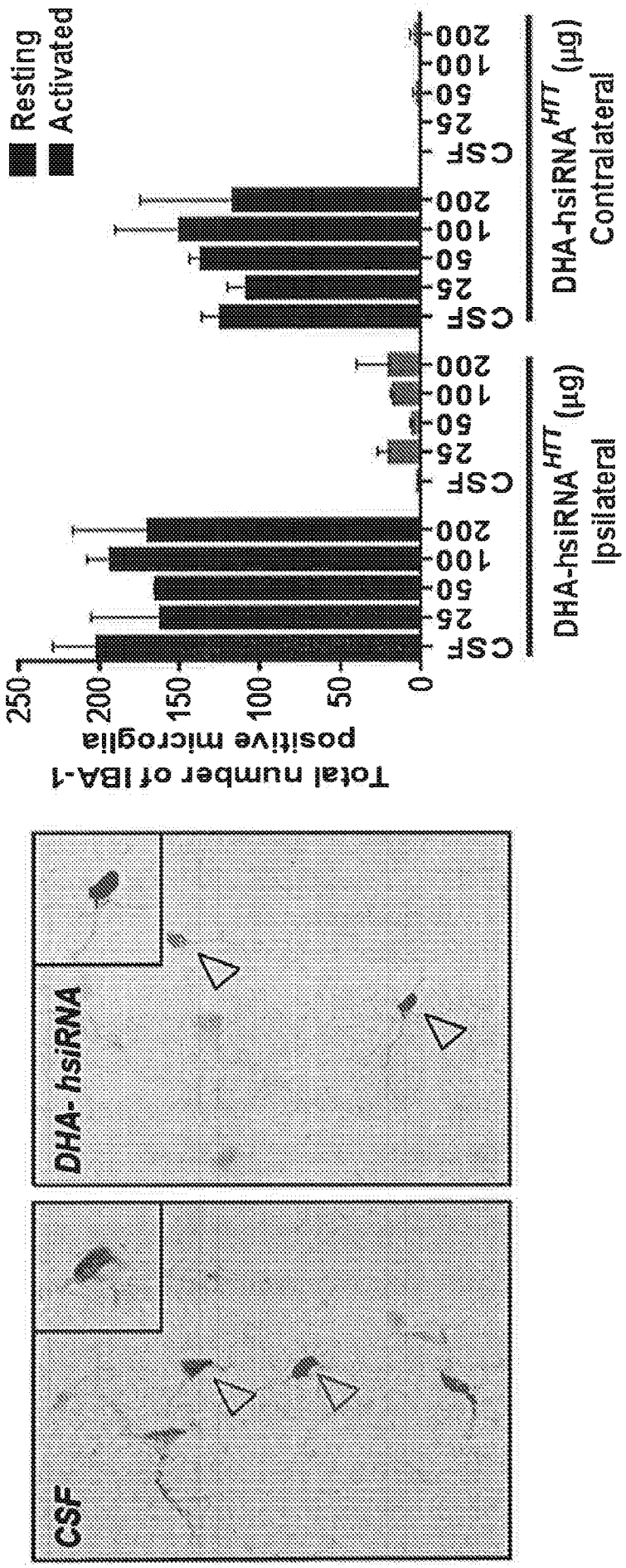
CSF
DHA- hsiRNA
Total number of IBA-1 positive microglia
250
200
150
100
50
0
Resting
Activated
CSF
25
50
100
200
DHA-hsiRNA^{HTT} (µg)
Ipsilateral
DHA-hsiRNA^{HTT} (µg)
Contralateral

Fig. 51

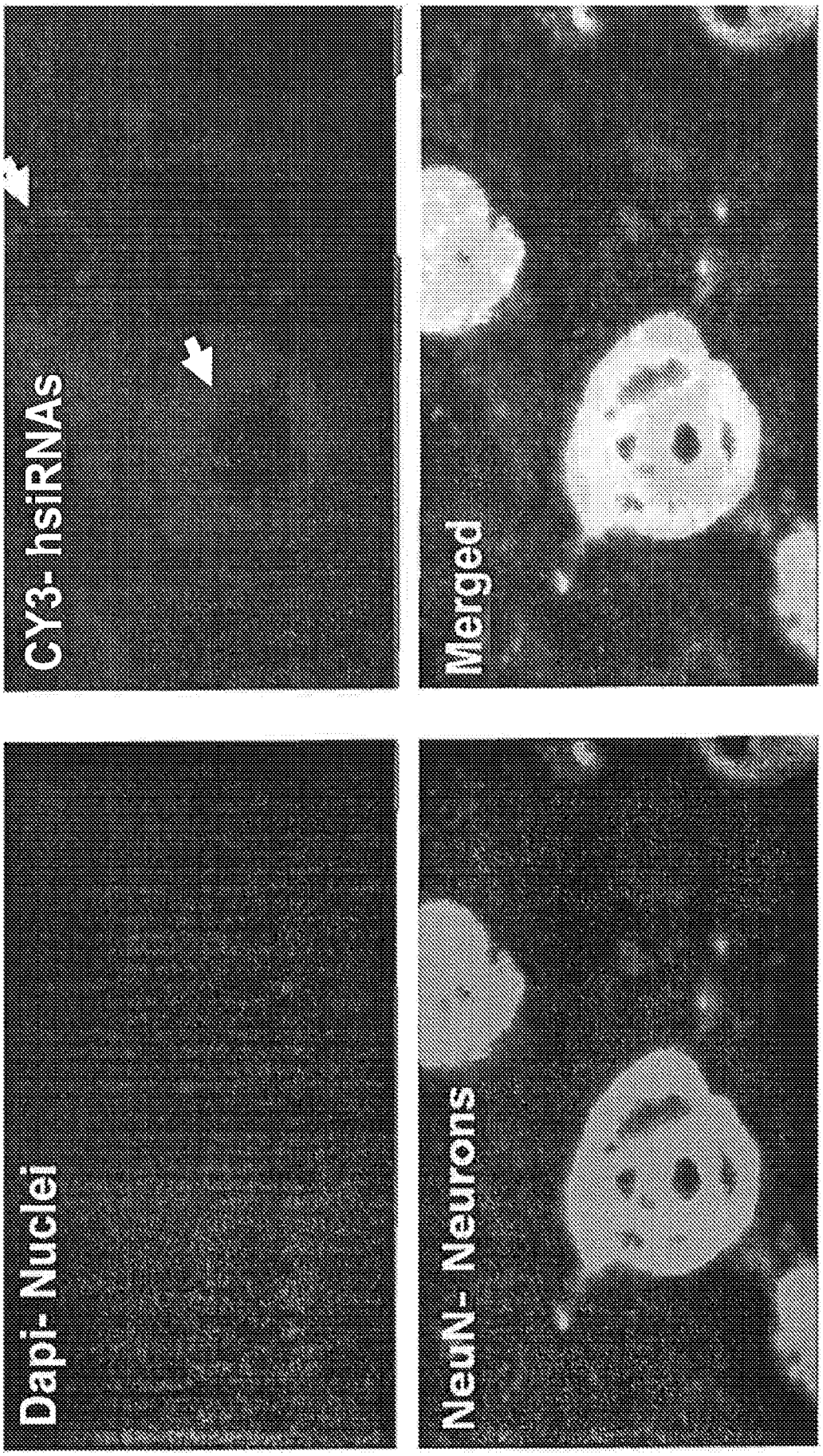

Fig. 52

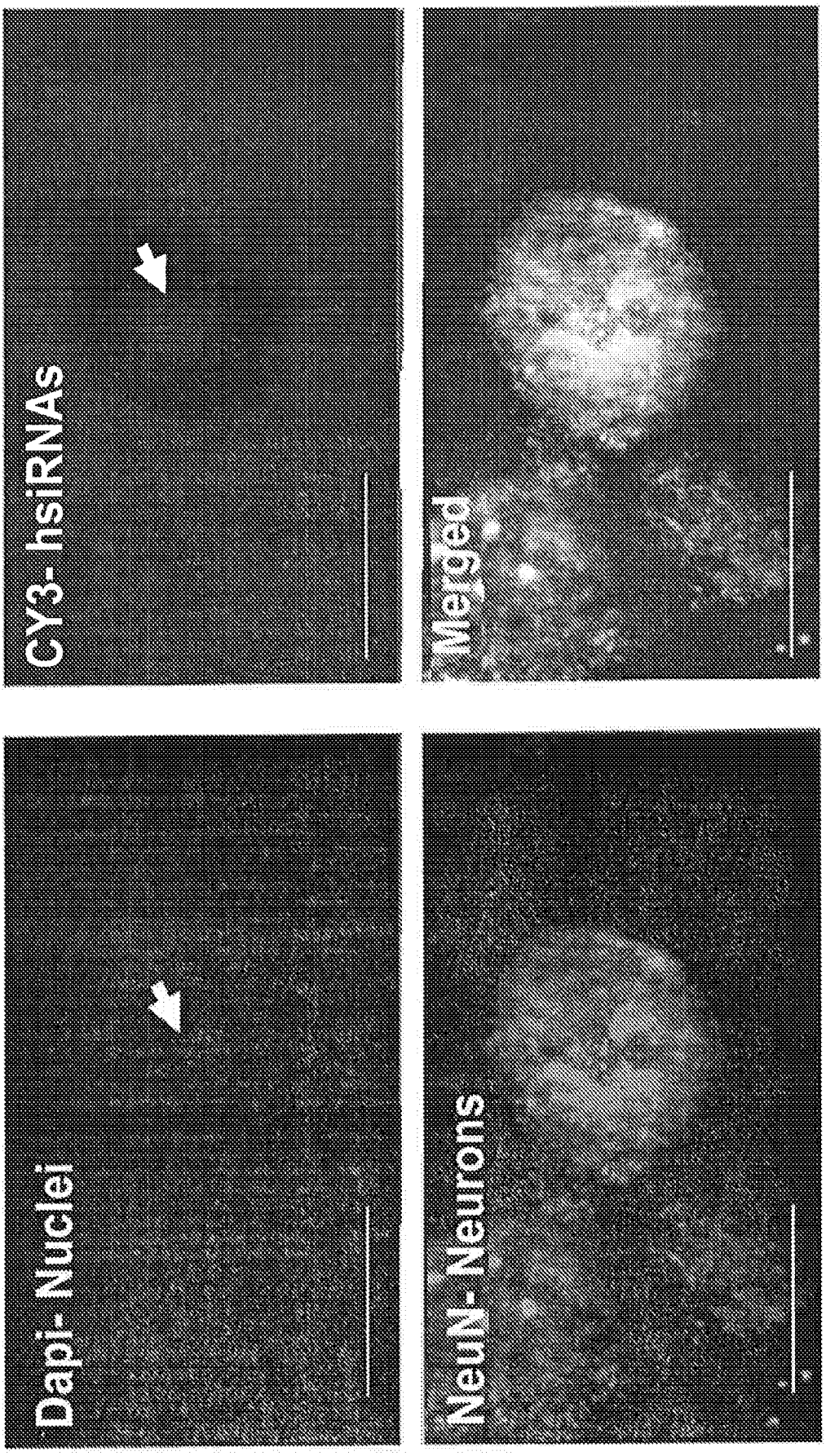

Fig. 53

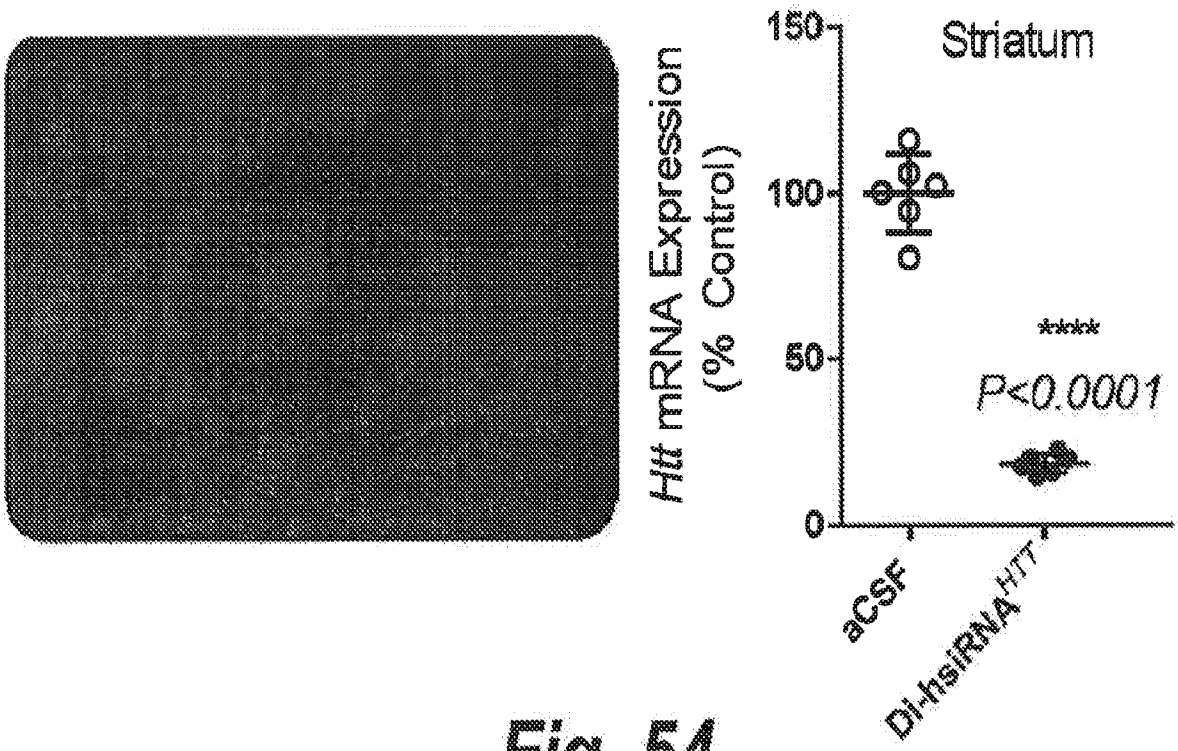
Striatum
Htt mRNA Expression
(% Control)
150
100
50
0
****
P<0.0001
aCSF
DI-HttsiRNA HTT

Fig. 54

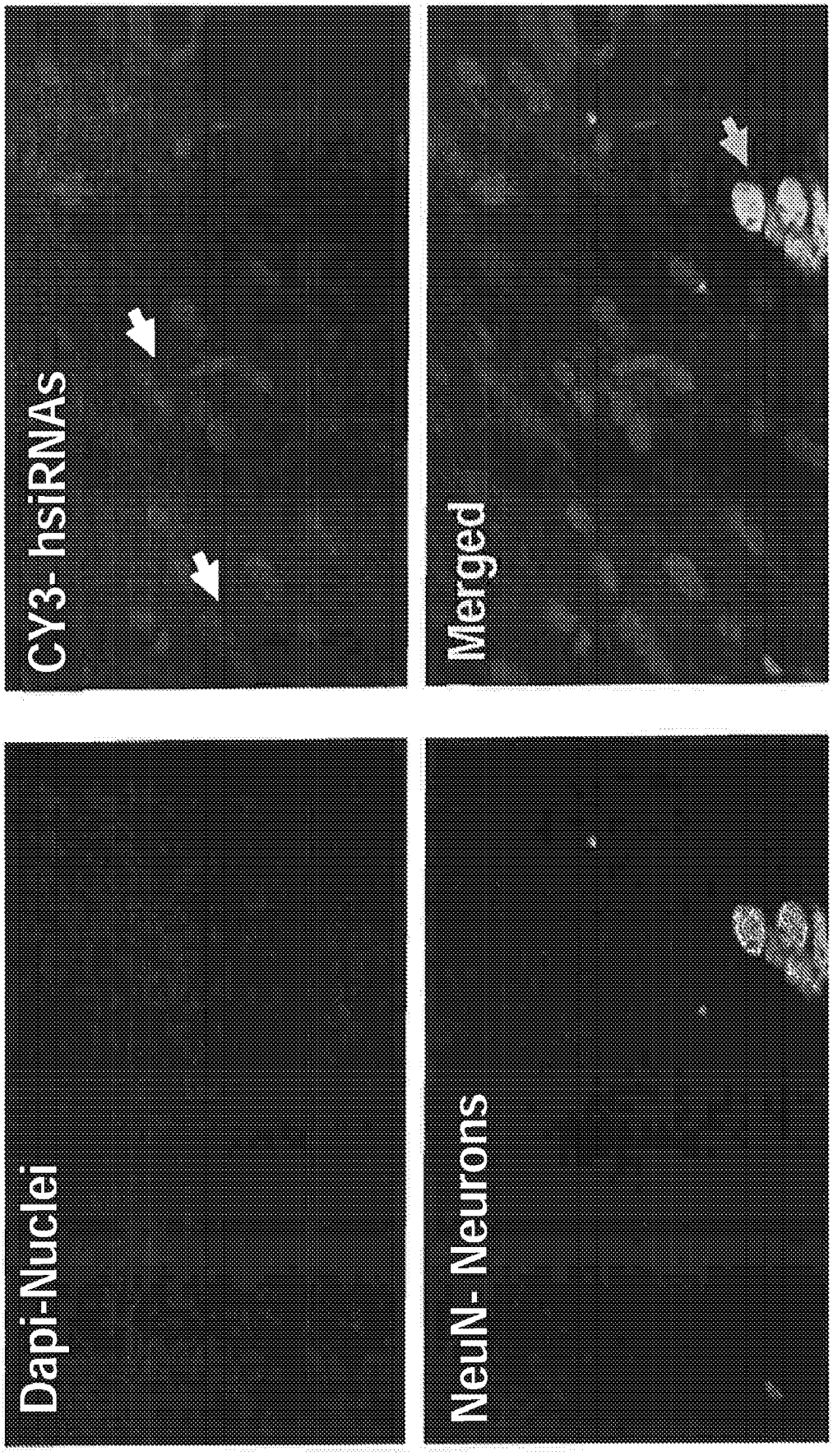

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Fig. 55

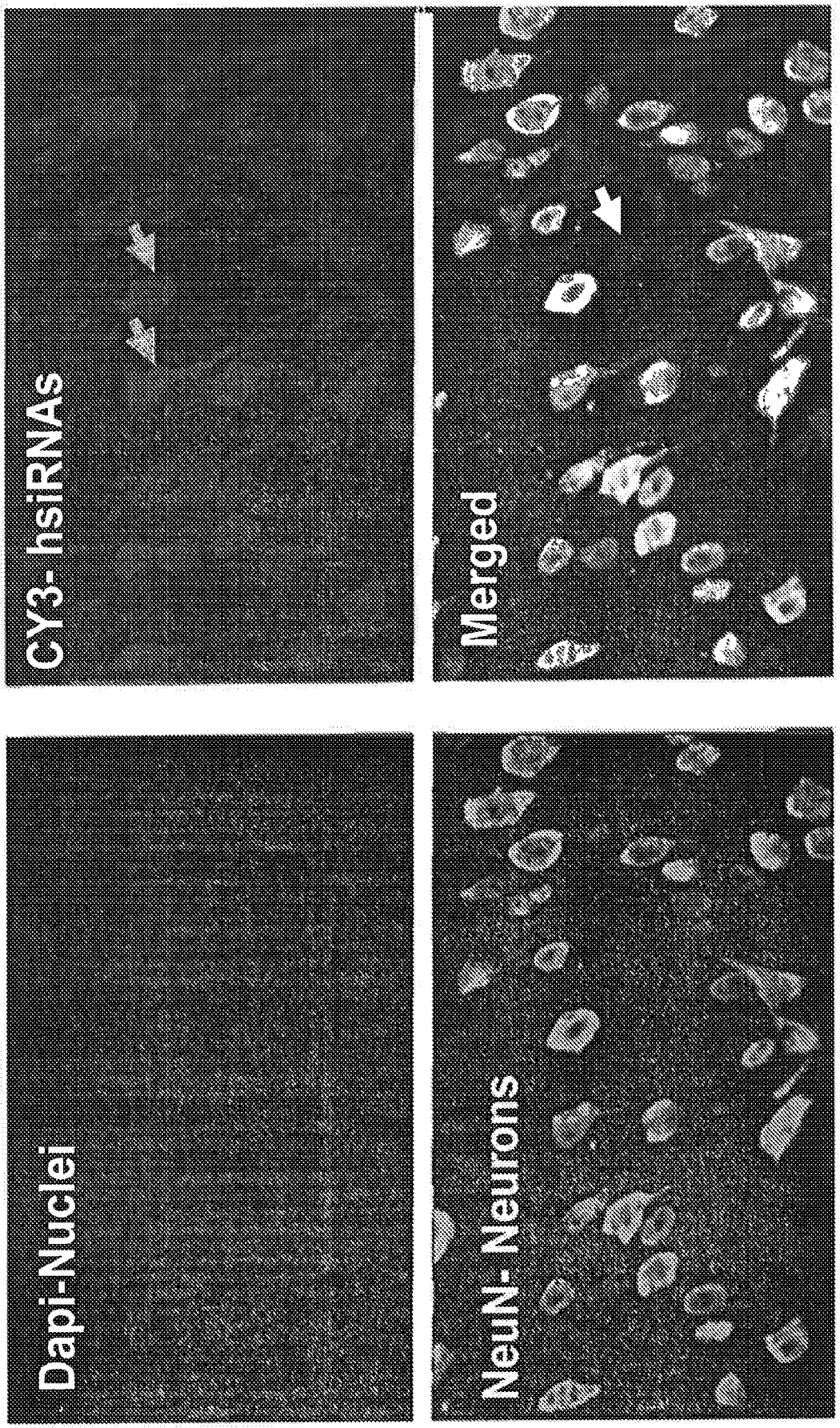


Fig. 56

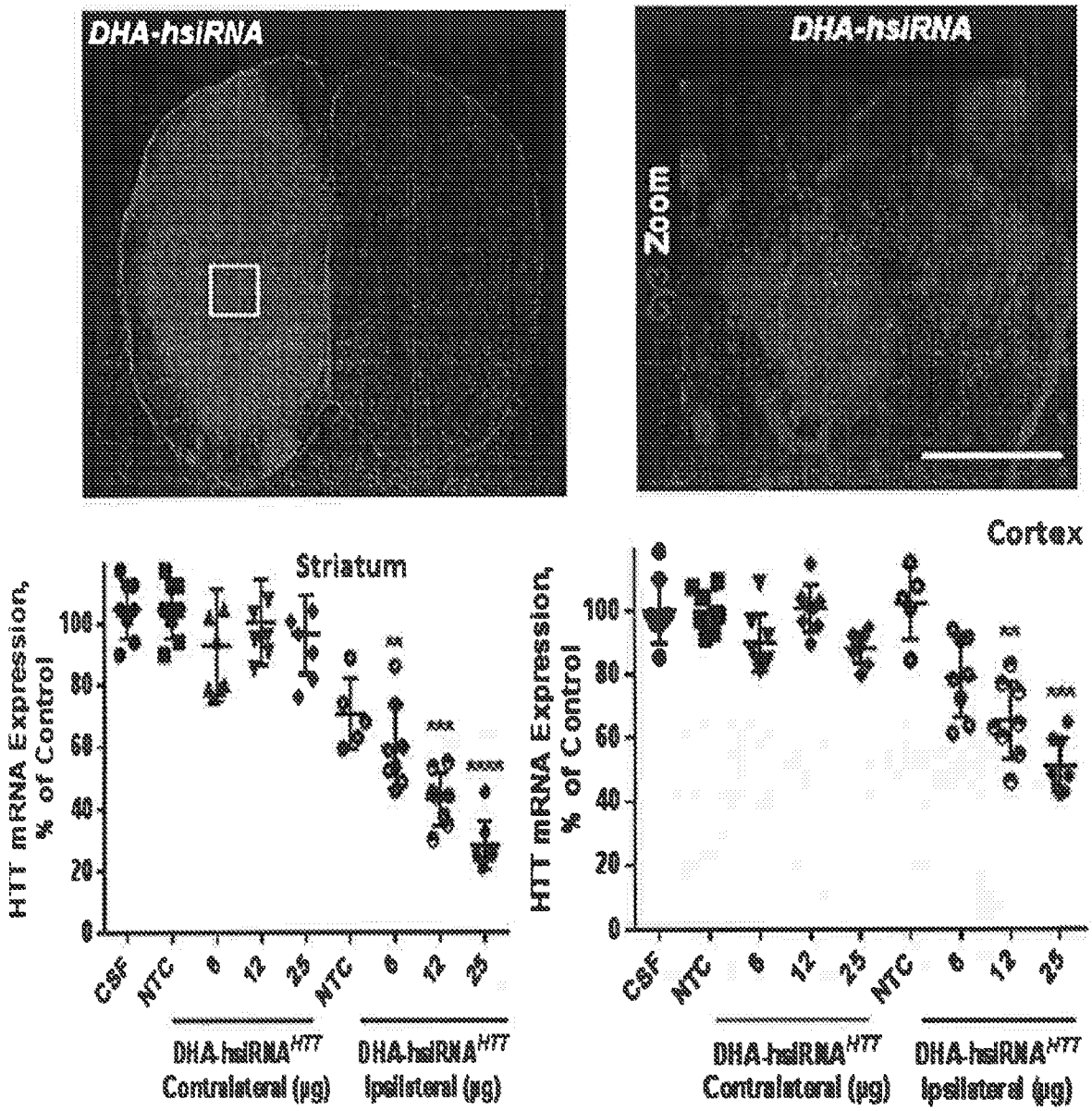

DHA-hsiRNA
DHA-hsiRNA
Zoom
Striatum
Cortex
HTT mRNA Expression, % of Control
HTT mRNA Expression, % of Control
CSF
NTC
6
12
25
NTC
6
12
25
DHA-hsiRNA^HTT Contralateral (µg)
DHA-hsiRNA^HTT Ipsilateral (µg)

Fig. 57

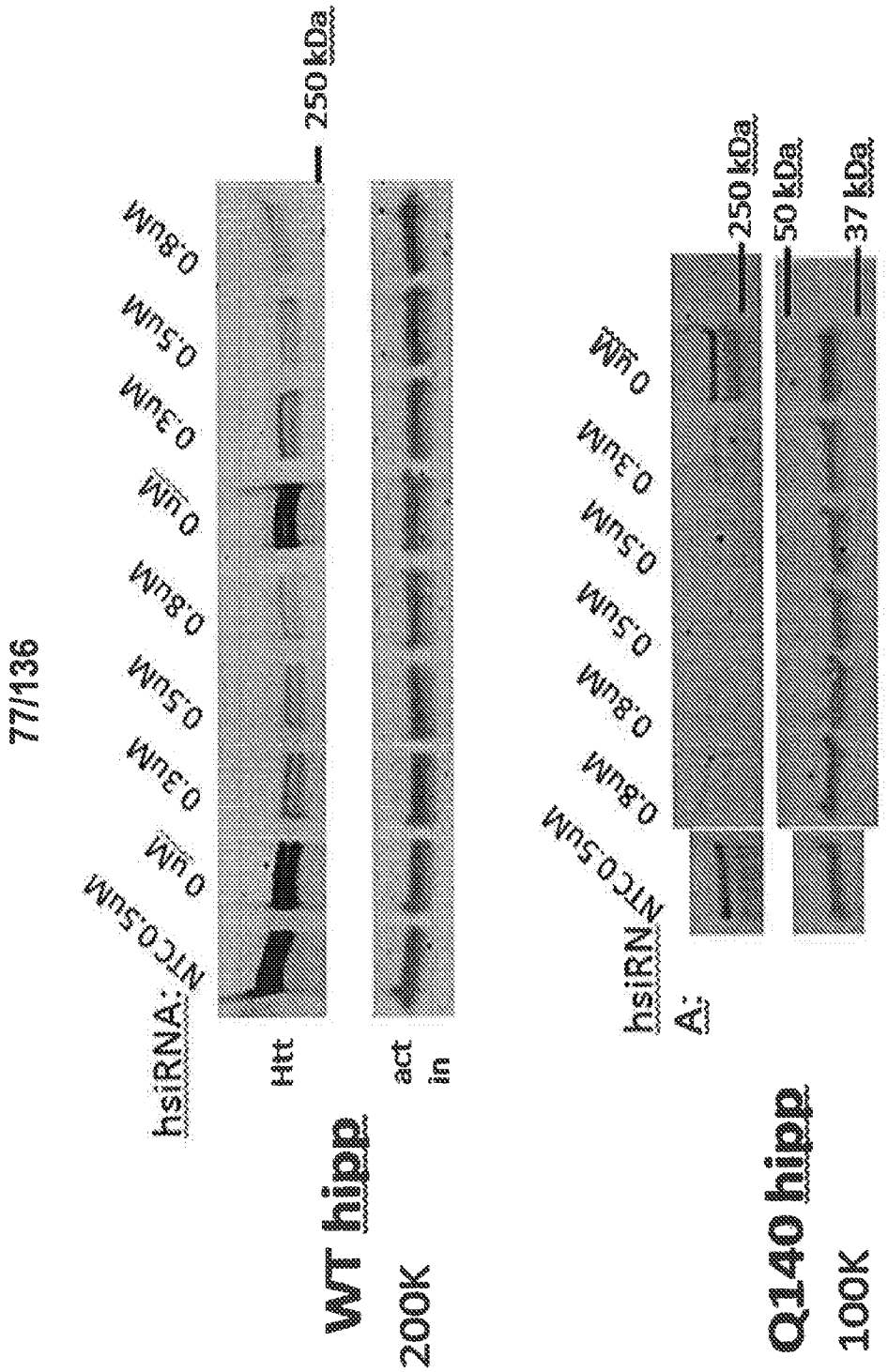

77/136
hsiRNA:
NTC0.5uM
0uM
0.3uM
0.5uM
0.8uM
0uM
0.3uM
0.5uM
0.8uM
250 kDa
Htt
actin
WT hipp
200K
hsiRNA:
NTC0.5uM
0.8uM
0.5uM
0.5uM
0.3uM
0uM
250 kDa
50 kDa
37 kDa
Q140 hipp
100K

Fig. 58

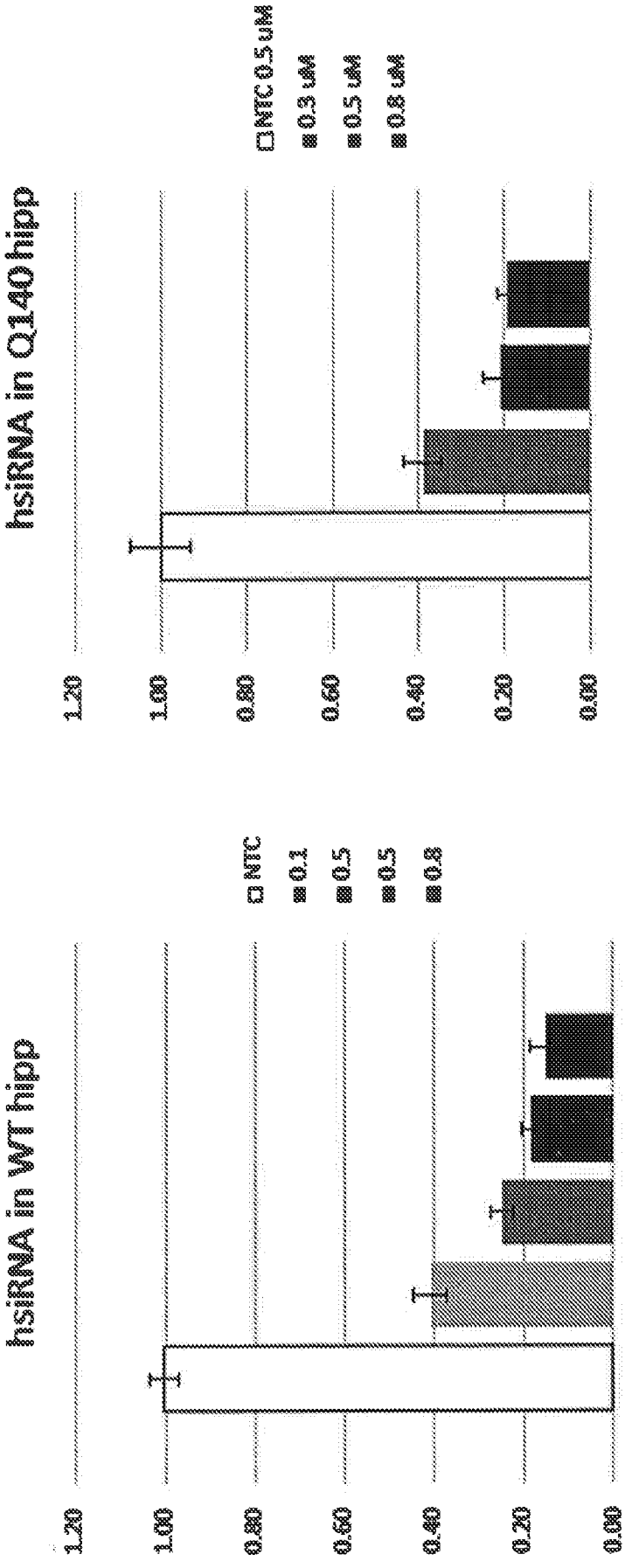

hsiRNA in Q140 hipp
NTC 0.5 uM
0.3 uM
0.5 uM
0.8 uM
1.20
1.00
0.80
0.60
0.40
0.20
0.00
hsiRNA in WT hipp
NTC
0.1
0.5
0.5
0.8
1.20
1.00
0.80
0.60
0.40
0.20
0.00

Fig. 59

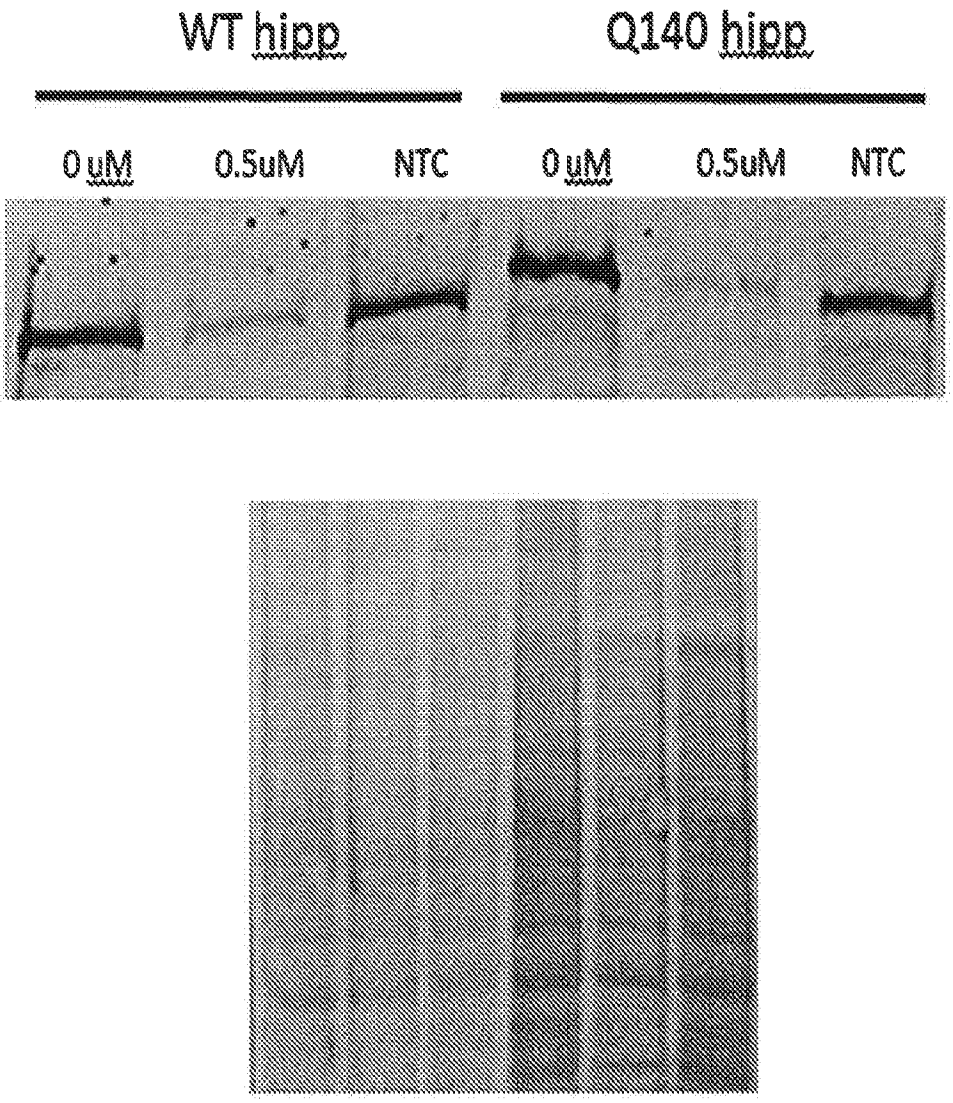


Fig. 60

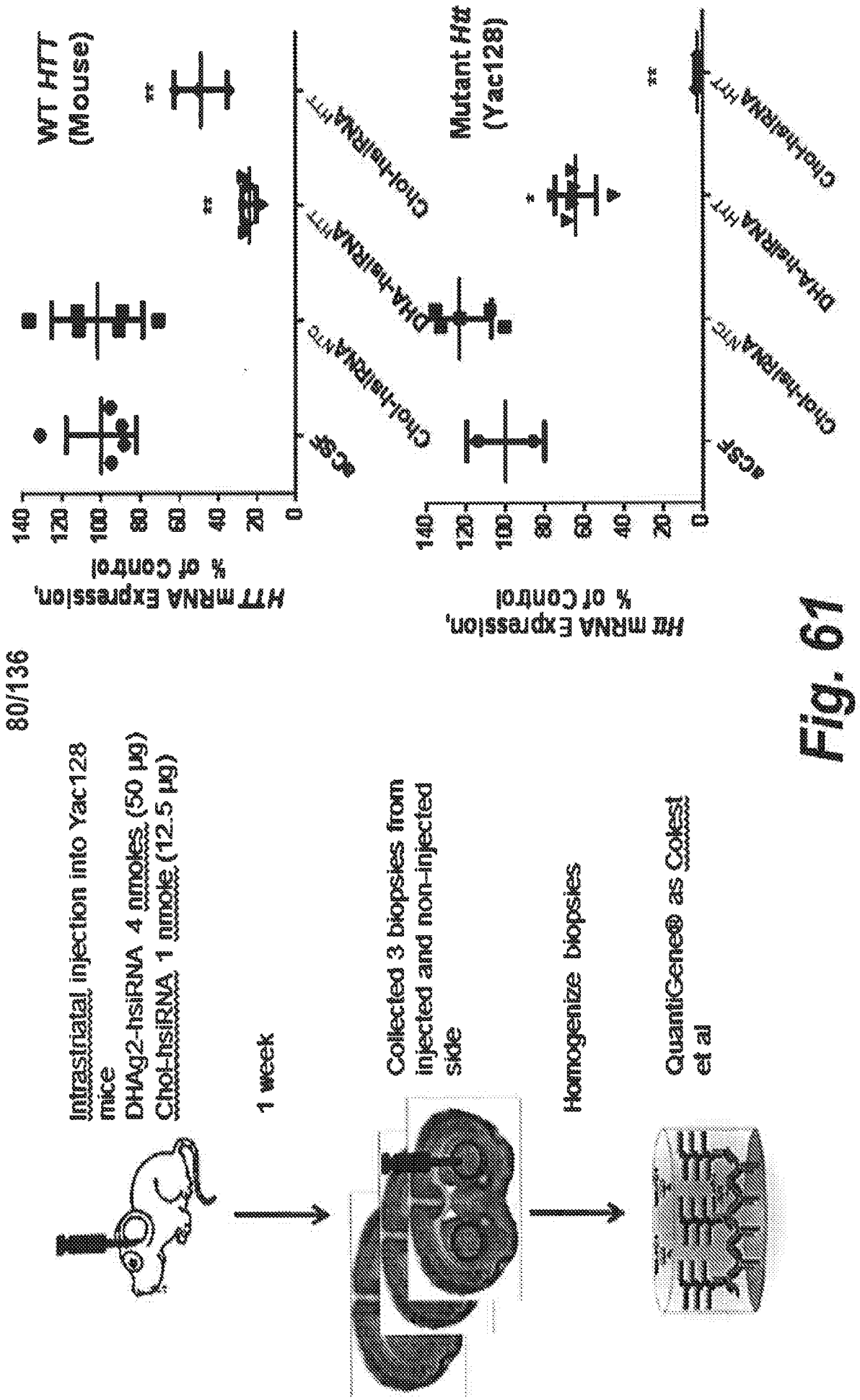

80/136
Intrastriatal injection into Yac128 mice
DHAg2-hsiRNA 4 nmoles (50 µg)
Chol-hsiRNA 1 nmole (12.5 µg)
1 week
Collected 3 biopsies from injected and non-injected side
Homogenize biopsies
QuantiGene® as Colest et al
WT HTT (Mouse)
Mutant Htt (Yac128)
HTT mRNA Expression, % of control
Htt mRNA Expression, % of control
aCSF
Chol-hsiRNA NTC
DHA-hsiRNA HTT
Chol-hsiRNA HTT
DHA-hsiRNA NTC
0
20
40
60
80
100
120
140
*
**

Fig. 61

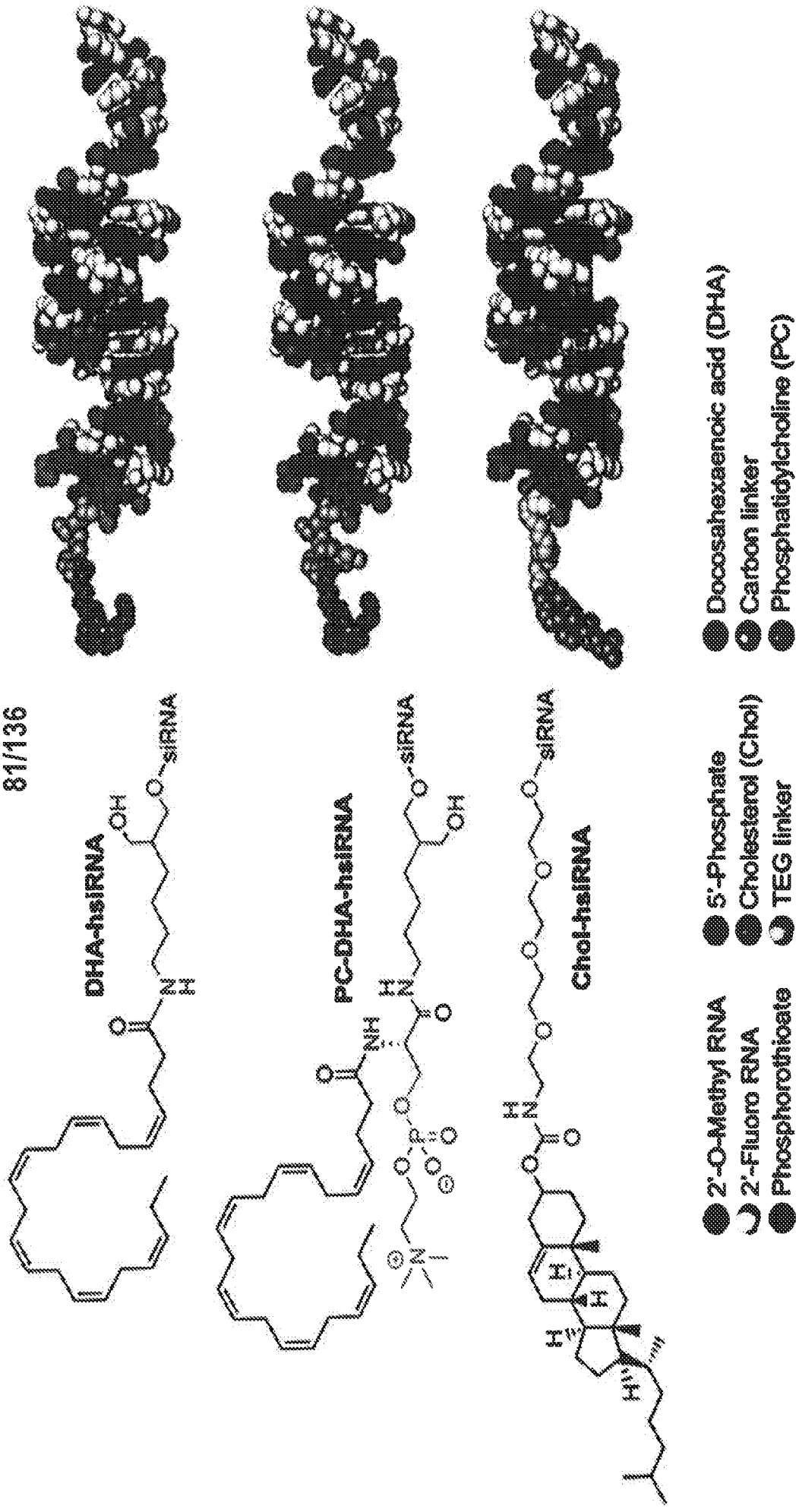

81/136
DHA-hsiRNA
PC-DHA-hsiRNA
Chol-hsiRNA
siRNA
2'-O-Methyl RNA
2'-Fluoro RNA
Phosphorothioate
5'-Phosphate
Cholesterol (Chol)
TEG linker
Docosahexaenoic acid (DHA)
Carbon linker
Phosphatidylcholine (PC)

Fig. 62

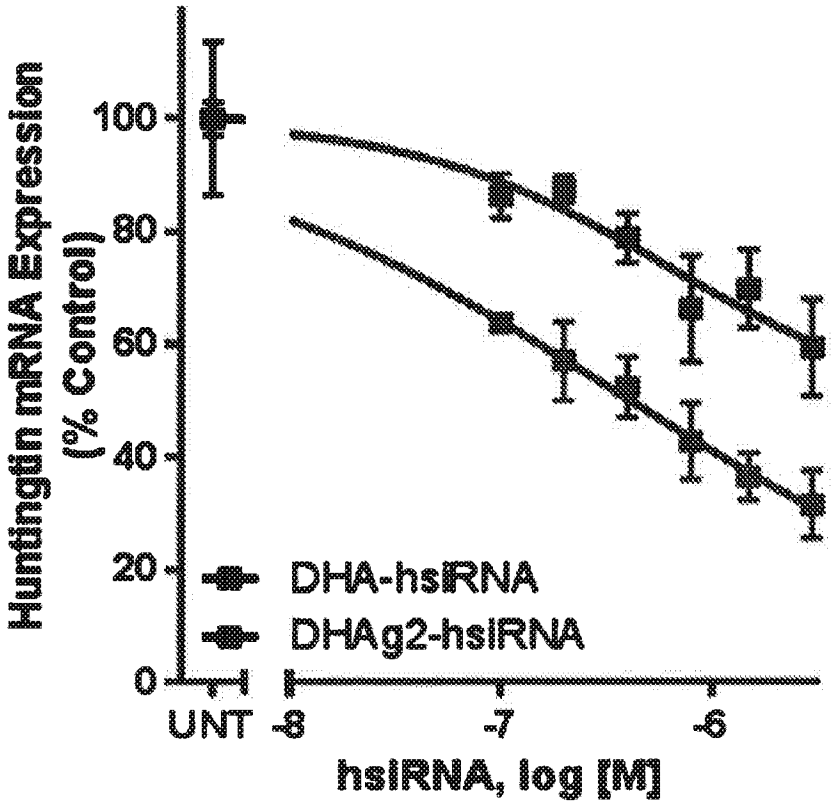


Fig. 63A

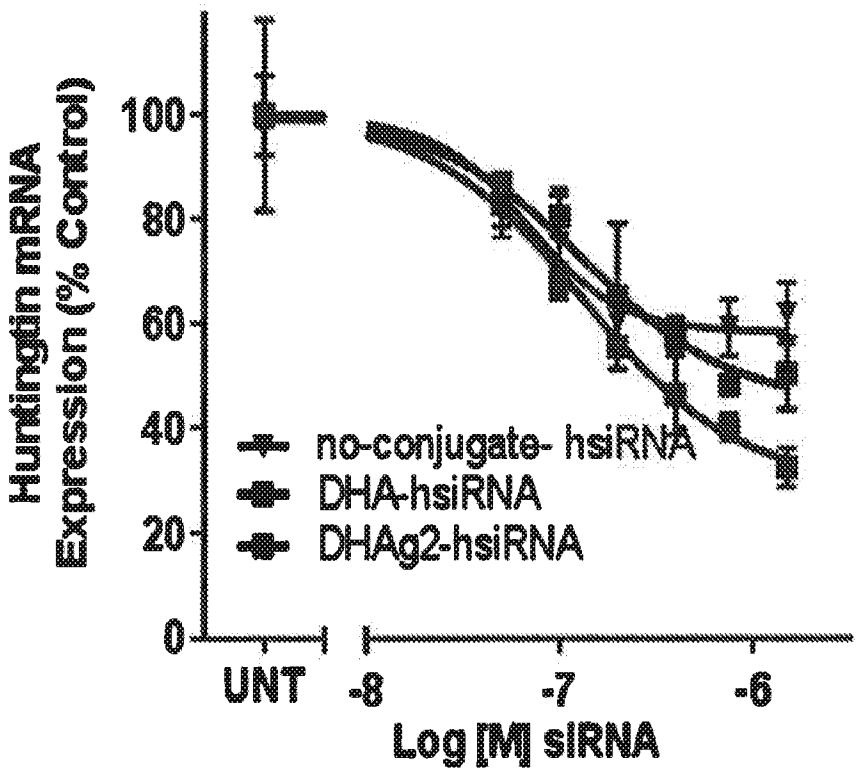


Fig. 63B

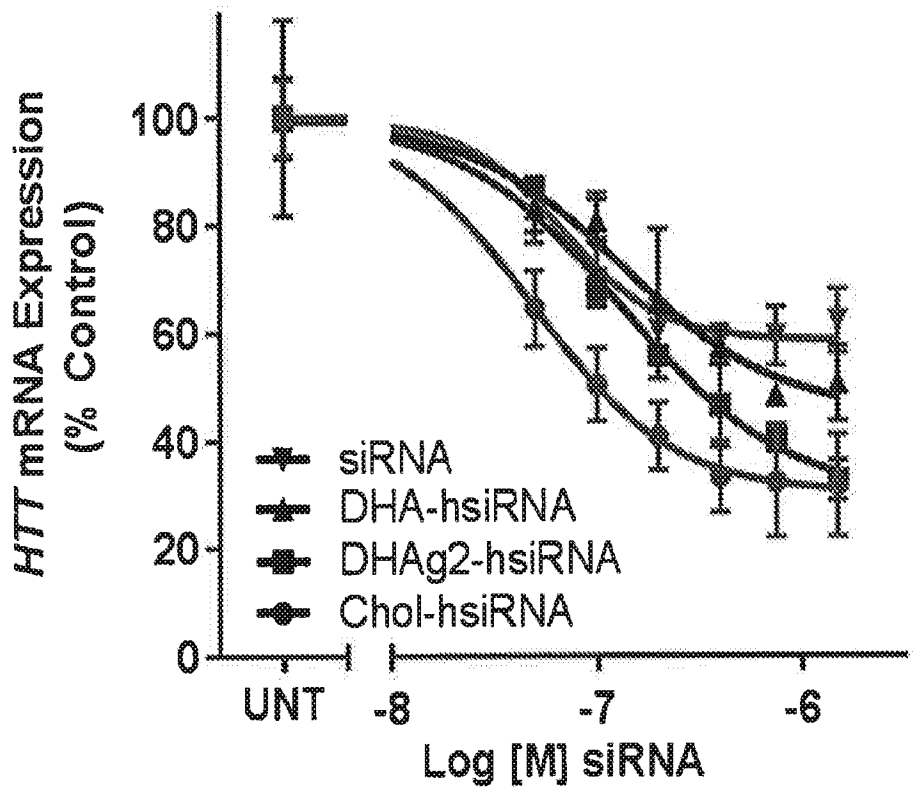

HTT mRNA Expression
(% Control)
100
80
60
40
20
0
siRNA
DHA-hsiRNA
DHAg2-hsiRNA
Chol-hsiRNA
UNT
-8
-7
-6
Log [M] siRNA

Fig. 64

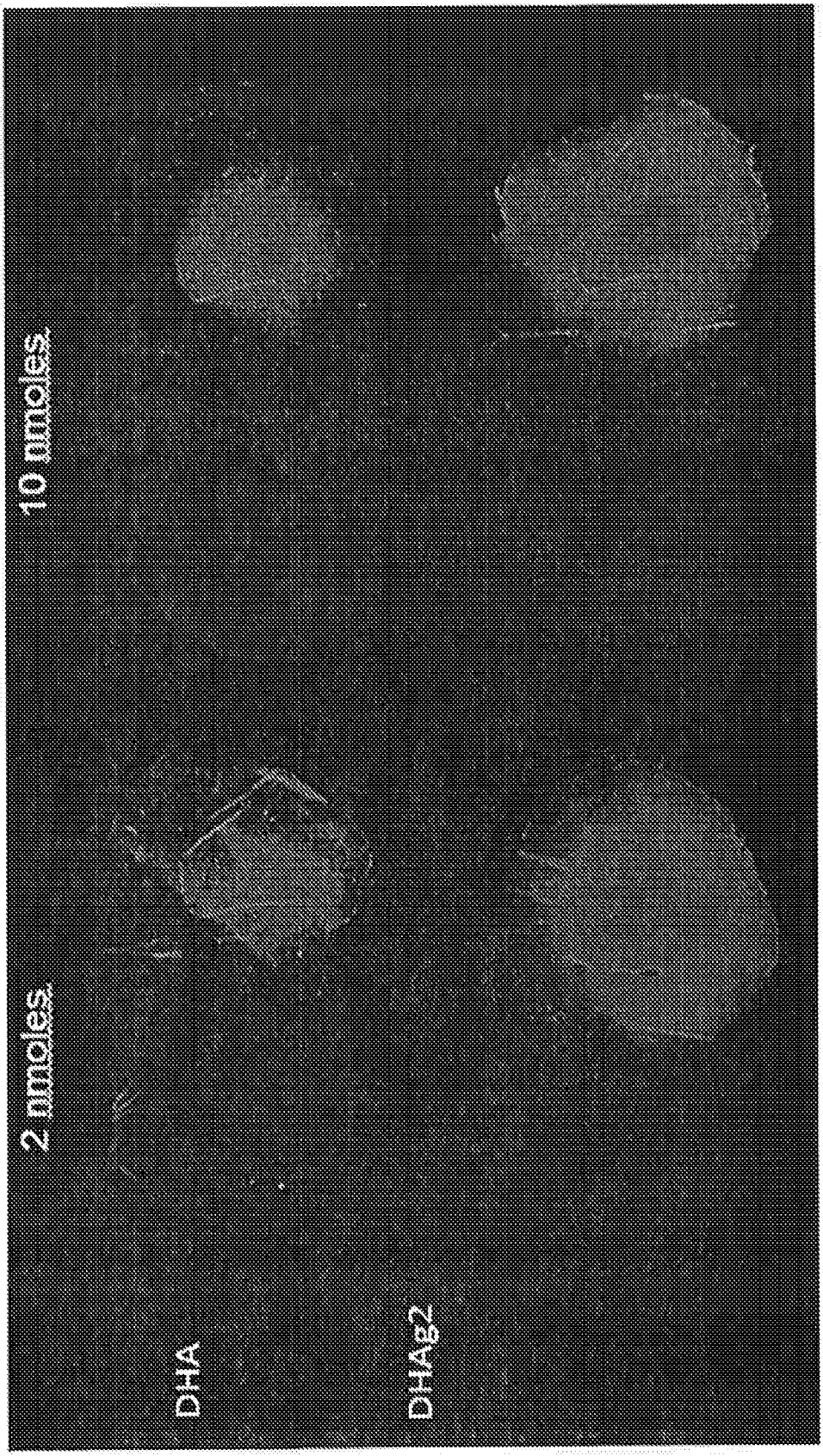


Fig. 65

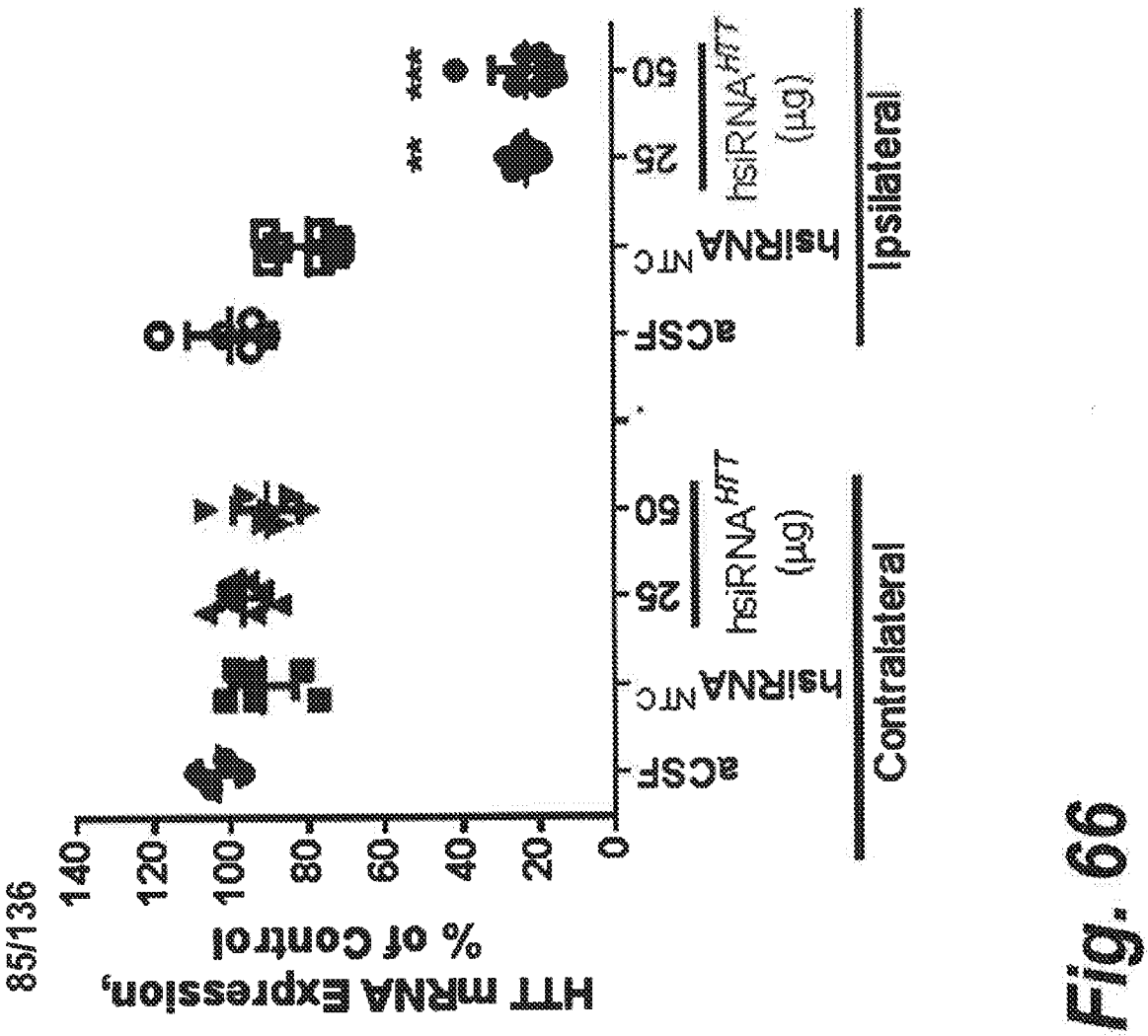

85/136
HTT mRNA Expression, % of control
140
120
100
80
60
40
20
0
aCSF
NTC
hsiRNA
25
50
hsiRNA^HTT (μg)
Contralateral
Ipsilateral
*
**
***
Intrastriatal injection 2 or 4 nmoles (25 or 50 μg)
Wait 1 week
Collected 3 biopsies from injected and non-injected side
Homogenize biopsies
Perform branched DNA assay for mRNA expression

Fig. 66

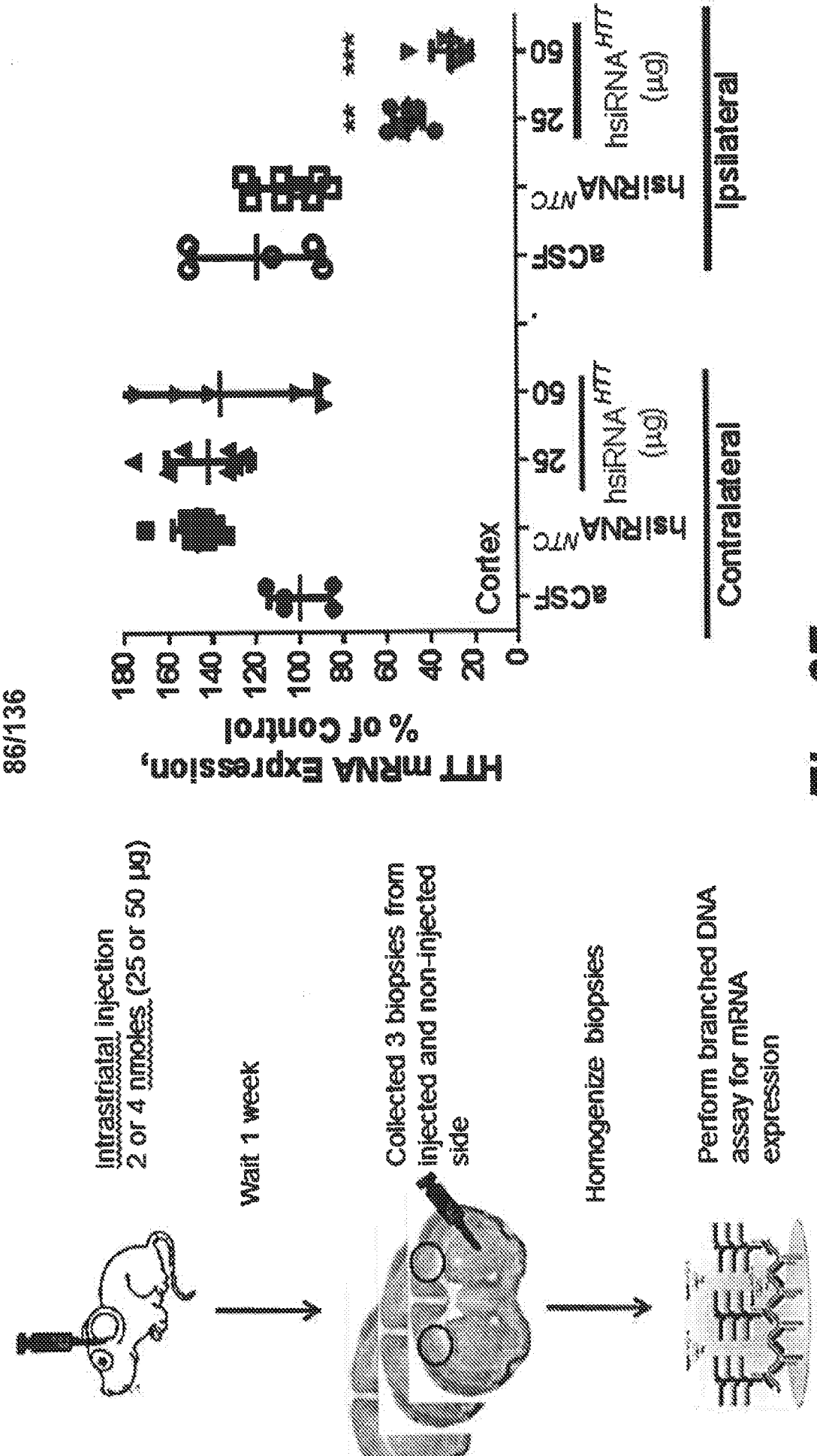

86/136
HTT mRNA Expression, % of control
180
160
140
120
100
80
60
40
20
0
Cortex
aCSF
hsiRNA NTC
hsiRNA HTT (µg)
25
50
Contralateral
Ipsilateral
**
***
Intrastriatal injection 2 or 4 nmoles (25 or 50 µg)
Wait 1 week
Collected 3 biopsies from injected and non-injected side
Homogenize biopsies
Perform branched DNA assay for mRNA expression

Fig. 67

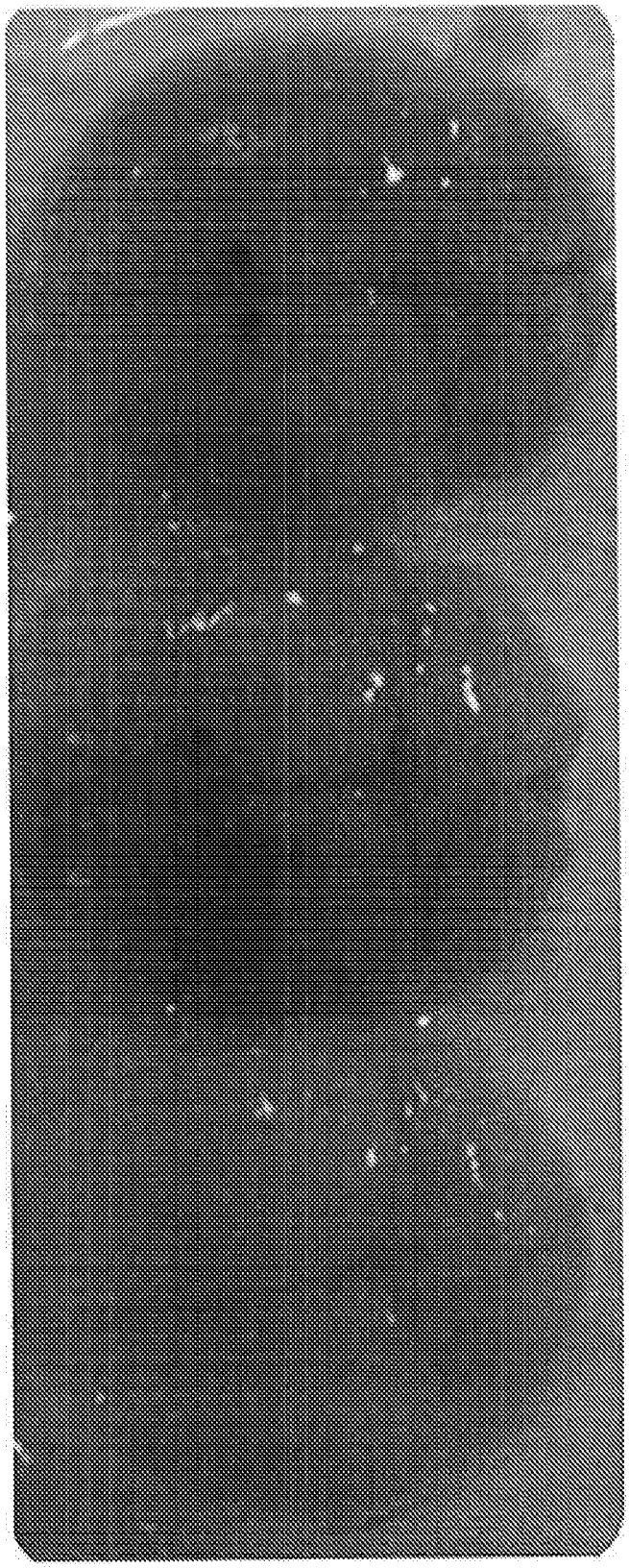


Fig. 68

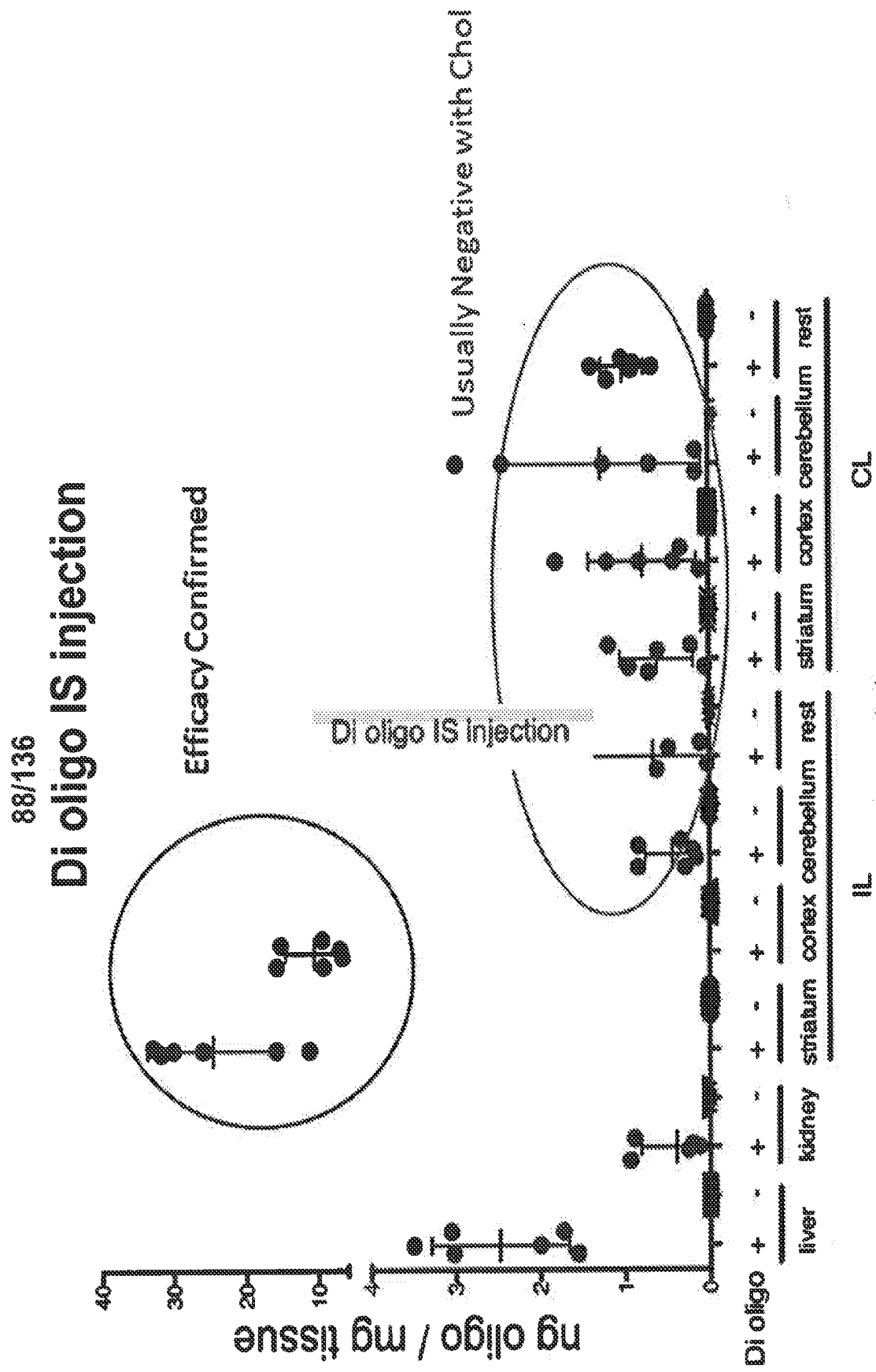

88/136
Di oligo IS injection
Efficacy Confirmed
Usually Negative with Chol
Di oligo IS Injection
ng oligo / mg tissue
40
30
20
10
4
3
2
1
0
Di oligo + - + - + - + - + - + - + - + -
liver kidney striatum cortex cerebellum rest striatum cortex cerebellum rest
IL
CL

Fig. 69

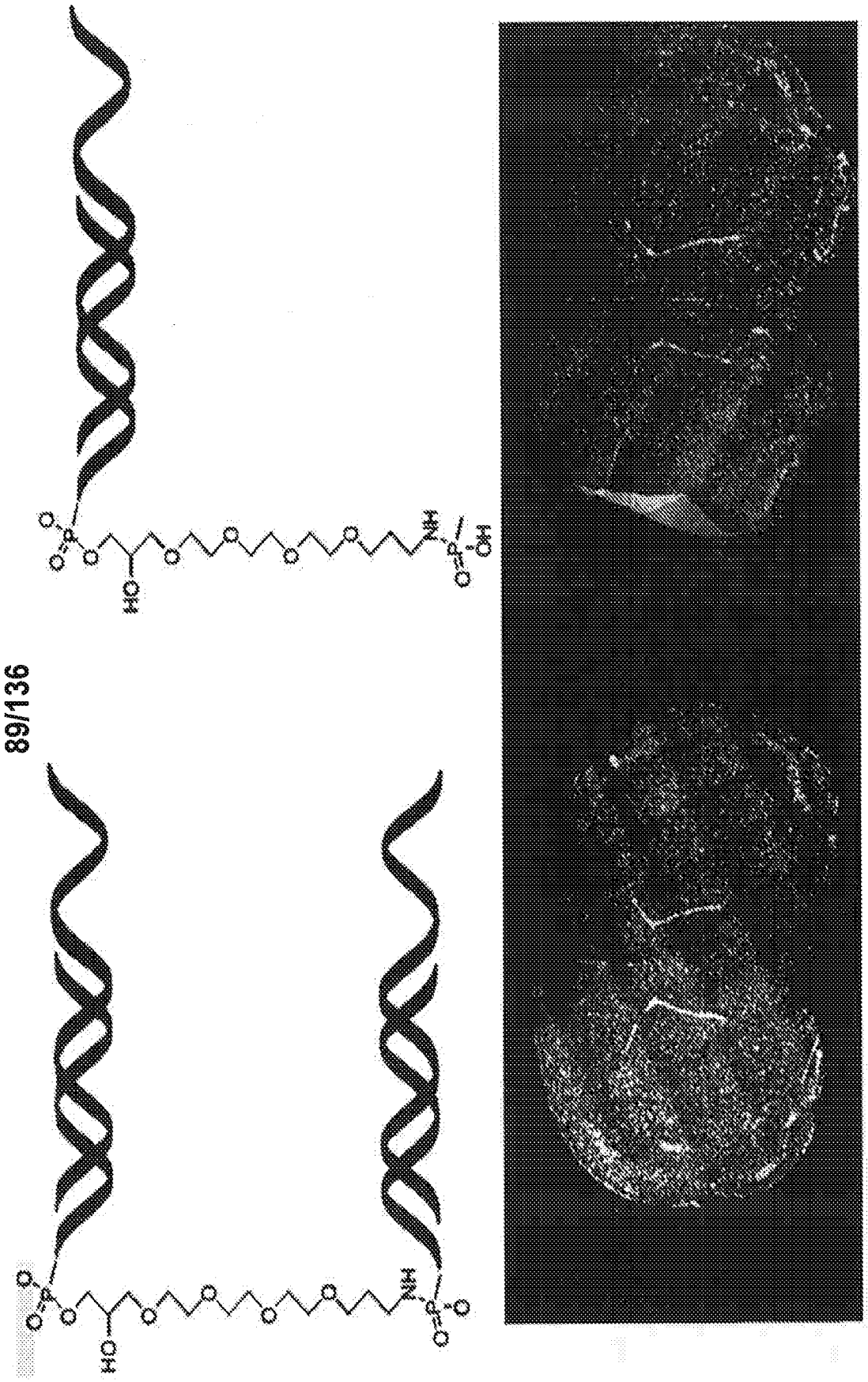
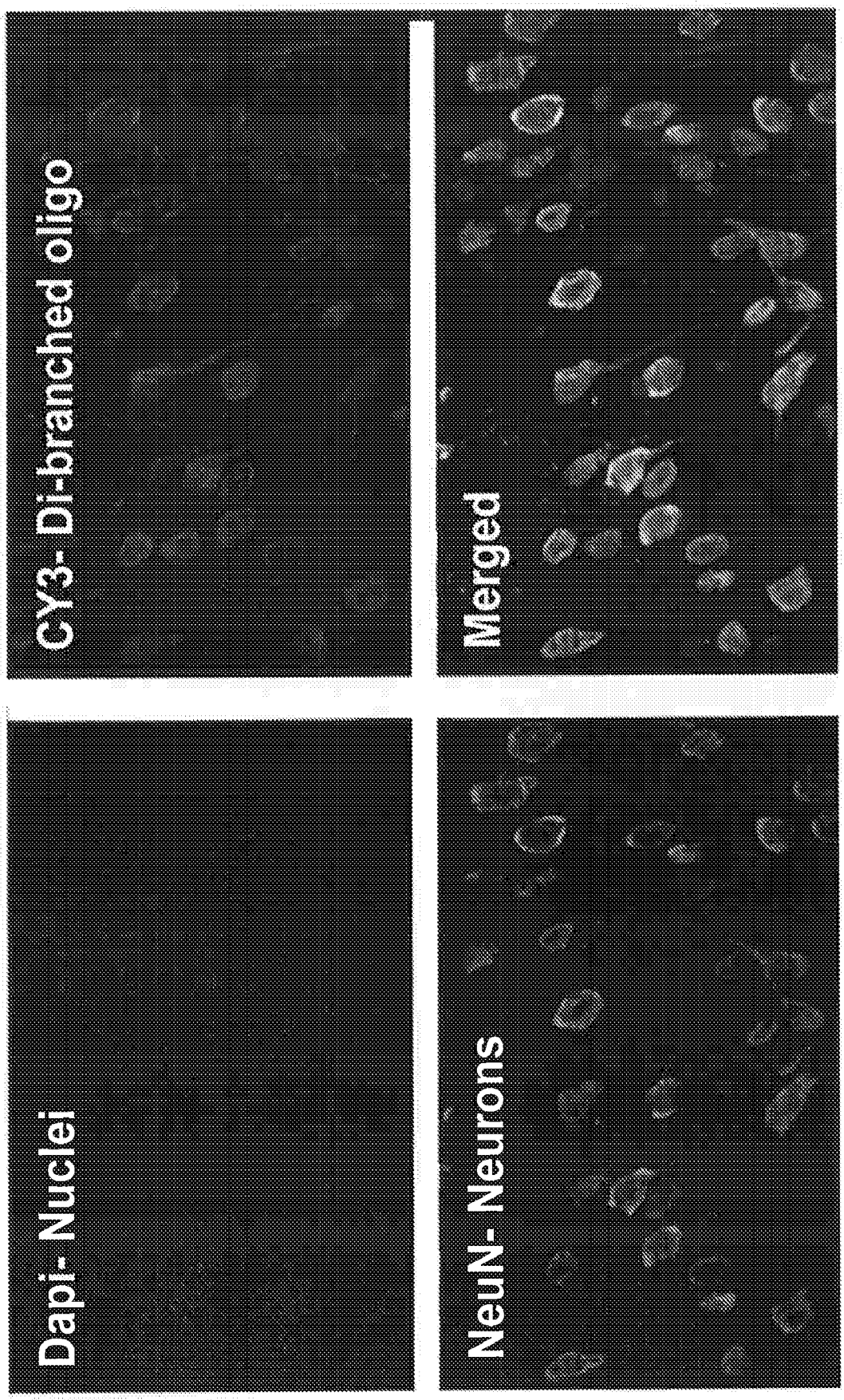


Fig. 70

90/136
FVBN WT Mice
Intrastriatal Unilateral injection
2 nmol /2 µL (4 nmol as)
Di-branched hsiRNA CY3 conjugates in aCSF
7 days
post-injection
Tissue punches collected from the injected
and non-injected sides of the brain
2 mm tissue punches of
Liver and Kidney samples
300 µm brain
slices
Tissue processed through a QIAGEN
Tissue Lyser II
Quantigene® branched-DNA assay
Tecan fluorescence plate
reader

Fig. 71



91/136

Fig. 72

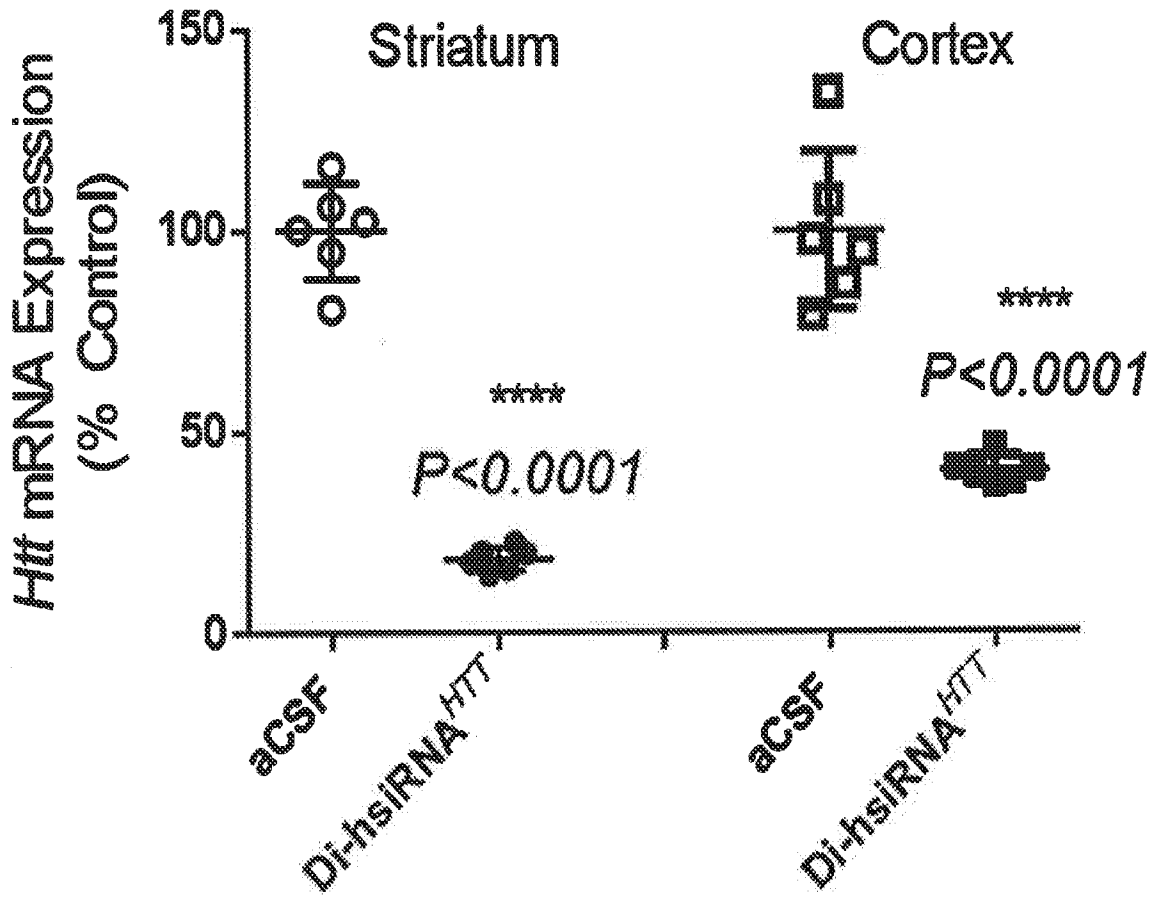
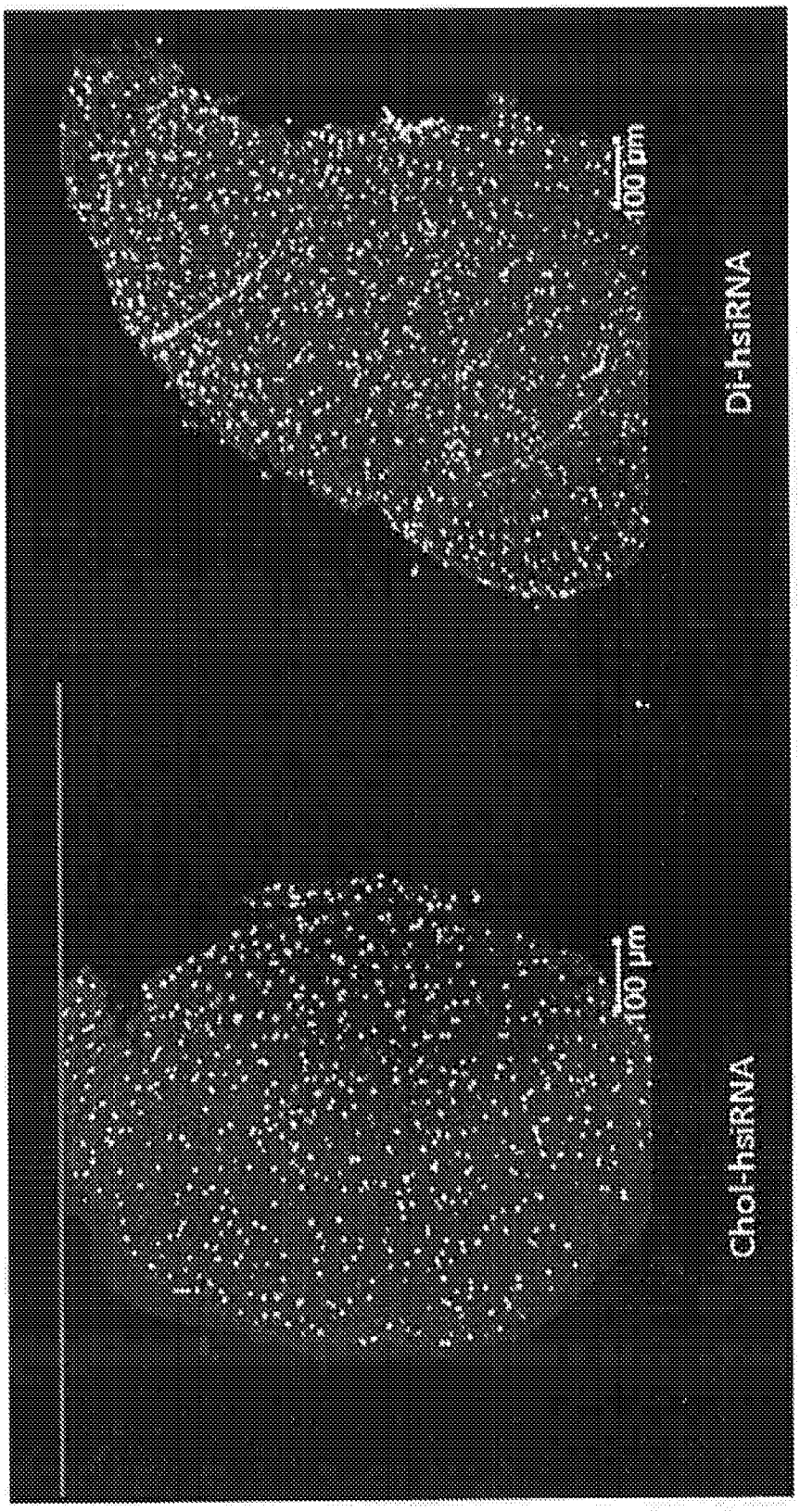

Htt mRNA Expression
(% Control)
150
100
50
0
Striatum
Cortex
****
P<0.0001
****
P<0.0001
aCSF
Di-hsiRNA^HTT
aCSF
Di-hsiRNA^HTT

Fig. 73



93/136

Fig. 74

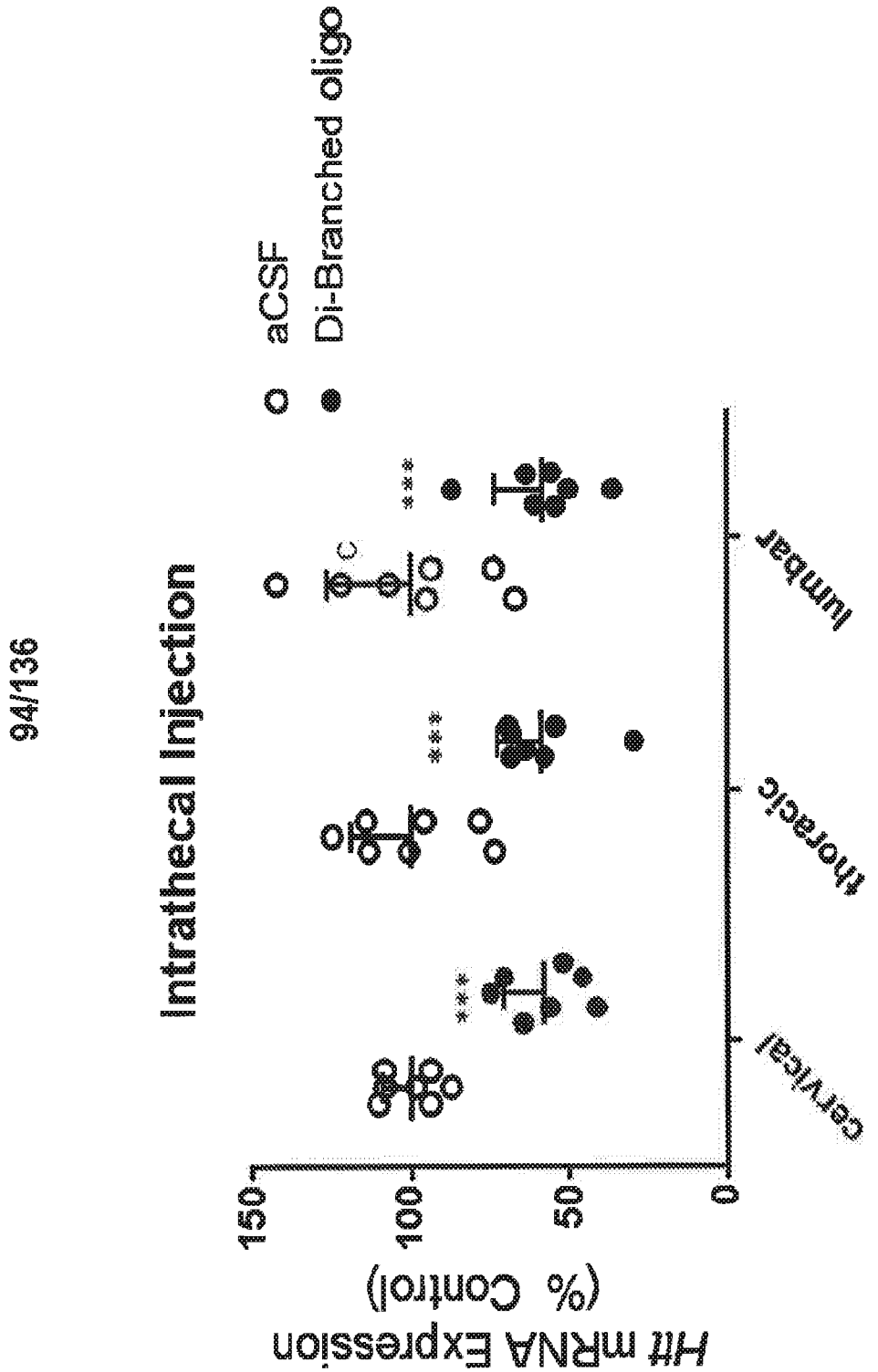

94/136
Intrathecal Injection
aCSF
Di-Branched oligo
Htt mRNA Expression
(% Control)
150
100
50
0
cervical
thoracic
lumbar
***
***
***

Fig. 75

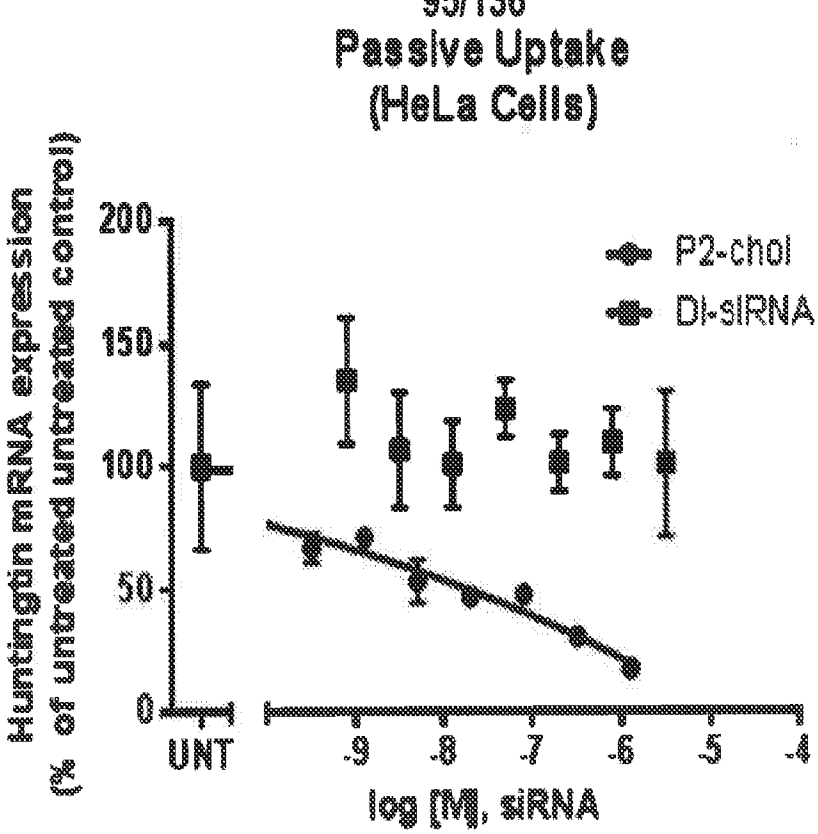


Fig. 76A

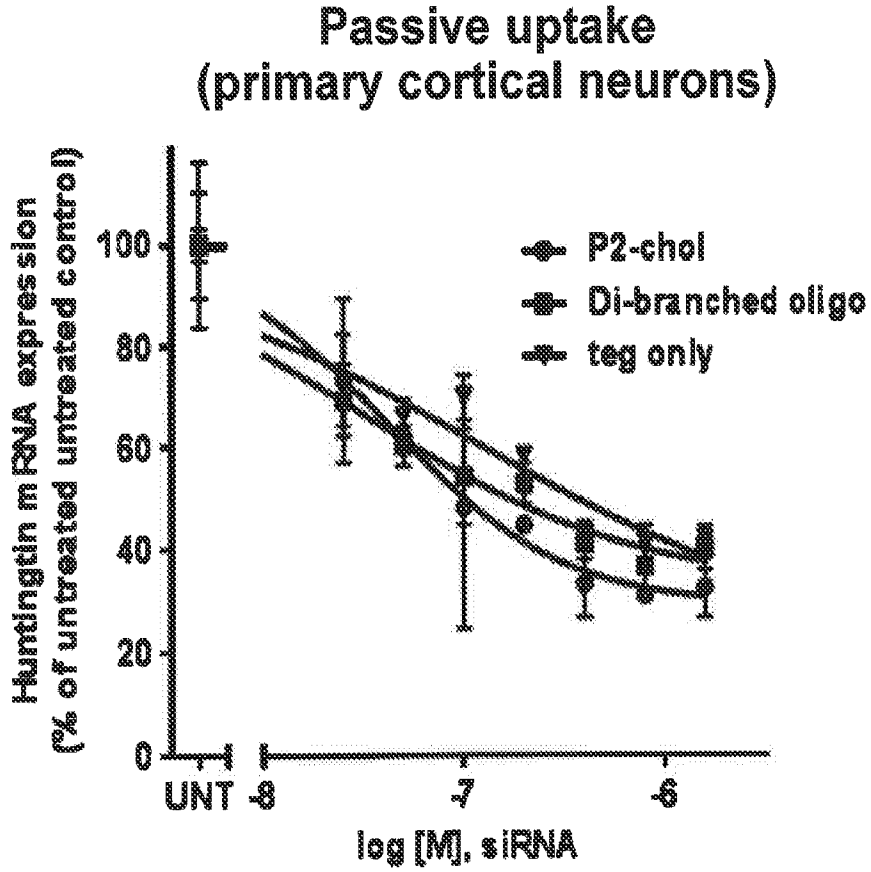


Fig. 76B

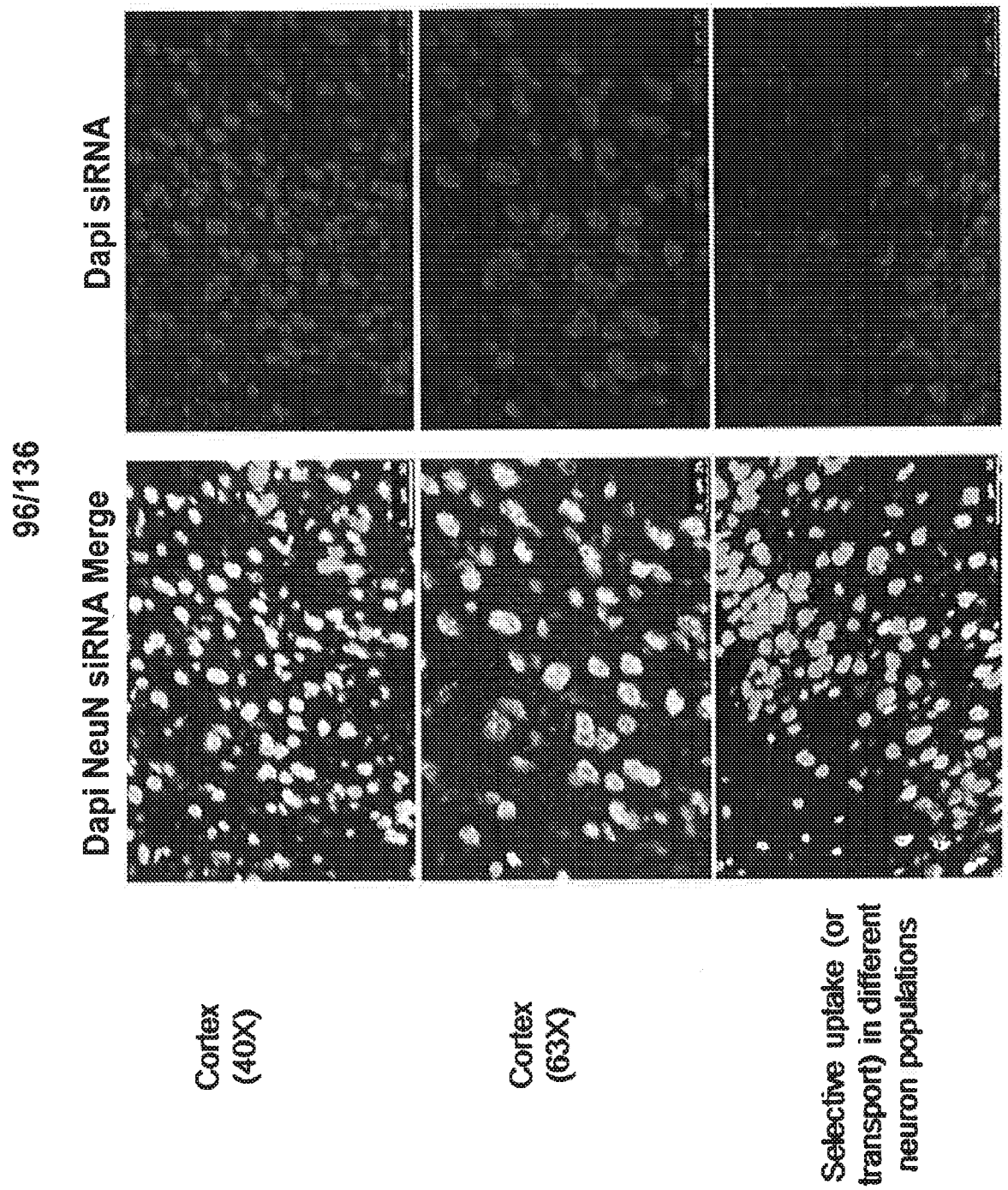

Dapi NeuN siRNA Merge
Dapi siRNA
Cortex (40X)
Cortex (63X)
Selective uptake (or transport) in different neuron populations

Fig. 77

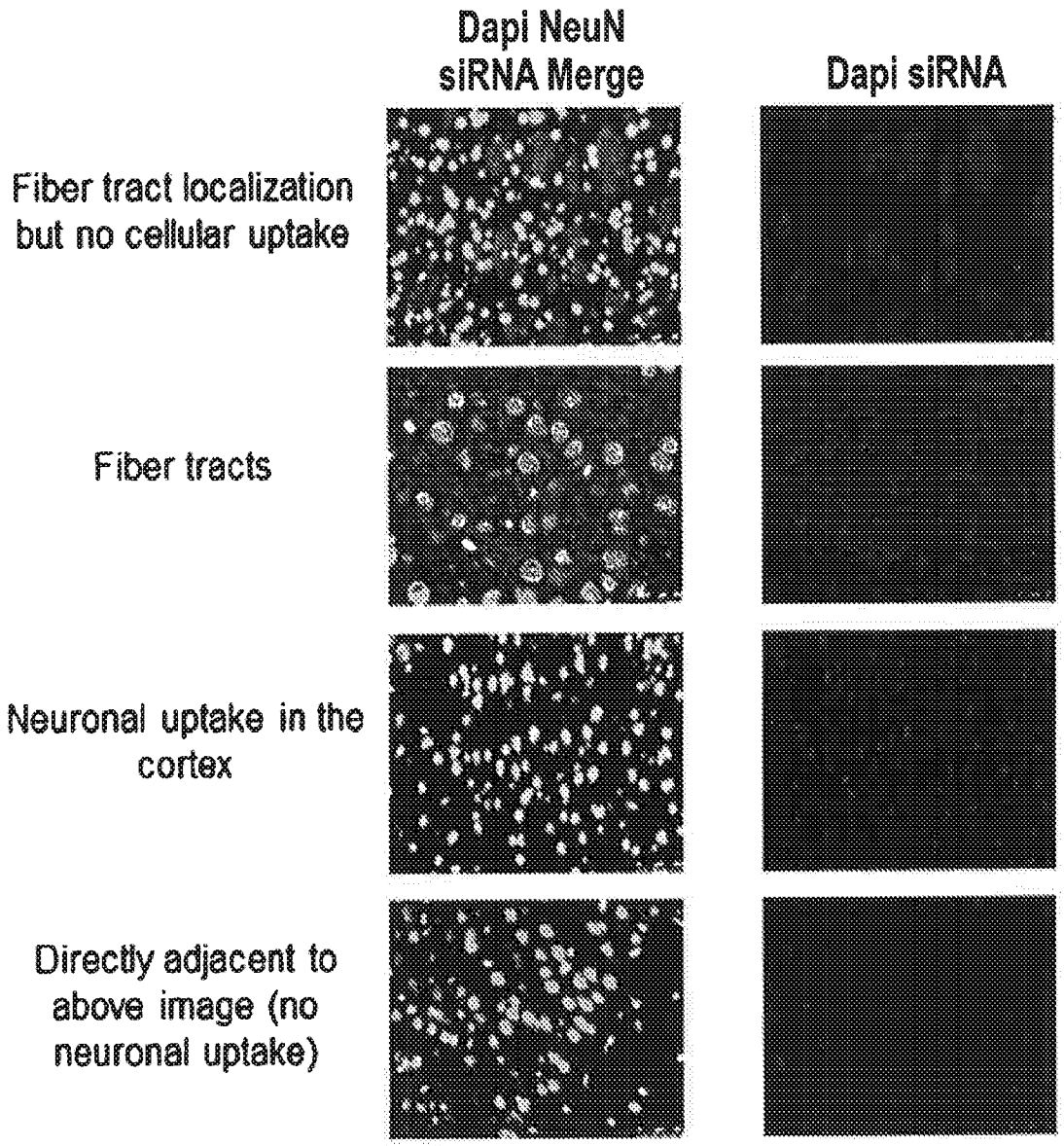

Dapi NeuN siRNA Merge
Dapi siRNA
Fiber tract localization but no cellular uptake
Fiber tracts
Neuronal uptake in the cortex
Directly adjacent to above image (no neuronal uptake)

Fig. 78

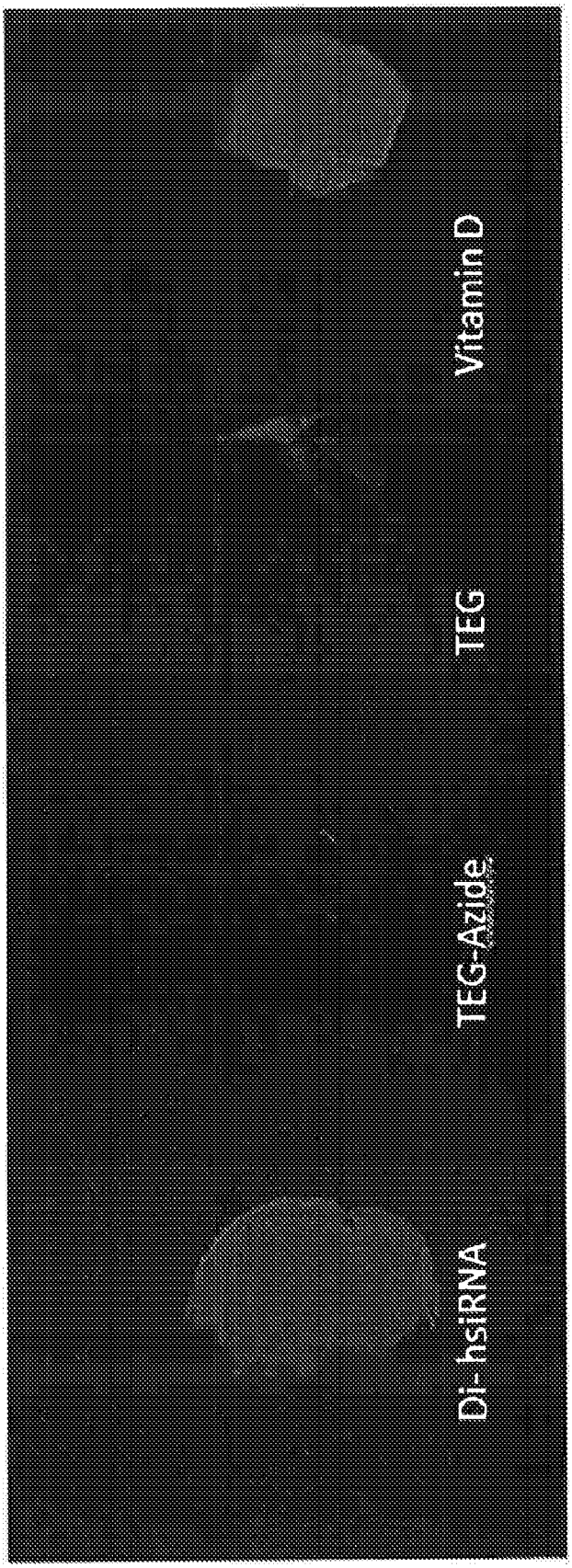


Fig. 79

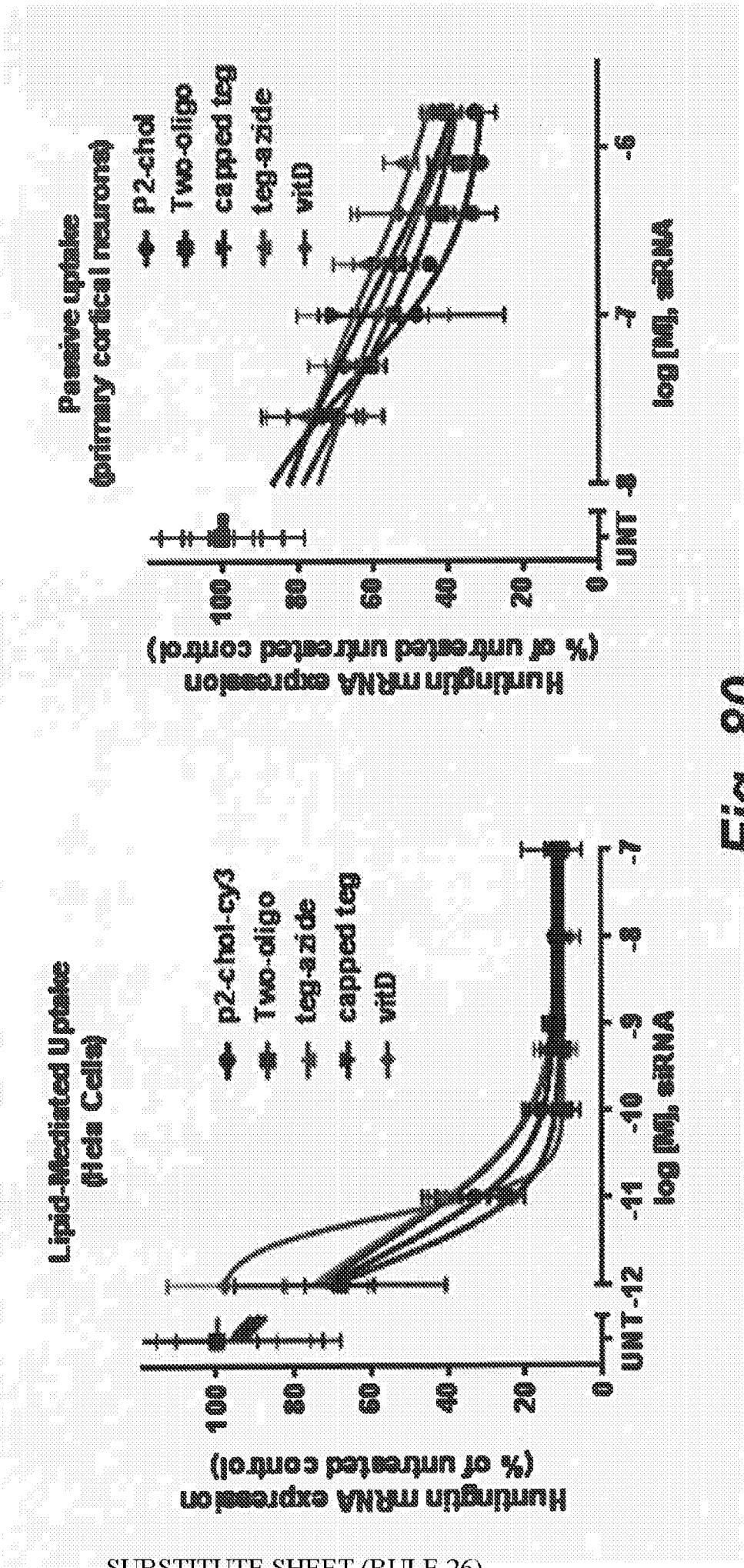
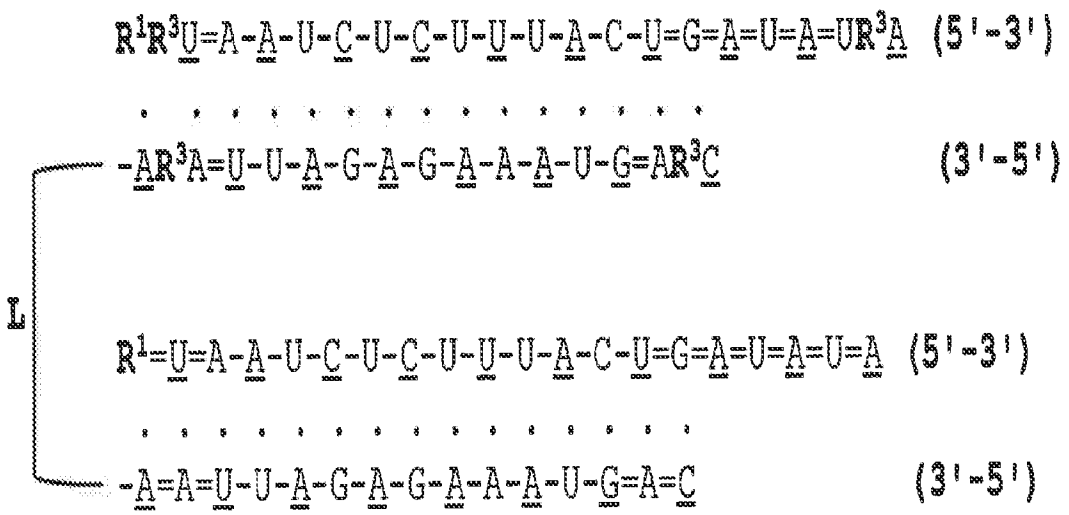

Lipid-Mediated Uptake
(Hela Cells)
p2-chol-cy3
Two-oligo
teg-azide
capped teg
vitD
Huntingtin mRNA expression
(% of untreated control)
100
80
60
40
20
0
UNT-12
-11
-10
-9
-8
-7
log [M], siRNA
Passive Uptake
(primary cortical neurons)
P2-chol
Two-oligo
capped teg
teg-azide
vitD
Huntingtin mRNA expression
(% of untreated untreated contrd)
100
80
60
40
20
0
UNT
-8
-7
-6
log [M], siRNA

Fig. 80



Legend

X- *2'-deoxy-2'-fluoro*

X- *2'-O-methyl*

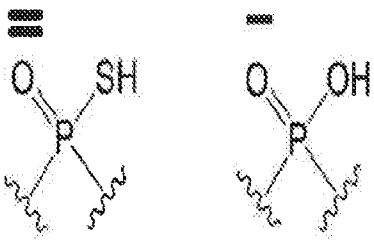


Fig. 81

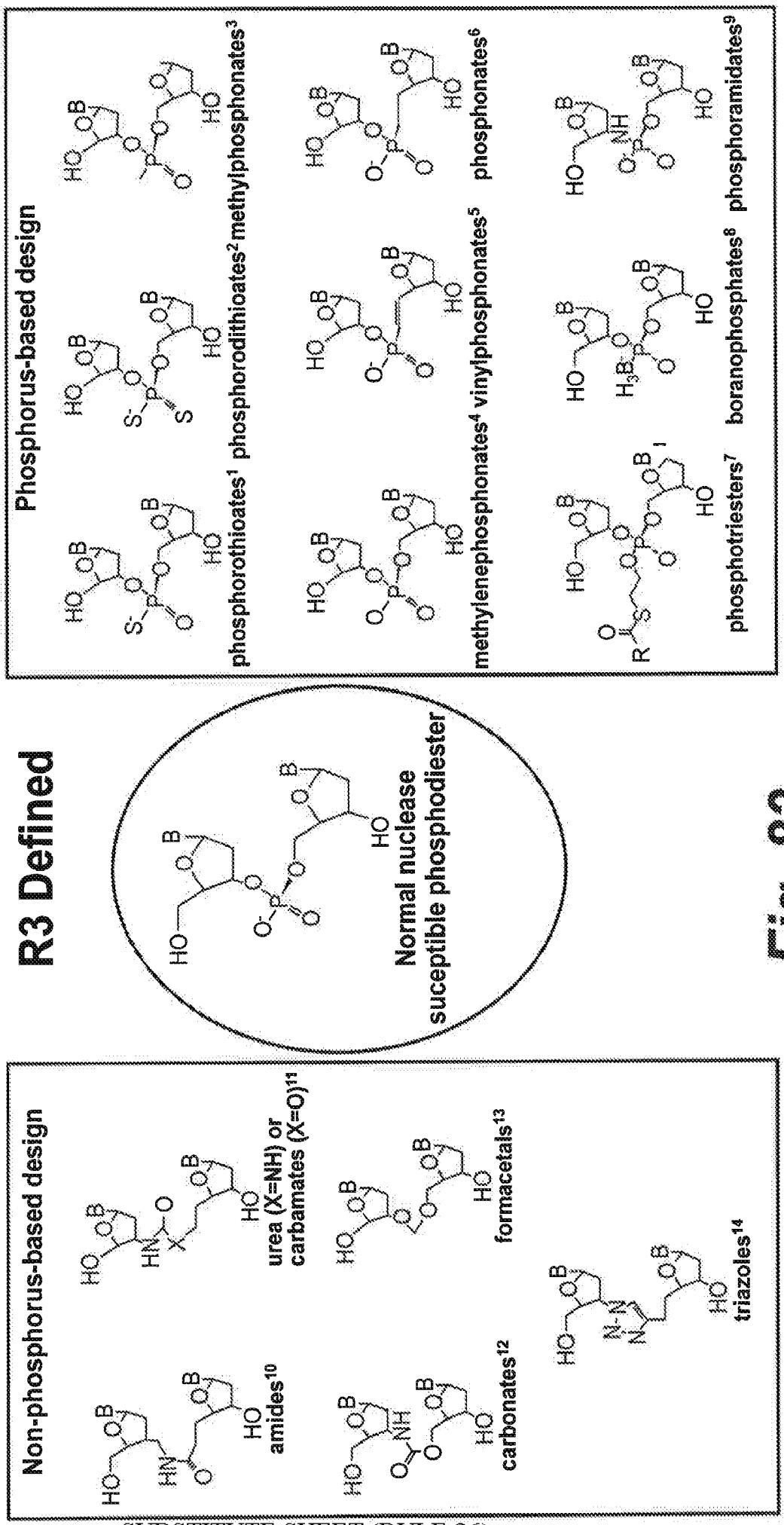

Phosphorus-based design
phosphorothioates[1]
phosphorodithioates[2]
methylphosphonates[3]
methylenephosphonates[4]
vinylphosphonates[5]
phosphonates[6]
phosphotriesters[7]
boranophosphates[8]
phosphoramidates[9]
R3 Defined
Normal nuclease
suceptible phosphodiester
Non-phosphorus-based design
amides[10]
urea (X=NH) or
carbamates (X=O)[11]
carbonates[12]
formacetals[13]
triazoles[14]

Fig. 82

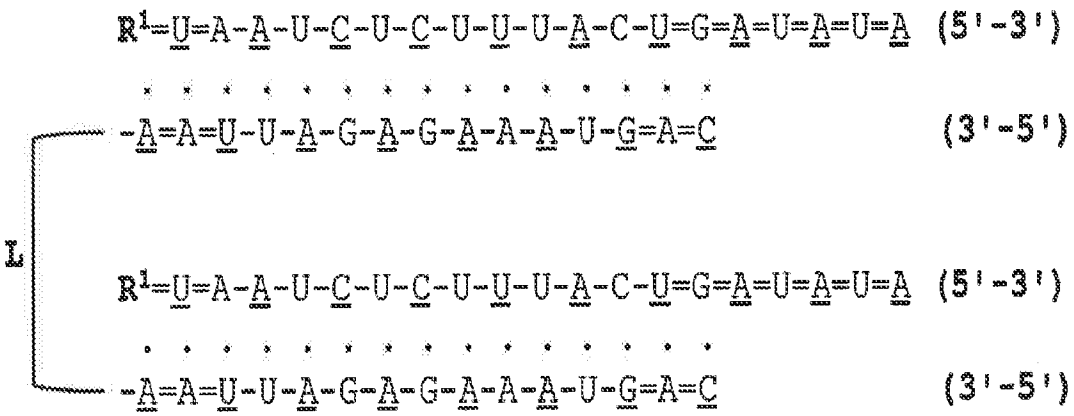
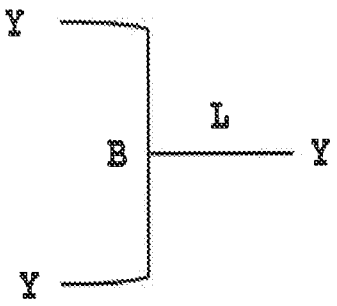
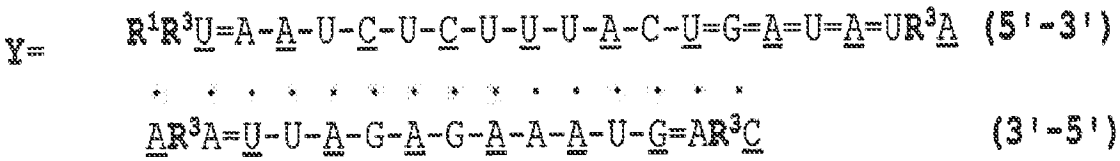


Fig. 83





Legend

X- *2'-deoxy-2'-fluoro*

X- *2'-O-methyl*

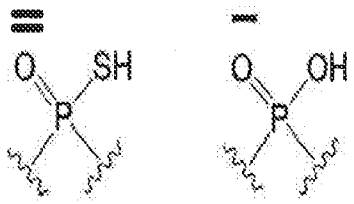
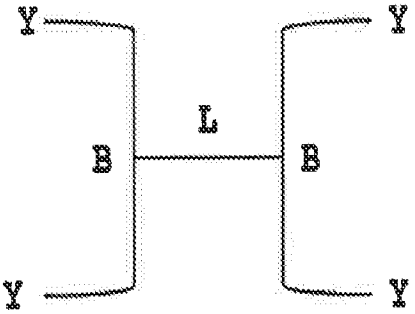


Fig. 84



Y= R^1R^3U=A-A-U-C-U-C-U-U-U-A-C-U=G=A=U=A=UR^3A (5'-3')

. . . . . .

AR^3A=U-U-A-G-A-G-A-A-A-U-G=AR^3C (3'-5')

Legend

X- *2'-deoxy-2'-fluoro*

X- *2'-O-methyl*

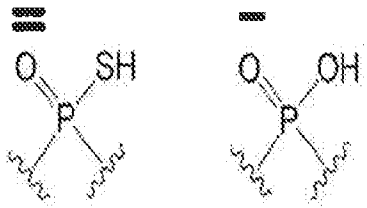


Fig. 85

Y= R^1=U=A-A-U-C-U-C-U-U-U-A-C-U=G=A=U=A=U=A (5'-3')

. . . . . .

A=A=U-U-A-G-A-G-A-A-A-U-G=A=C (3'-5')

Fig. 86

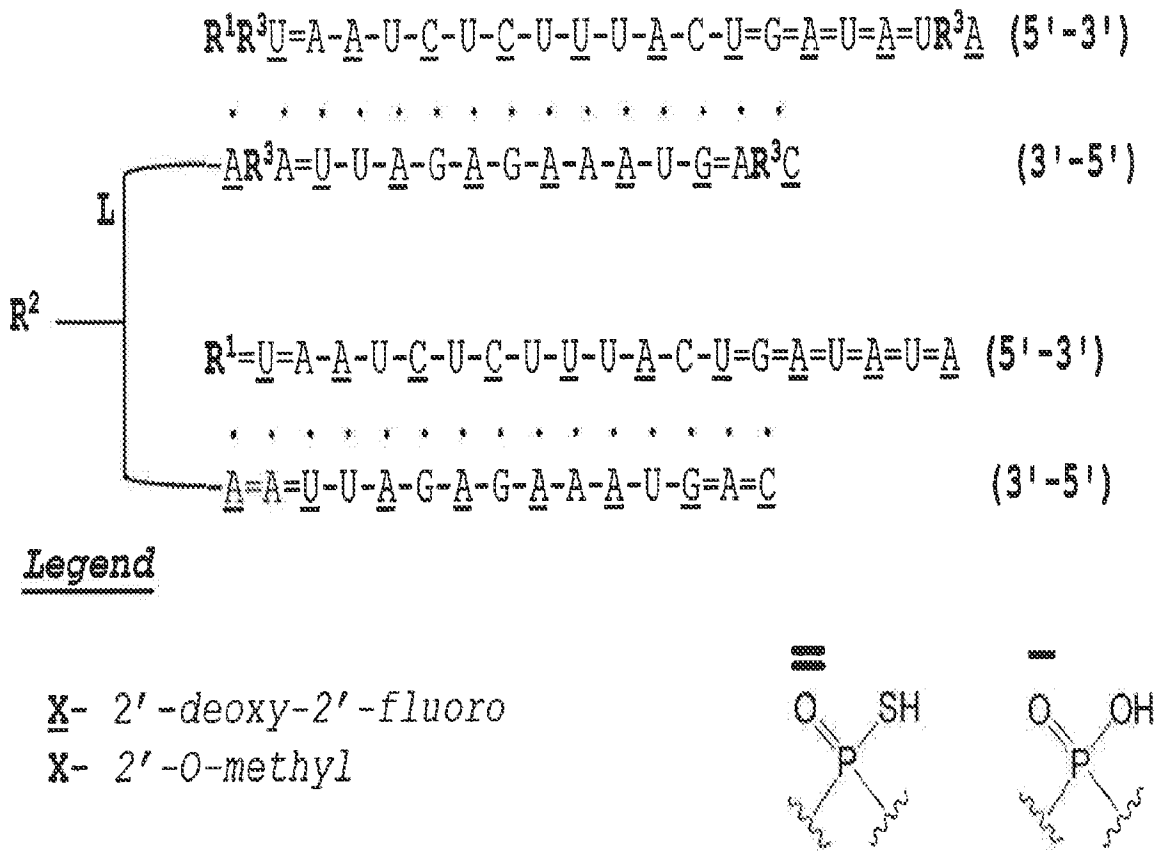

R1R3U=A-A-U-C-U-C-U-U-U-A-C-U=G=A=U=A=UR3A (5'-3')
AR3A=U-U-A-G-A-G-A-A-A-U-G=AR3C (3'-5')
L
R2
R1=U=A-A-U-C-U-C-U-U-U-A-C-U=G=A=U=A=U=A (5'-3')
A=A=U-U-A-G-A-G-A-A-A-U-G=A=C (3'-5')
Legend
X- 2'-deoxy-2'-fluoro
X- 2'-O-methyl
O
SH
P
O
OH
P

Fig. 87

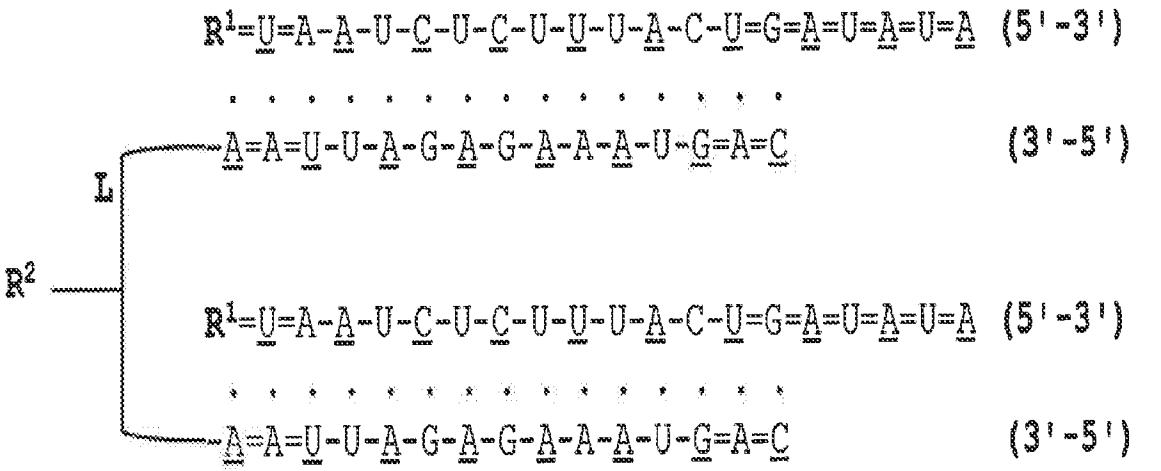

R1=U=A-A-U-C-U-C-U-U-U-A-C-U=G=A=U=A=U=A (5'-3')
A=A=U-U-A-G-A-G-A-A-A-U-G=A=C (3'-5')
L
R2
R1=U=A-A-U-C-U-C-U-U-U-A-C-U=G=A=U=A=U=A (5'-3')
A=A=U-U-A-G-A-G-A-A-A-U-G=A=C (3'-5')

Fig. 88

(5'-3') R^1R^3U=A-A-U-C-U-C-U-U-U-A-C-U=G=A=U=A=UR^3A

. . . . . .

(3'-5') R^2-AR^3A=U-U-A-G-A-G-A-A-A-U-G=AR^3C

Legend

X- *2'-deoxy-2'-fluoro*

X- *2'-O-methyl*

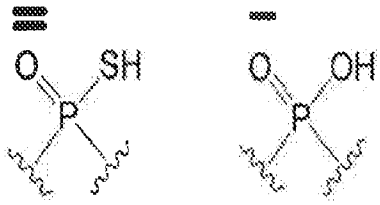


Fig. 89

(5'-3') R^1=U=A-A-U-C-U-C-U-U-U-A-C-U=G=A=U=A=U=A

. . . . . .

(3'-5') R^2-A=A=U-U-A-G-A-G-A-A-A-U-G=A=C

Fig. 90

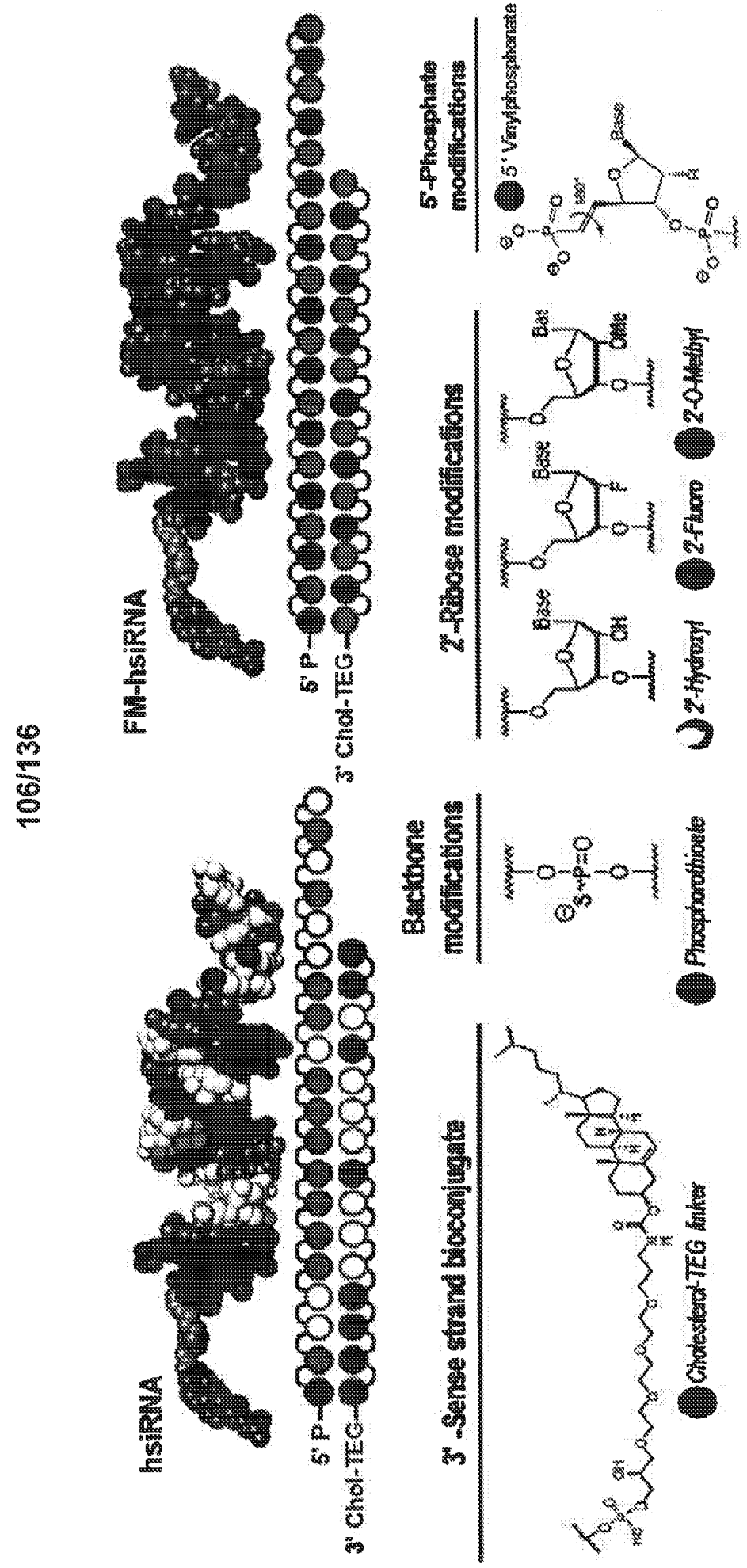

hsiRNA
5' P
3' Chol-TEG
FM-hsiRNA
5' P
3' Chol-TEG
3' -Sense strand bioconjugate
Cholesterol-TEG linker
Backbone modifications
Phosphorothioate
2'-Ribose modifications
2'-Hydroxyl
2'-Fluoro
2'-O-Methyl
5'-Phosphate modifications
5' Vinylphosphonate

Fig. 91A

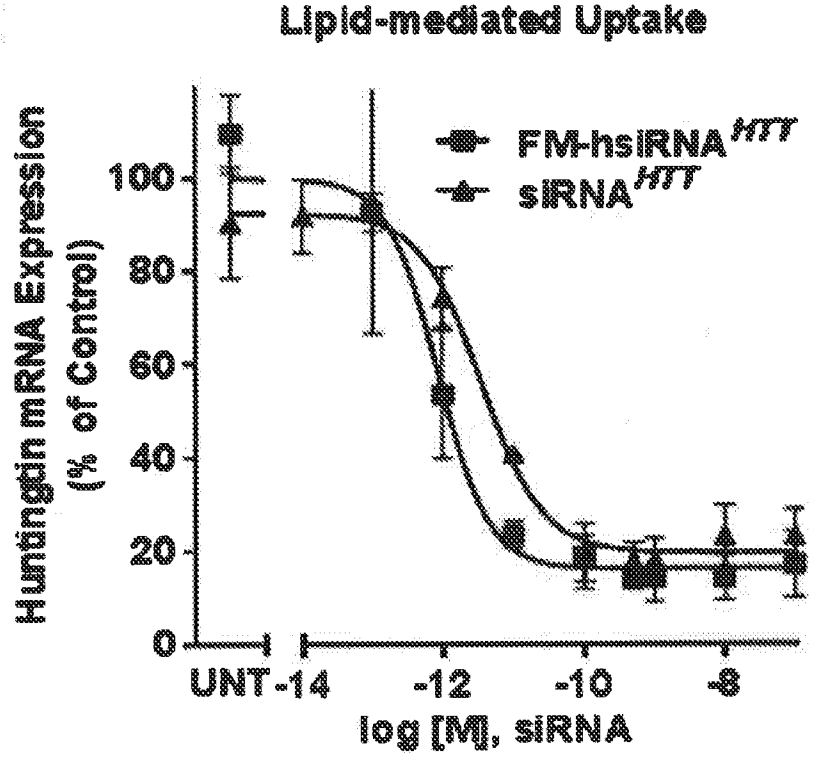


Fig. 91B

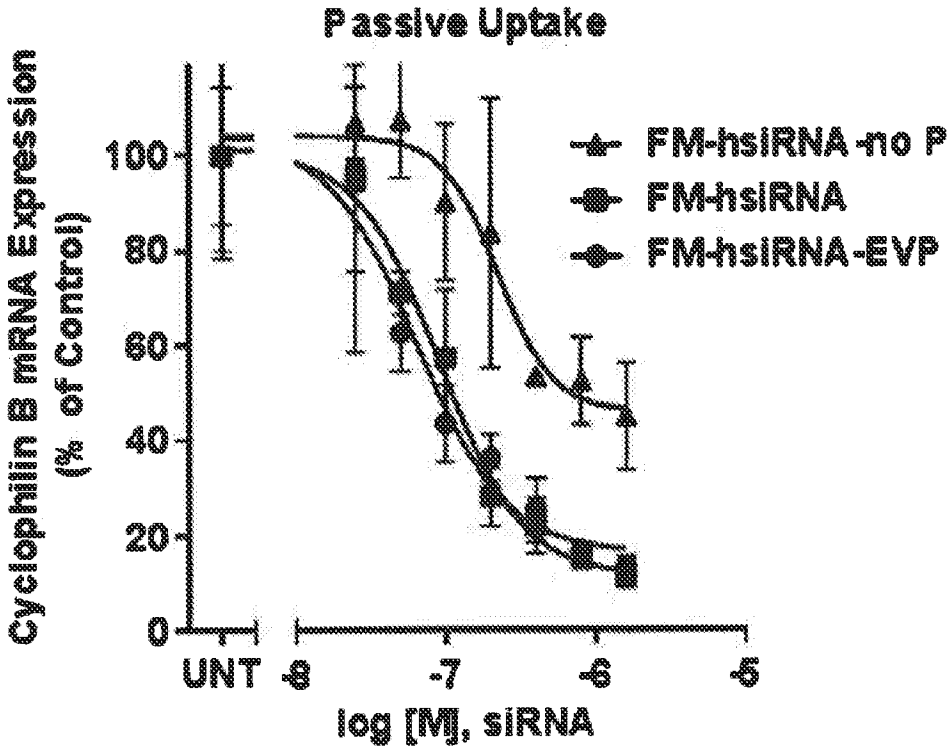


Fig. 91C

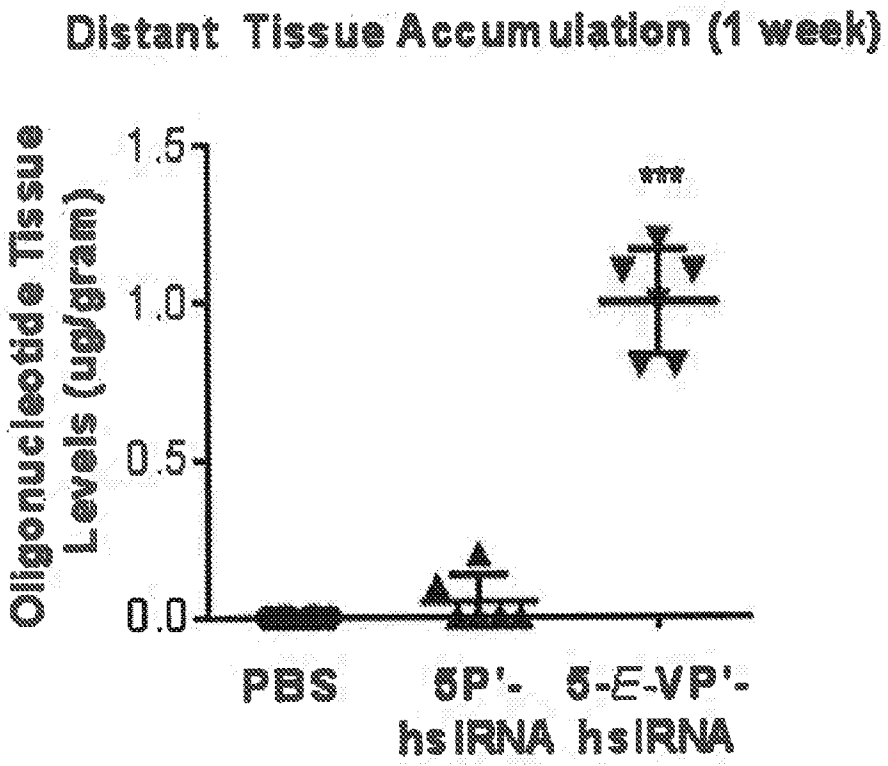

Distant Tissue Accumulation (1 week)
Oligonucleotide Tissue Levels (ug/gram)
1.5
1.0
0.5
0.0
***
PBS
5P'-hsIRNA
5-E-VP'-hsIRNA

Fig. 91D

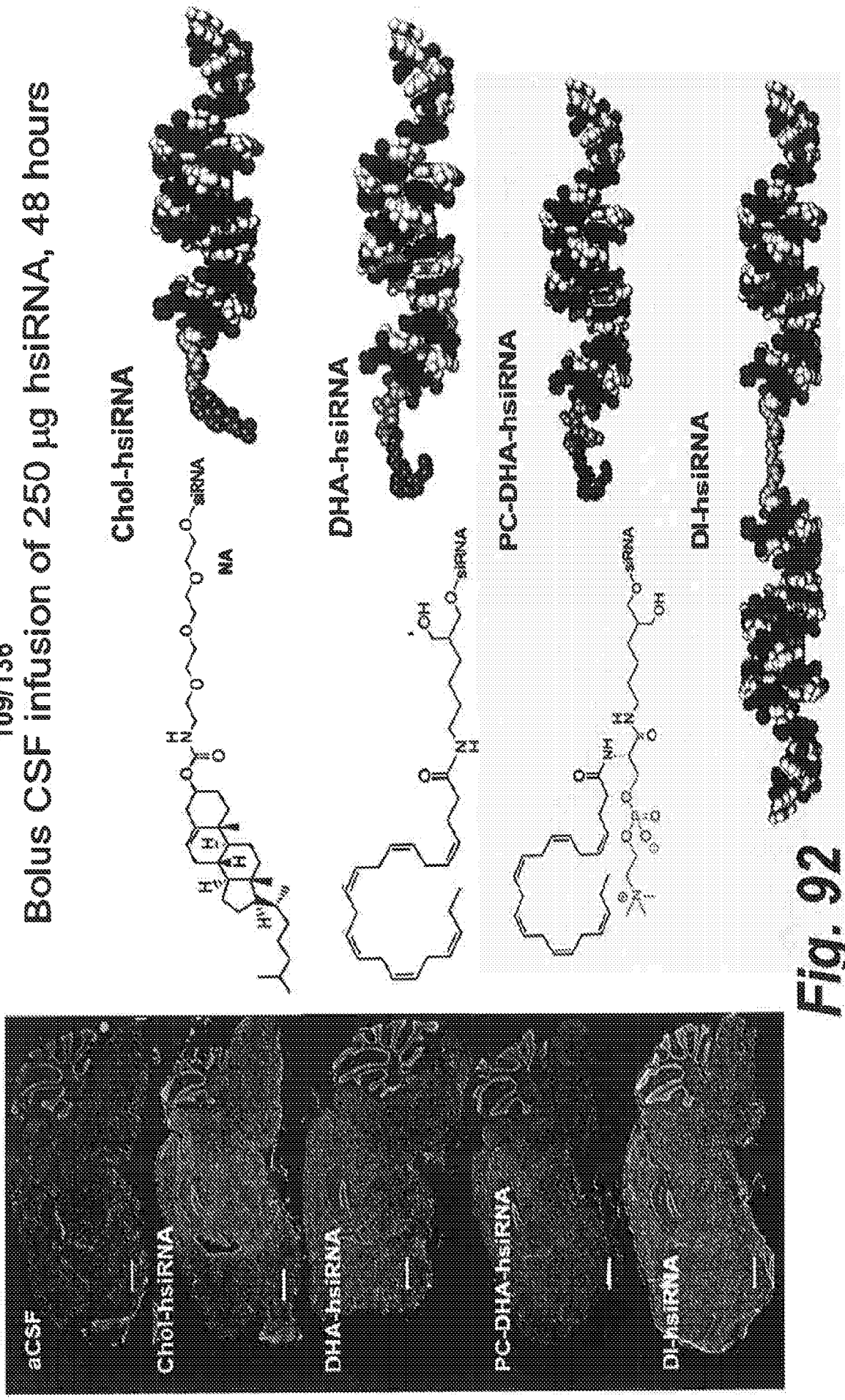

109/136
Bolus CSF infusion of 250 µg hsiRNA, 48 hours
aCSF
Chol-hsiRNA
DHA-hsiRNA
PC-DHA-hsiRNA
DI-hsiRNA
siRNA

Fig. 92

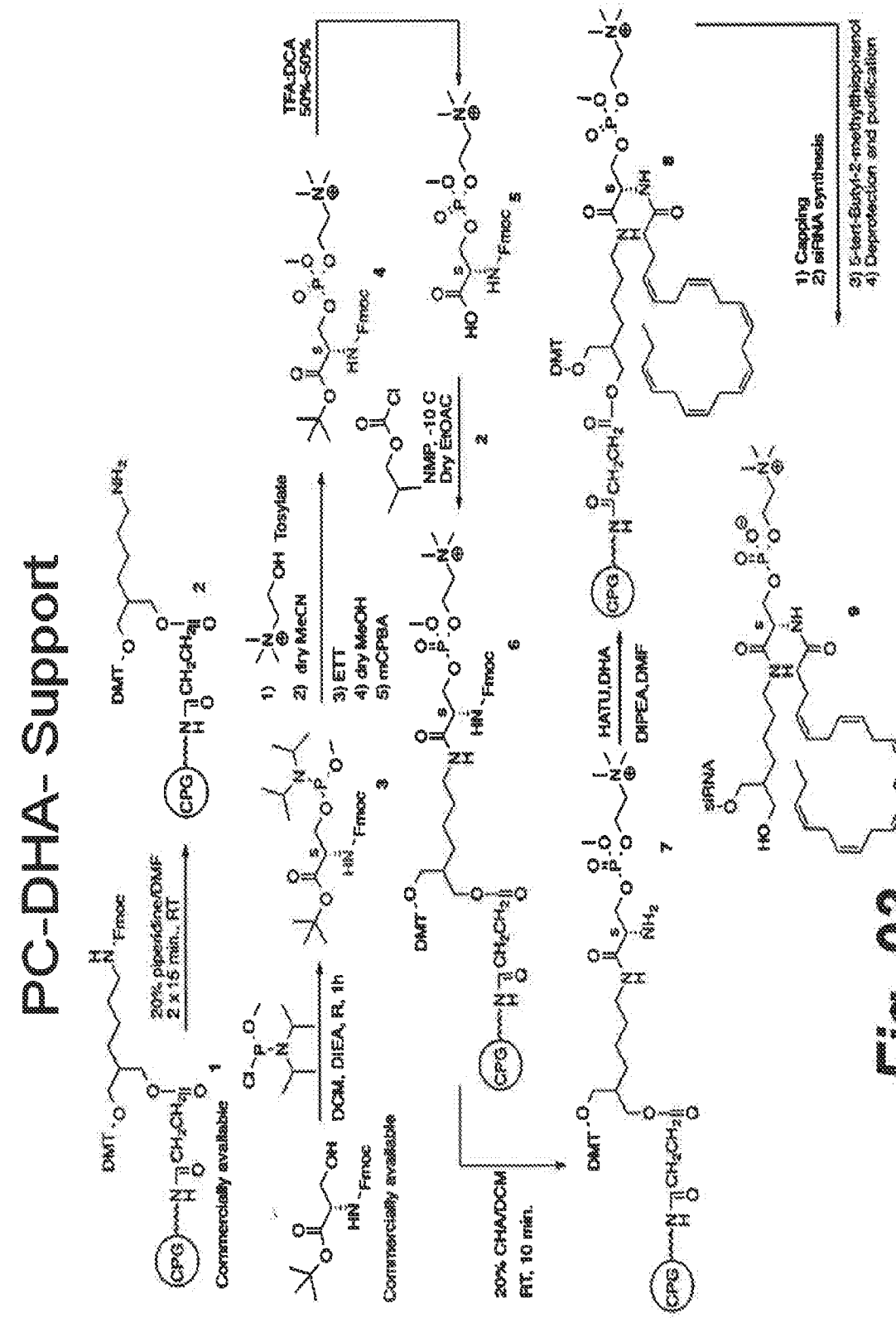

110/136
PC-DHA- Support
20% piperidine/DMF
2 x 15 min., RT
Commercially available
DCM, DIEA, R, 1h
Commercially available
20% CHA/DCM
RT, 10 min.
1) Tosylate
2) dry MeCN
3) ETT
4) dry MeOH
5) mCPBA
NMP, -10 C
Dry EtOAC
TFA:DCA
50%-50%
HATU,DHA
DIPEA,DMF
siRNA
1) Capping
2) siRNA synthesis
3) t-tert-Butyl-2-methylthiopriamol
4) Deprotection and purification

Fig. 93

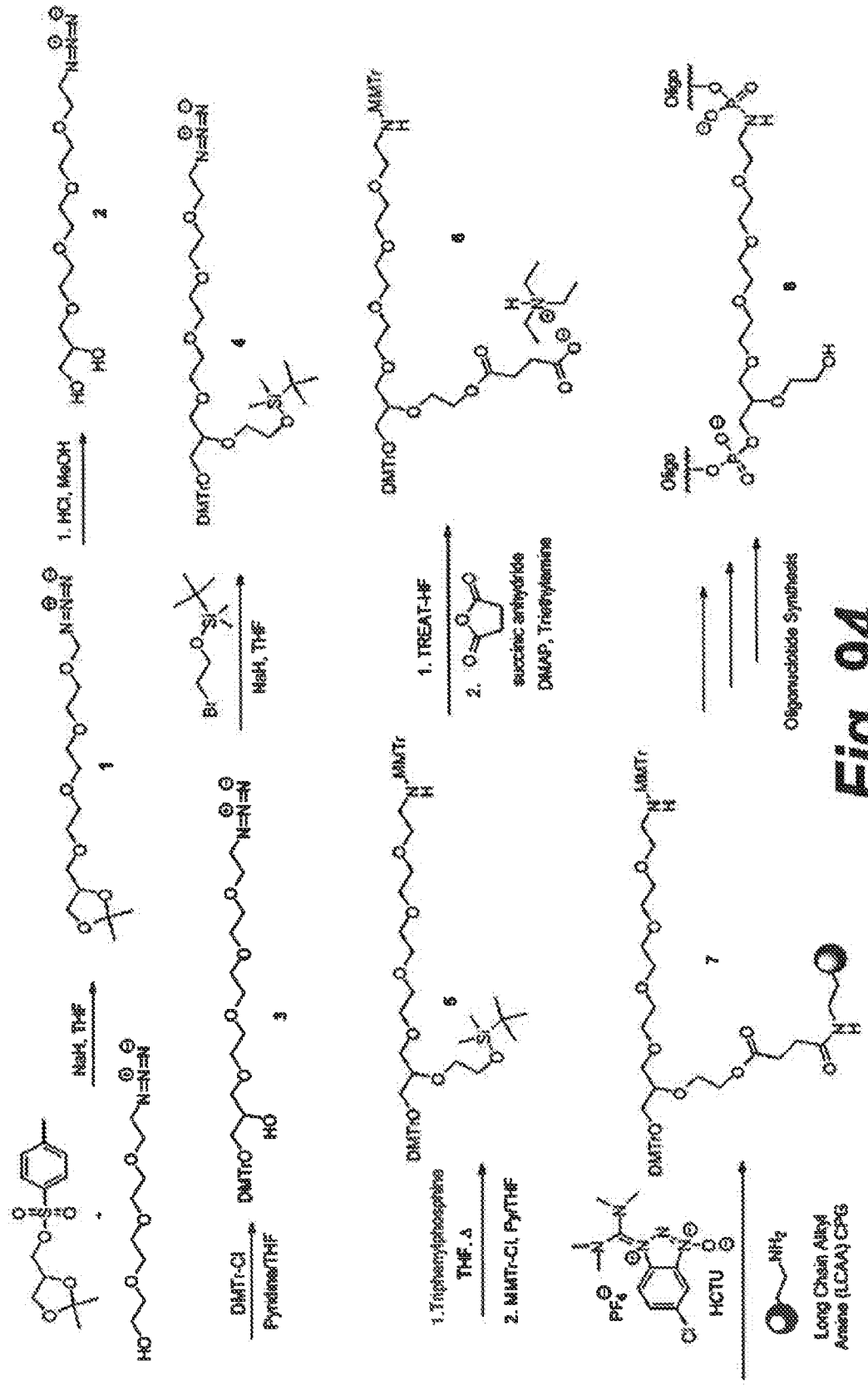

Di- Support
111/136
NaH, THF
1. HCl, MeOH
DMT-Cl
Pyridine/THF
NaH, THF
1. Triphenylphosphine
THF, Δ
2. MMT-Cl, PyrTHF
1. TREAT-HF
2.
succinic anhydride
DMAP, Triethylamine
HCTU
Long Chain Alkyl
Amine (LCAA) CPG
Oligonucleotide Synthesis

Fig. 94

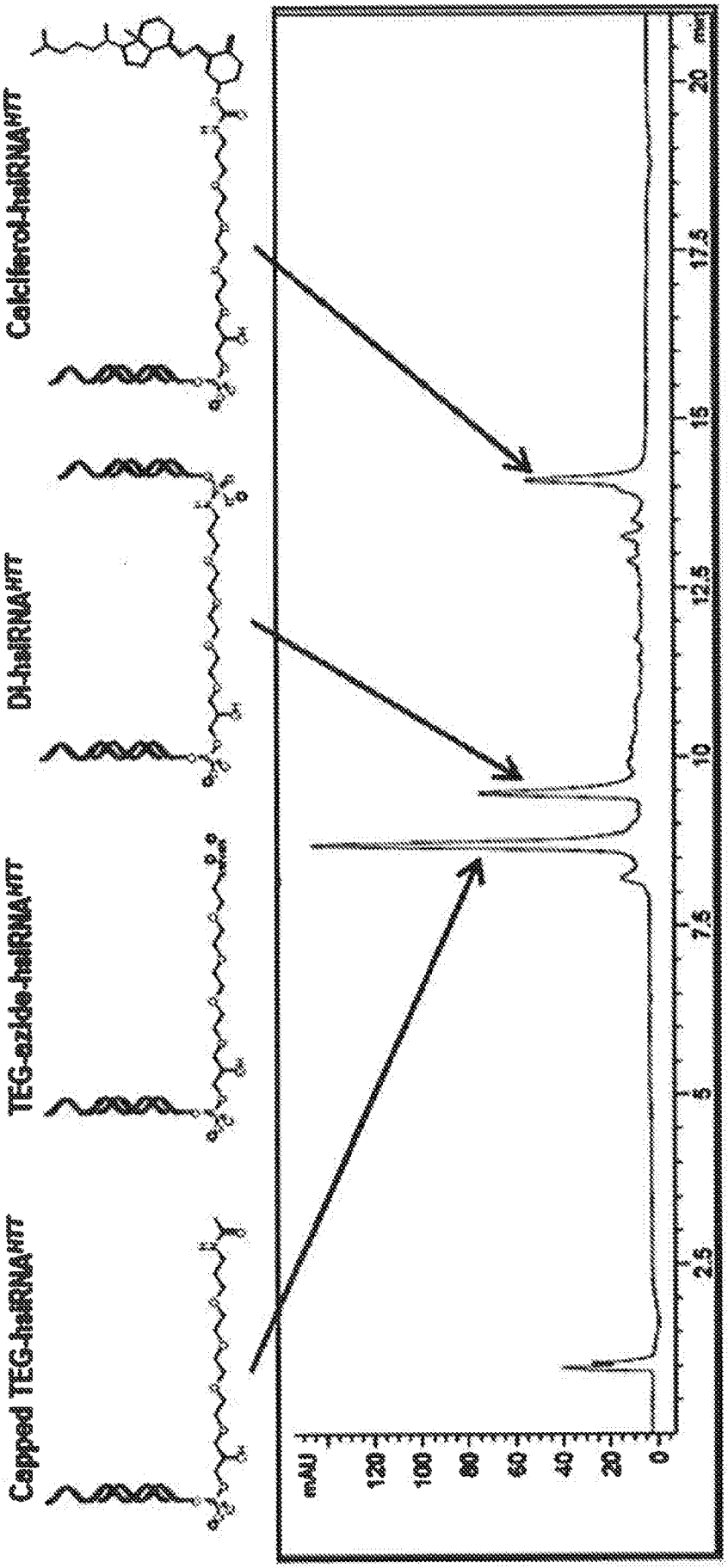

Capped TEG-hsiRNA^HTT
TEG-azido-hsiRNA^HTT
Di-hsiRNA^HTT
Cholesterol-hsiRNA^HTT
mAU
120
100
80
60
40
20
0
2.5
5
7.5
10
12.5
15
17.5
20
min

Fig. 95A

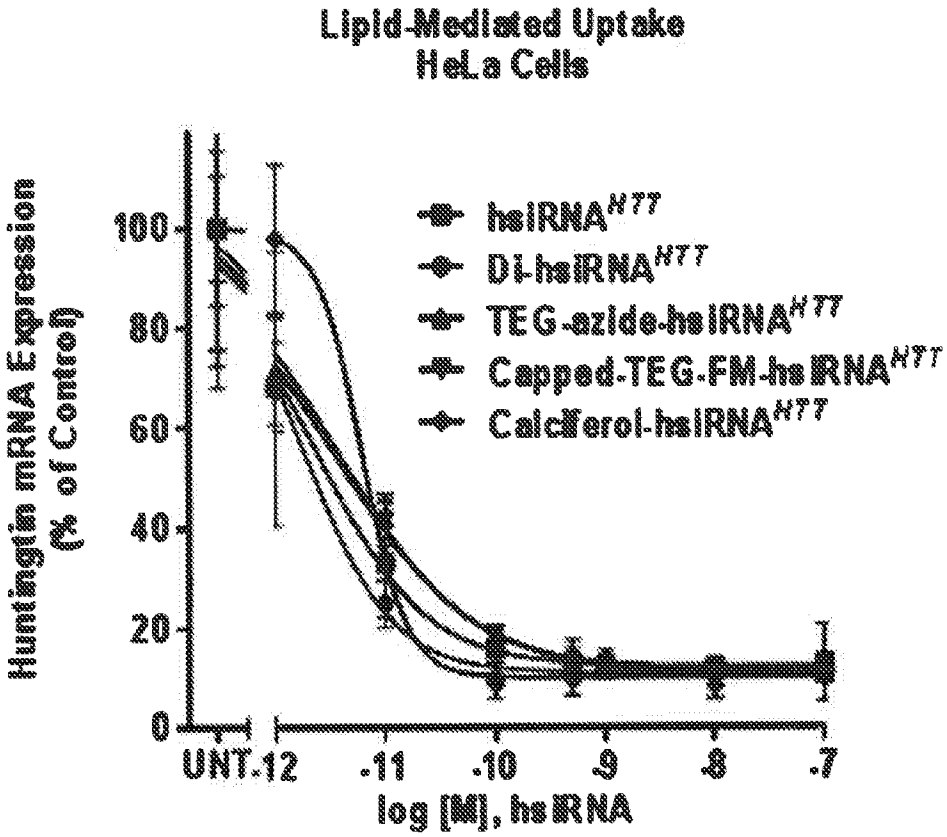

Lipid-Mediated Uptake
HeLa Cells
Huntingtin mRNA Expression
(% of Control)
100
80
60
40
20
0
UNT.
-12
-11
-10
-9
-8
-7
log [M], hsiRNA
hsiRNA^HTT
DI-hsiRNA^HTT
TEG-azide-hsiRNA^HTT
Capped-TEG-FM-hsiRNA^HTT
Calciferol-hsiRNA^HTT

Fig. 95B

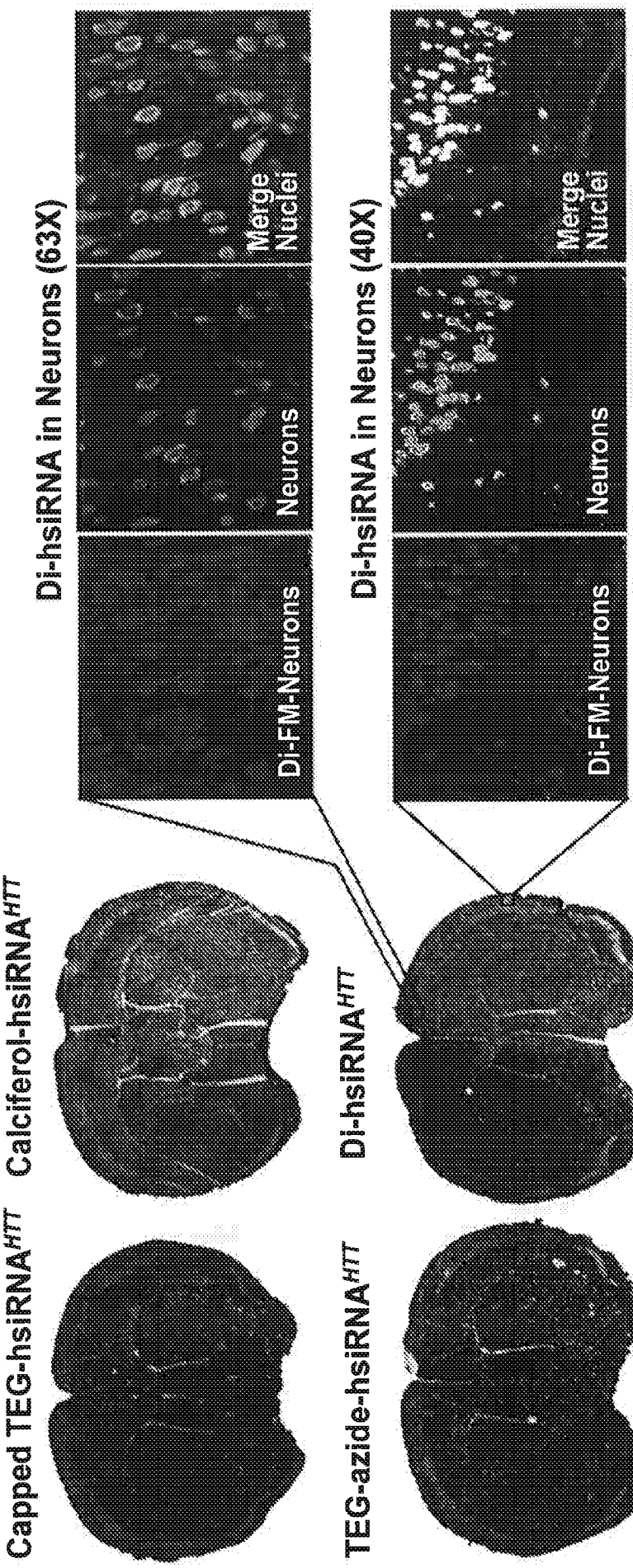

Capped TEG-hsiRNA^{HTT}
Calciferol-hsiRNA^{HTT}
TEG-azide-hsiRNA^{HTT}
Di-hsiRNA^{HTT}
Di-hsiRNA in Neurons (63X)
Di-hsiRNA in Neurons (40X)
Di-FM-Neurons
Neurons
Merge Nuclei

Fig. 95C

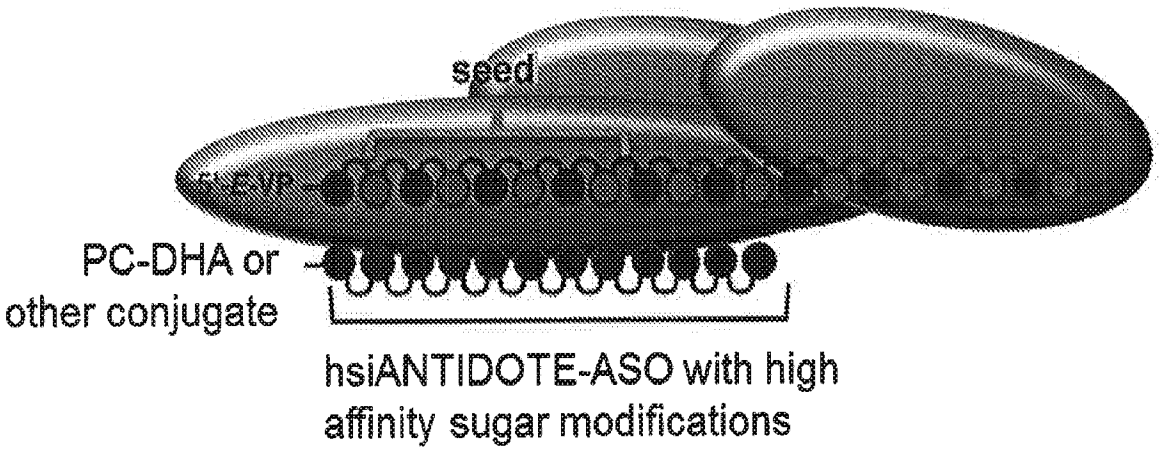

speed
PC-DHA or
other conjugate
hsiANTIDOTE-ASO with high
affinity sugar modifications

Fig. 96

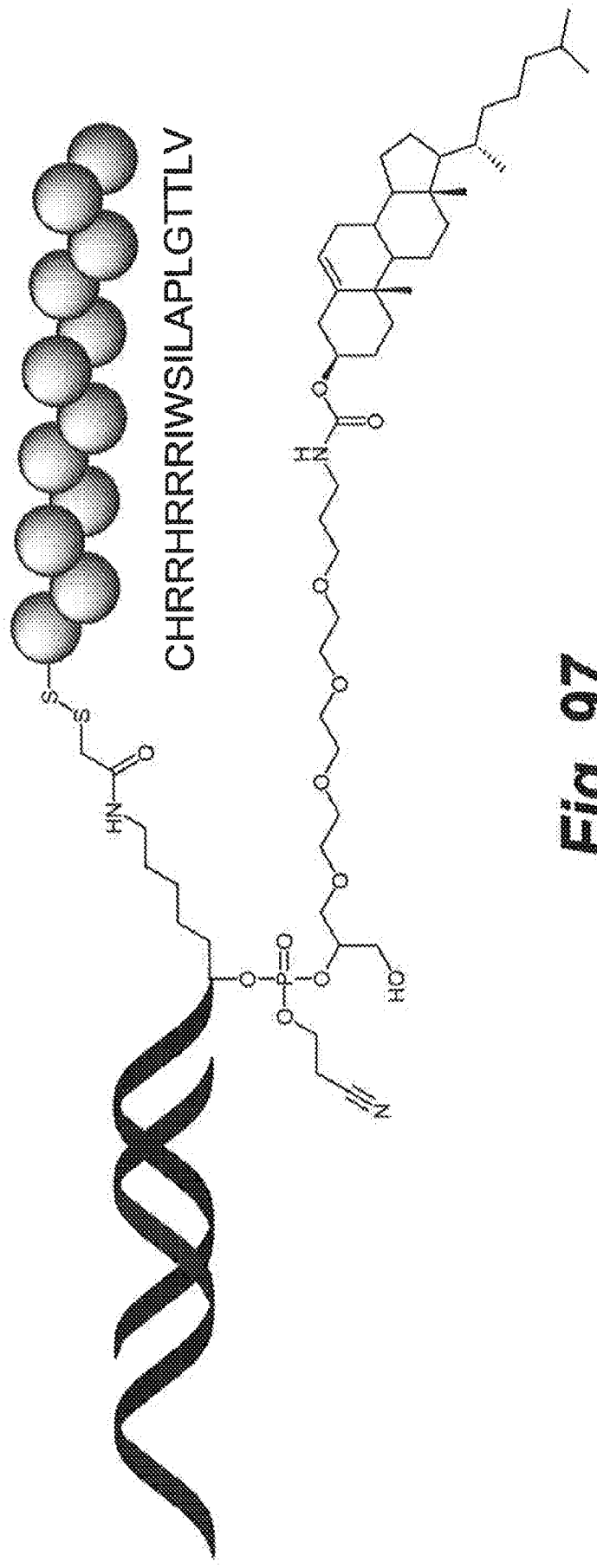


Fig. 97

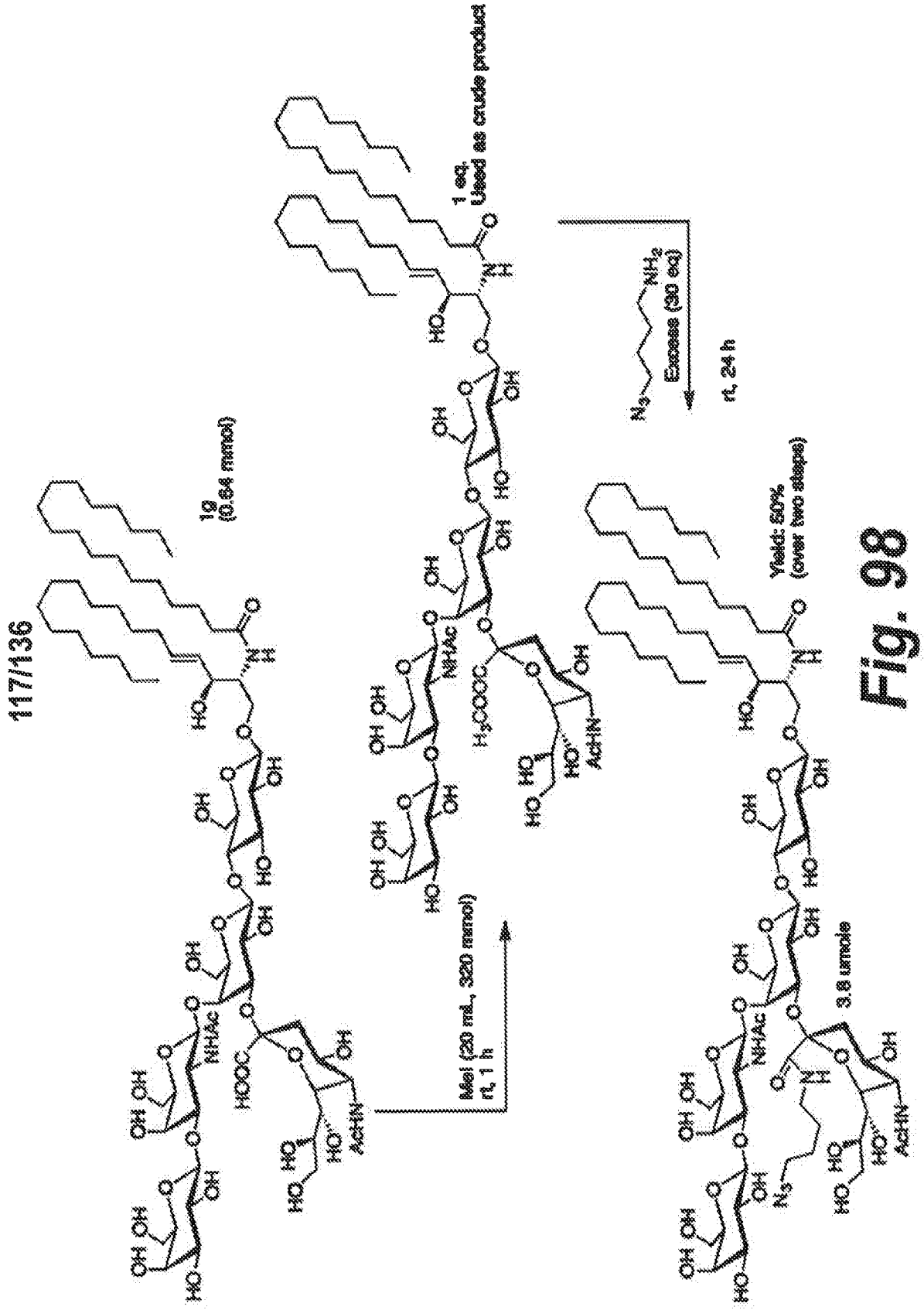

117/136
1g
(0.64 mmol)
MeI (220 mL, 320 mmol)
rt, 1 h
1 eq.
Used as crude product
Excess (30 eq)
rt, 24 h
Yield: 80%
(over two steps)
3.8 umole

Fig. 98

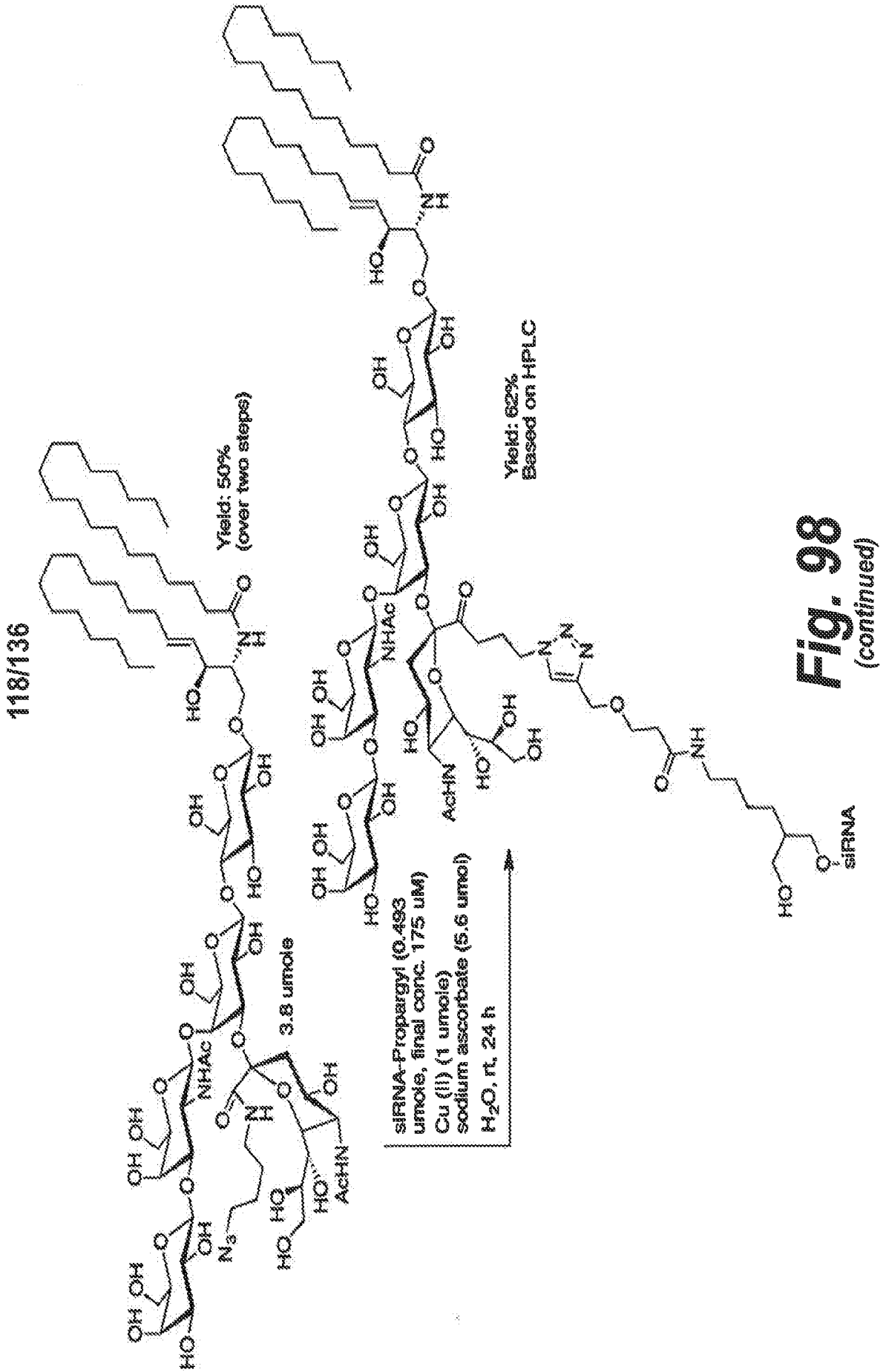


Fig. 98
(continued)

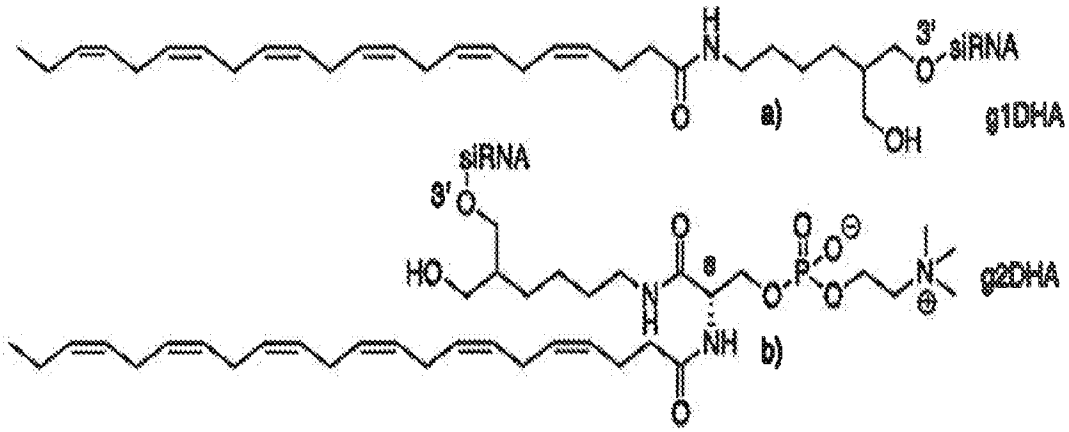


Fig. 99

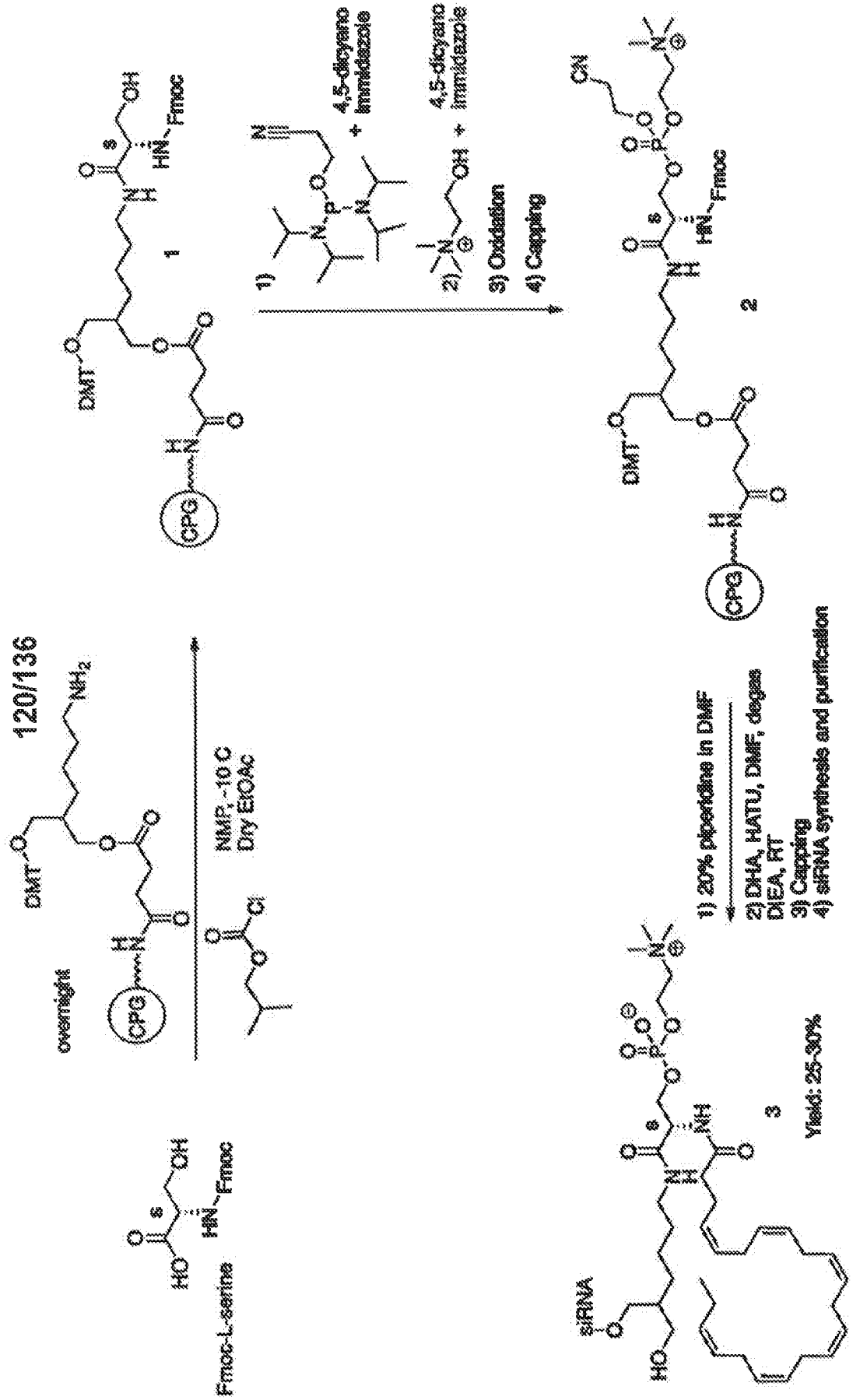

120/136
overnight
NMP, -10 C
Dry EtOAc
Fmoc-L-serine
1
2
3
1) 20% piperidine in DMF
2) DHA, HATU, DMF, degas
DIEA, RT
3) Capping
4) siRNA synthesis and purification
1) 4,5-dicyano imidazole
2) 4,5-dicyano imidazole
3) Oxidation
4) Capping
Yield: 25-30%

Fig. 100

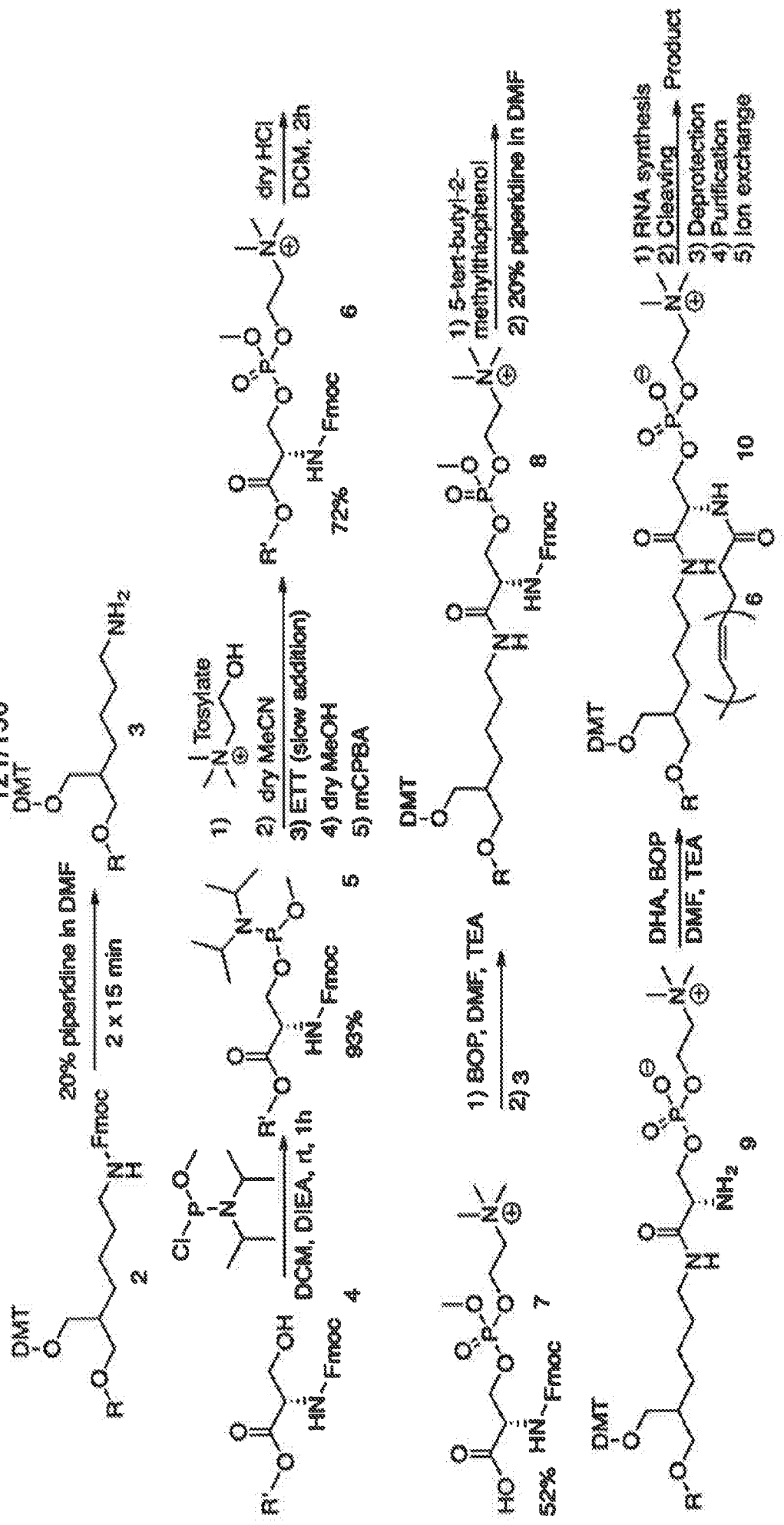

121/136
20% piperidine in DMF
2 x 15 min
DCM, DIEA, rt, 1h
1) Tosylate
2) dry MeCN
3) ETT (slow addition)
4) dry MeOH
5) mCPBA
dry HCl
DCM, 2h
72%
93%
1) BOP, DMF, TEA
2) 3
52%
1) 5-tert-butyl-2-methylthiophenol
2) 20% piperidine in DMF
DHA, BOP
DMF, TEA
1) RNA synthesis
2) Cleaving
Product
3) Deprotection
4) Purification
5) Ion exchange
R =
R' =

Fig. 101

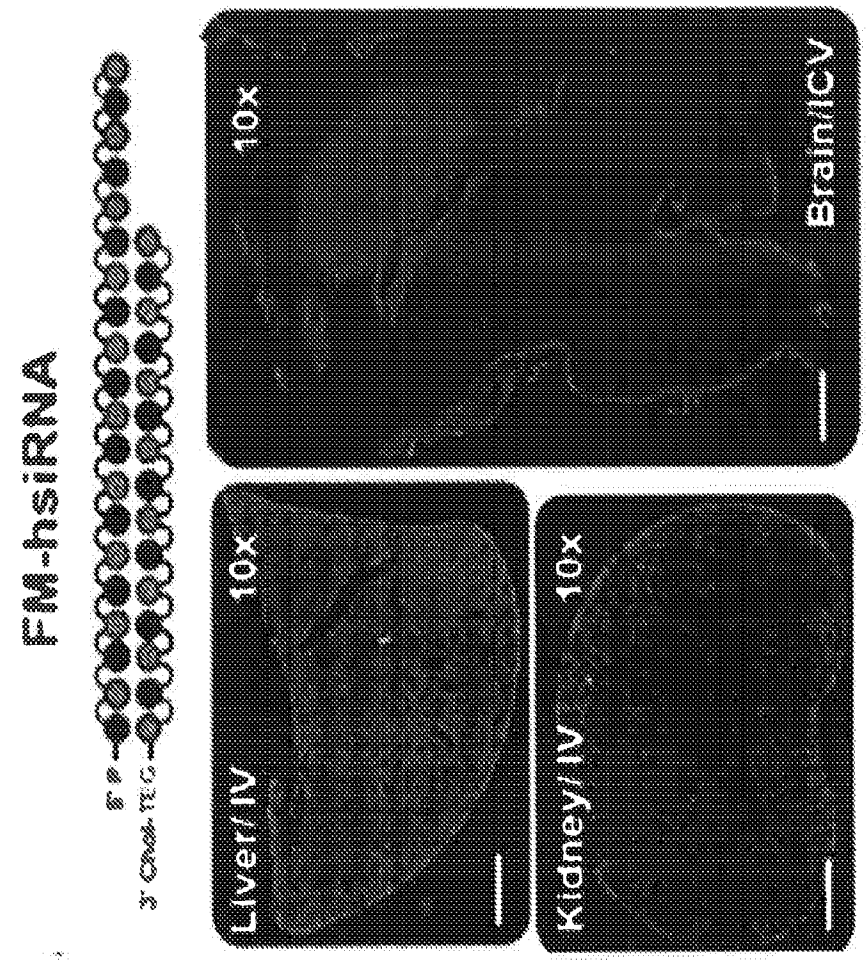


Fig. 102A

Fig. 102B

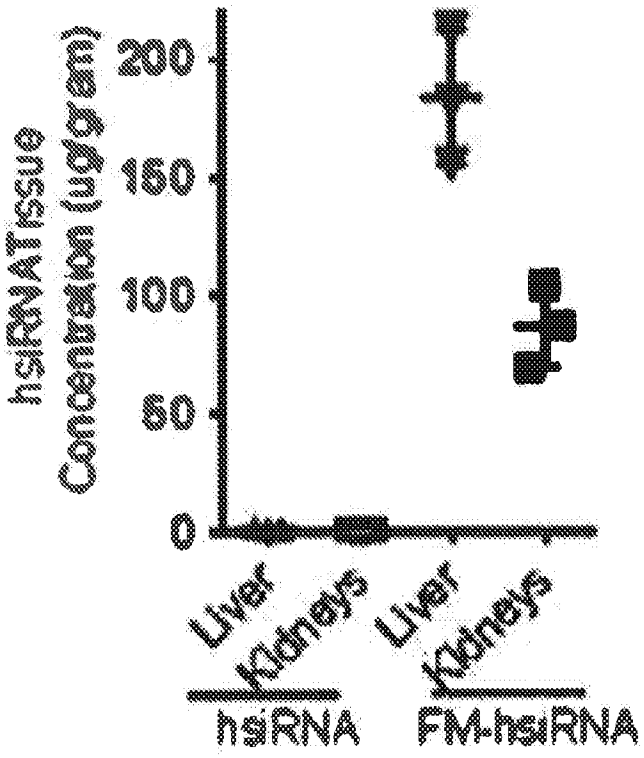


Fig. 102C

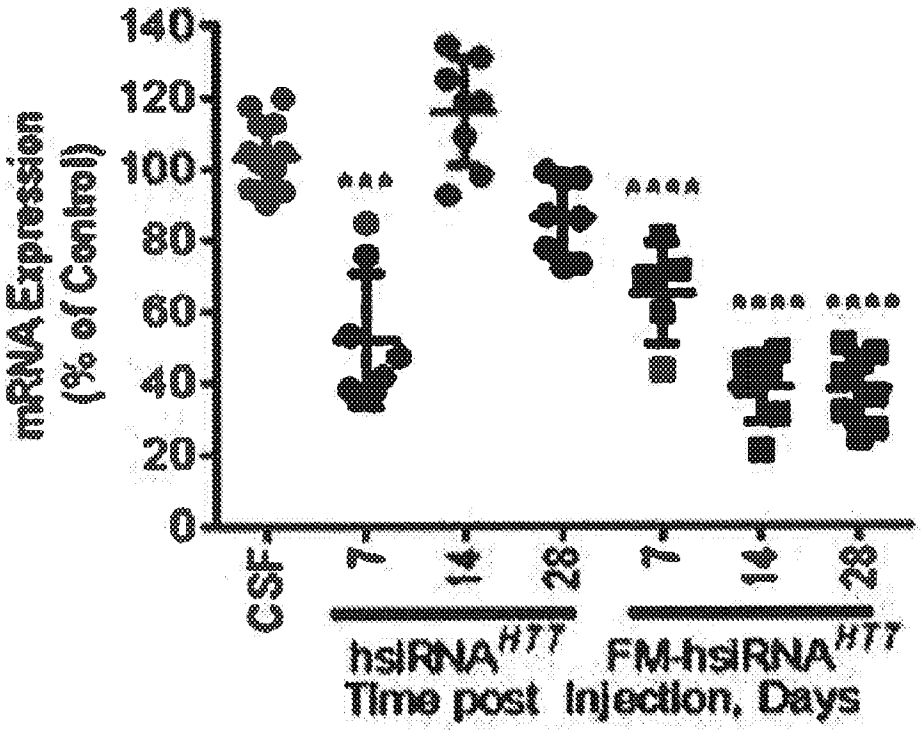


Fig. 102D

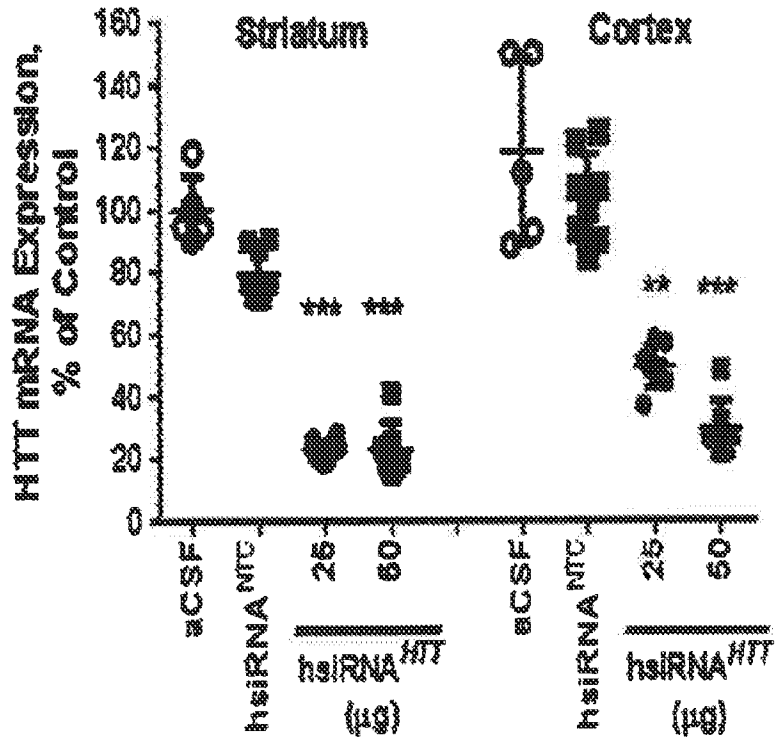


Fig. 103A

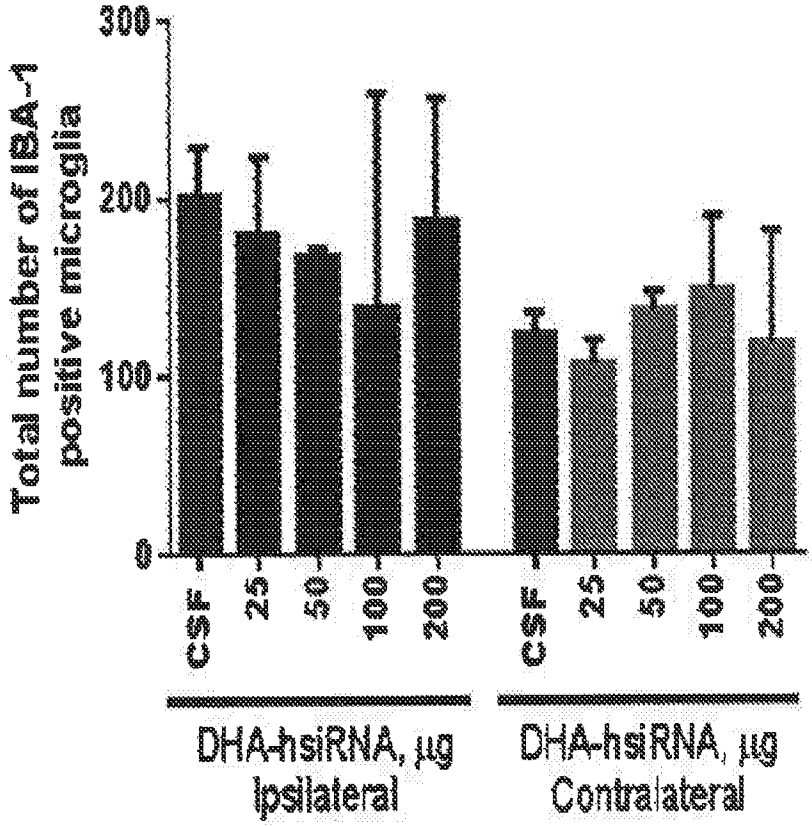


Fig. 103B

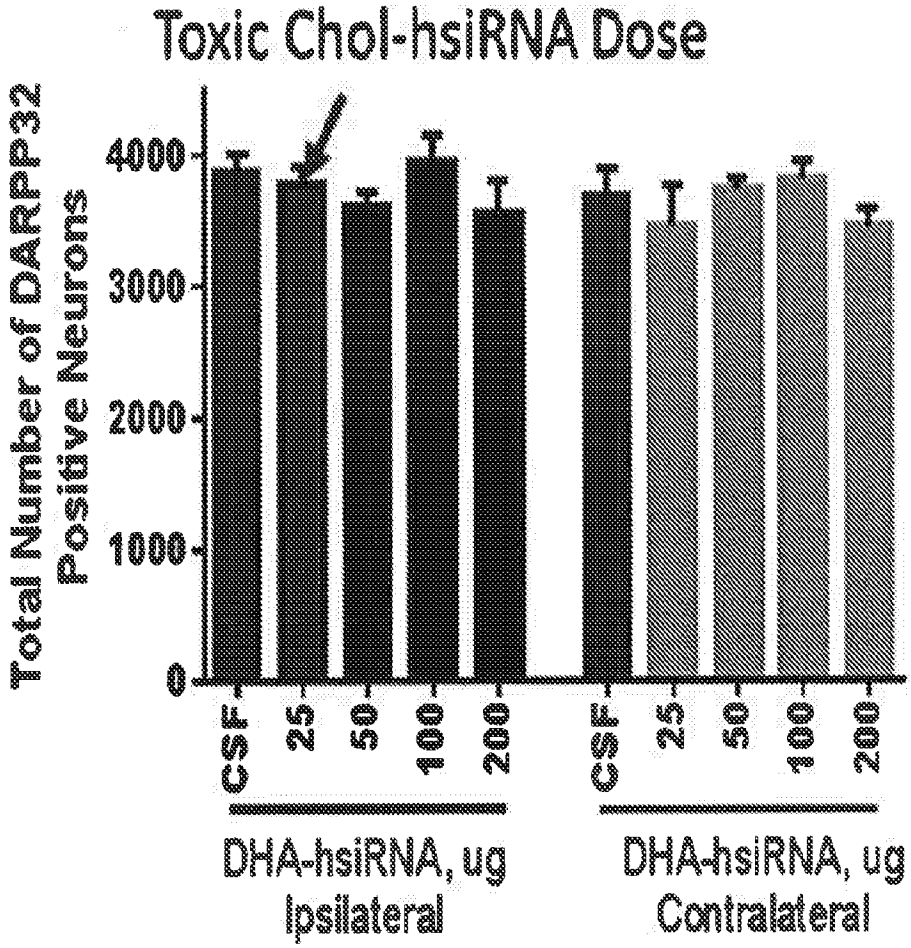

Toxic Chol-hsiRNA Dose
Total Number of DARPP32 Positive Neurons
4000
3000
2000
1000
0
CSF
25
50
100
200
CSF
25
50
100
200
DHA-hsiRNA, ug
Ipsilateral
DHA-hsiRNA, ug
Contralateral

Fig. 103C

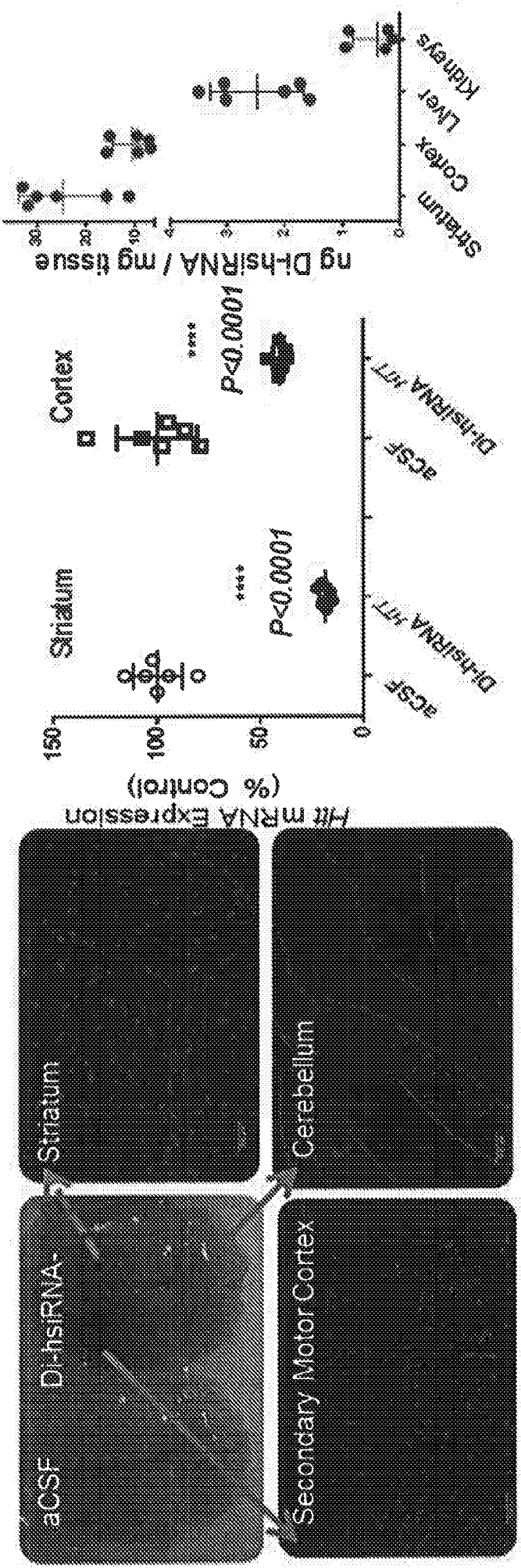

aCSF
Di-hsiRNA-
Striatum
Cerebellum
Secondary Motor Cortex
Htt mRNA expression
(% Control)
150
100
50
0
Striatum
Cortex
****
P<0.0001
aCSF
Di-hsiRNA HTT
ng Di-hsiRNA / mg tissue
Striatum
Cortex
Liver
Kidneys

Fig. 104

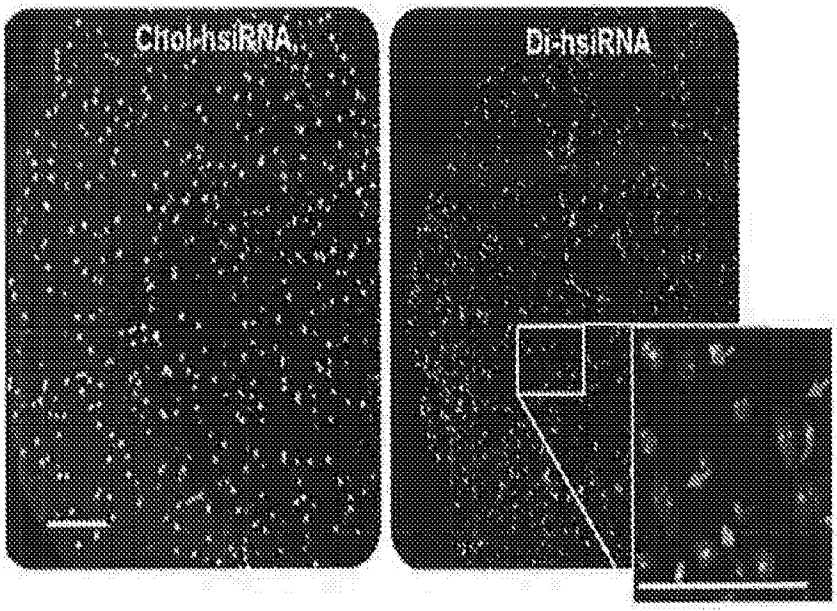


Fig. 105A

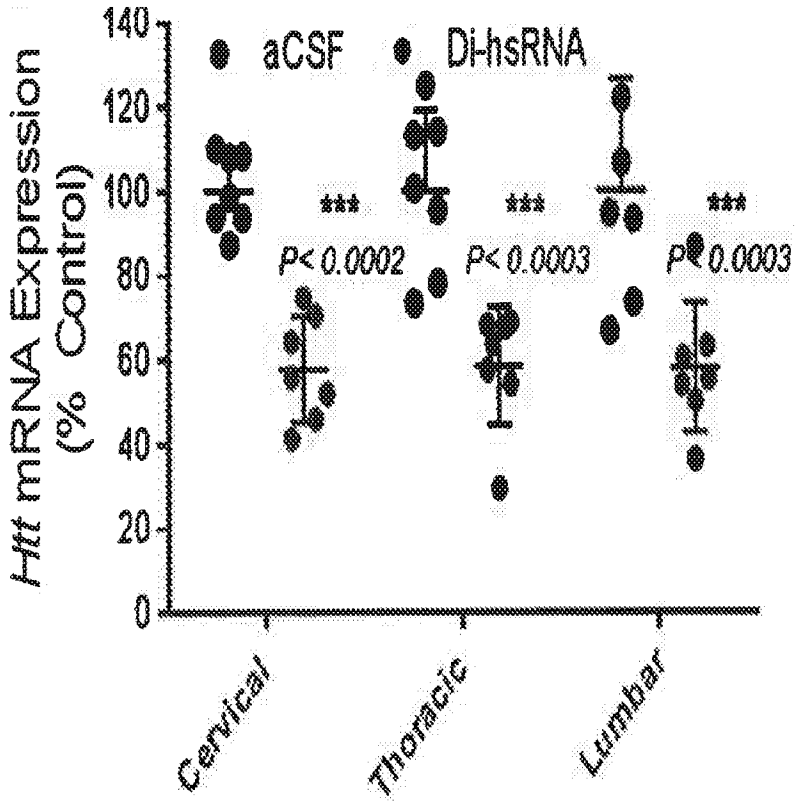


Fig. 105B

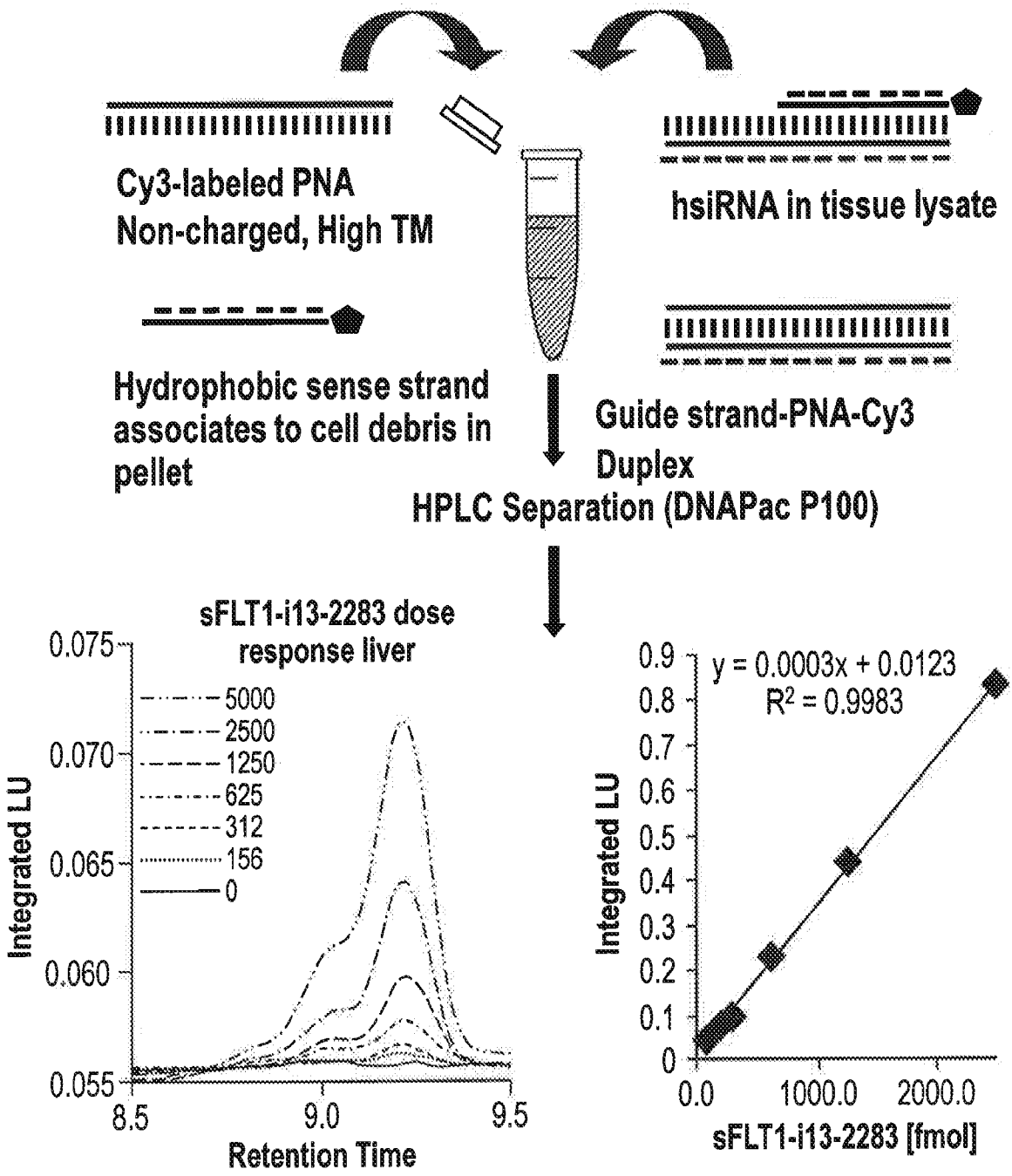

Cy3-labeled PNA
Non-charged, High TM
hsiRNA in tissue lysate
Hydrophobic sense strand associates to cell debris in pellet
Guide strand-PNA-Cy3 Duplex
HPLC Separation (DNAPac P100)
sFLT1-i13-2283 dose response liver
5000
2500
1250
625
312
156
0
Integrated LU
Retention Time
y = 0.0003x + 0.0123
R2 = 0.9983
sFLT1-i13-2283 [fmol]

Fig. 106

Uptake and efficacy of g2DHA-hsiRNA in kidney following a single injection (1 x 15 mg/Kg, 1 week)
DAPI
Cy3
DAPI-Cy3
PBS
hsiRNA-chol
hsiRNA-g2DHA
Tissue Accumulation (ng/mg)
2000
1000
0
129/136
Liver
Kidney
hsiRNA-Chol
hsiRNA-g2DHA
Pfil-113 mRNA Expression (% of Control)
120
100
80
60
40
20
0
PBS
Pfil-g2DHA
Liver
Kidney
g2DHA-hsiRNA accumulates in the proximal convoluted tubules
Glomerulus
Proximal tubule

Fig. 107

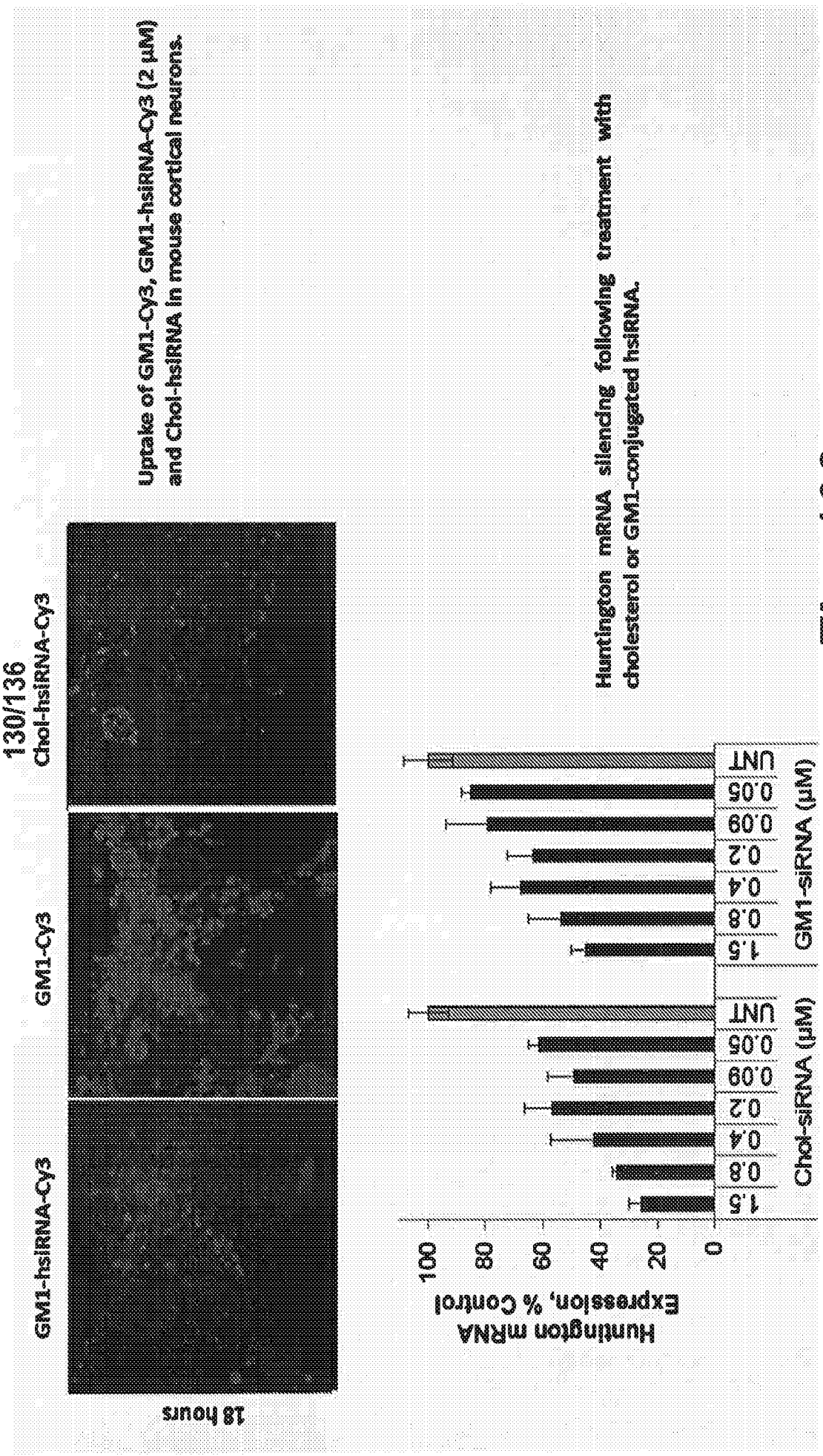

Uptake of GM1-Cy3, GM1-hsiRNA-Cy3 (2 μM) and Chol-hsiRNA in mouse cortical neurons.
130/136
GM1-hsiRNA-Cy3
GM1-Cy3
Chol-hsiRNA-Cy3
18 hours
Huntington mRNA silencing following treatment with cholesterol or GM1-conjugated hsiRNA.
Huntington mRNA Expression, % Control
100
80
60
40
20
0
Chol-siRNA (μM)
1.5
0.8
0.4
0.2
0.09
0.05
UNT
GM1-siRNA (μM)
1.5
0.8
0.4
0.2
0.09
0.05
UNT

Fig. 108

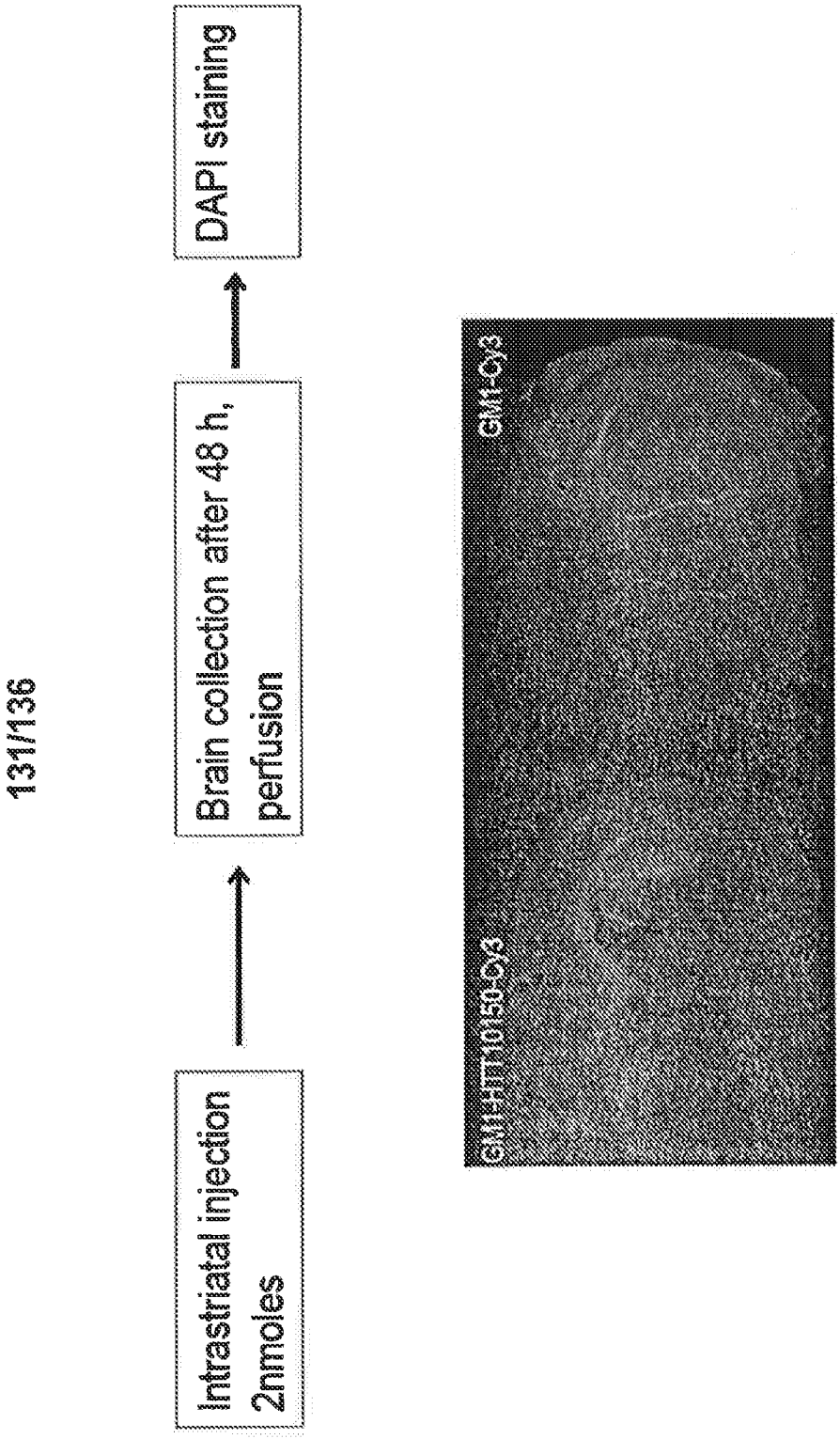

Intrastriatal injection
2nmoles
Brain collection after 48 h,
perfusion
DAPI staining
CM1-Cy3
CM1-HTT1015Q-Cy3

Fig. 109

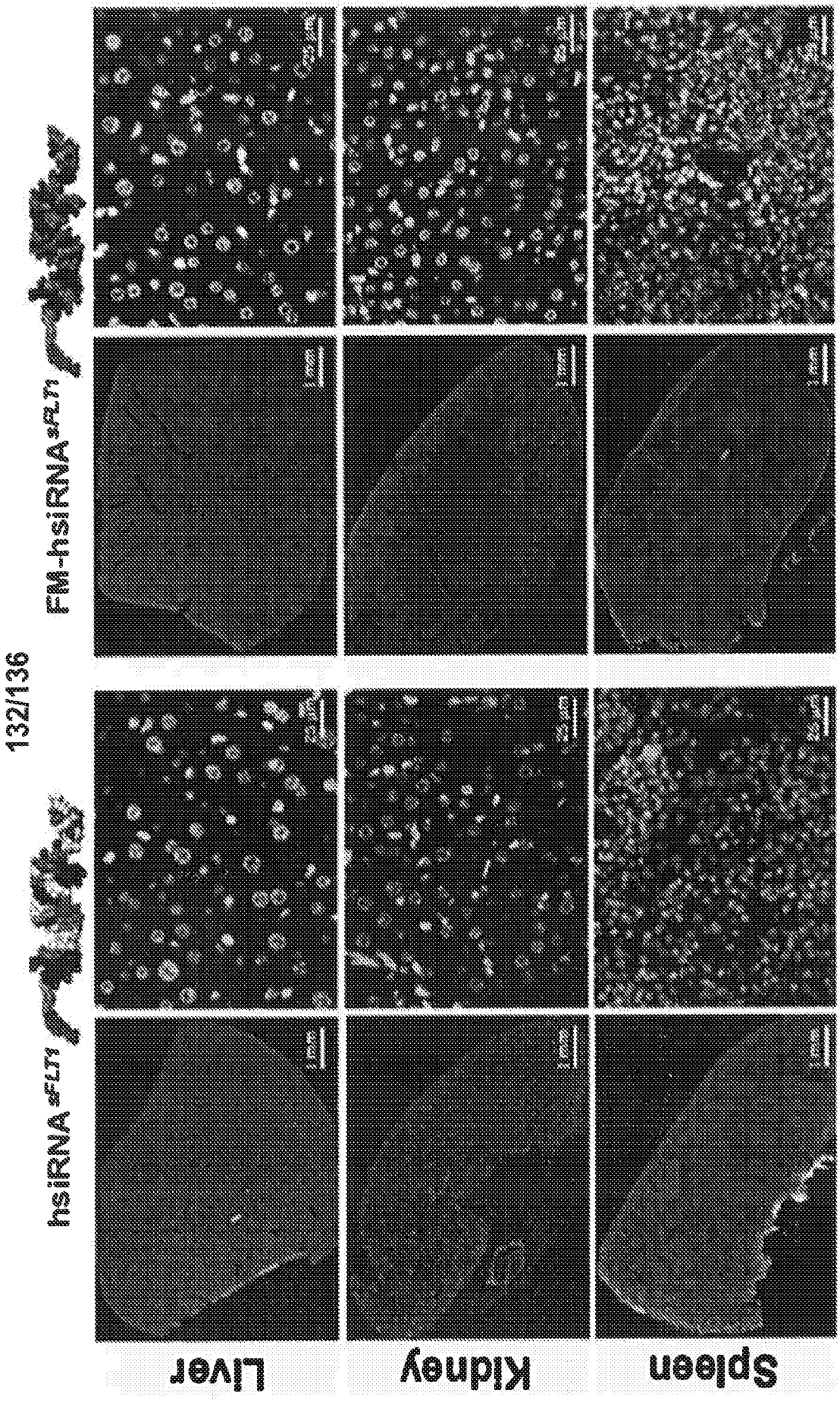


Fig. 110A

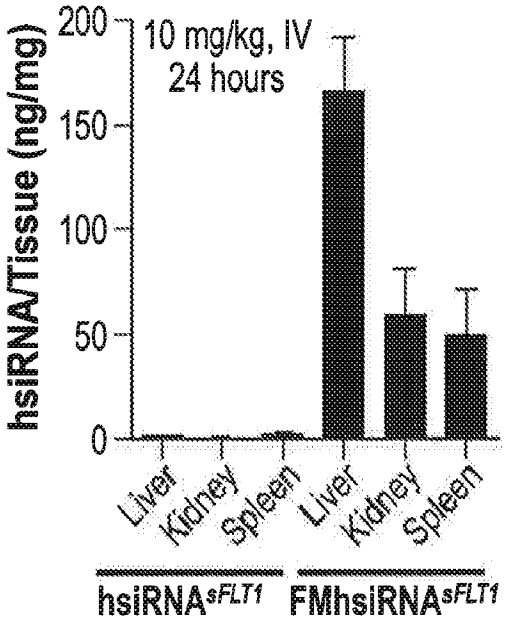


Fig. 110B

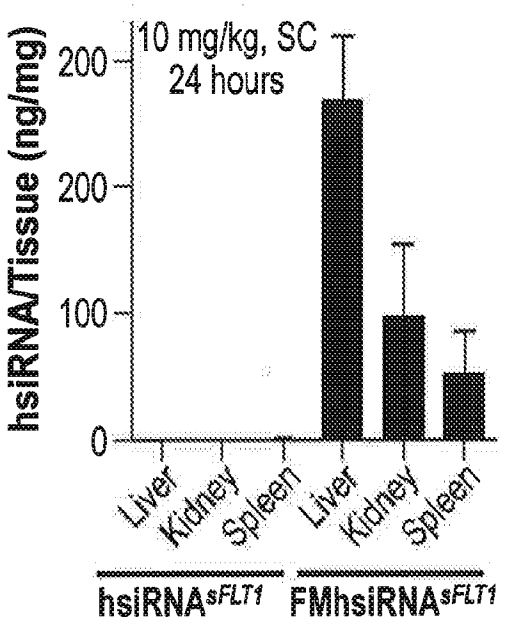


Fig. 110C

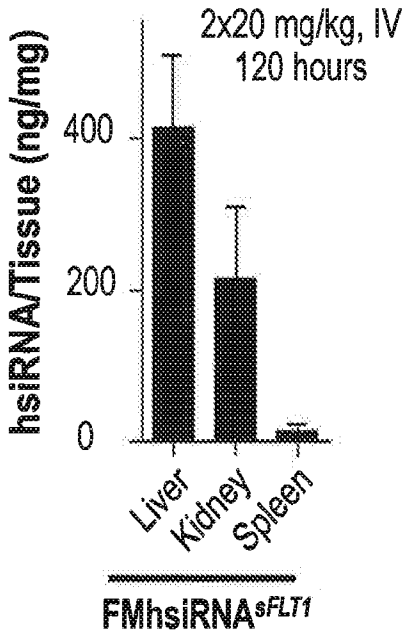


Fig. 110D

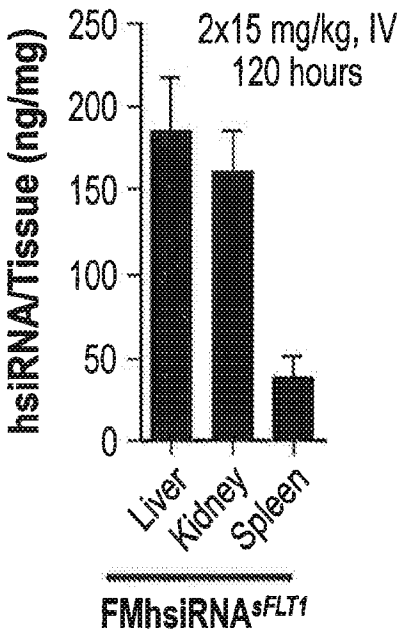


Fig. 110E

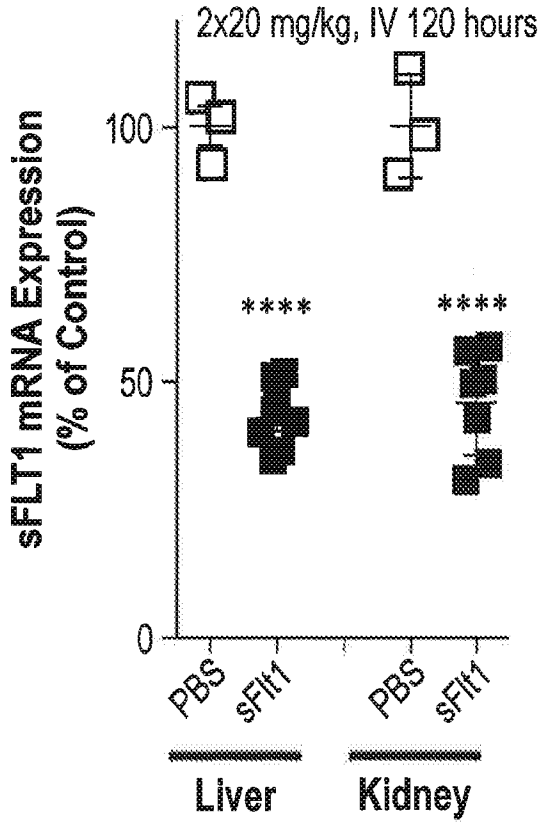


Fig. 110E

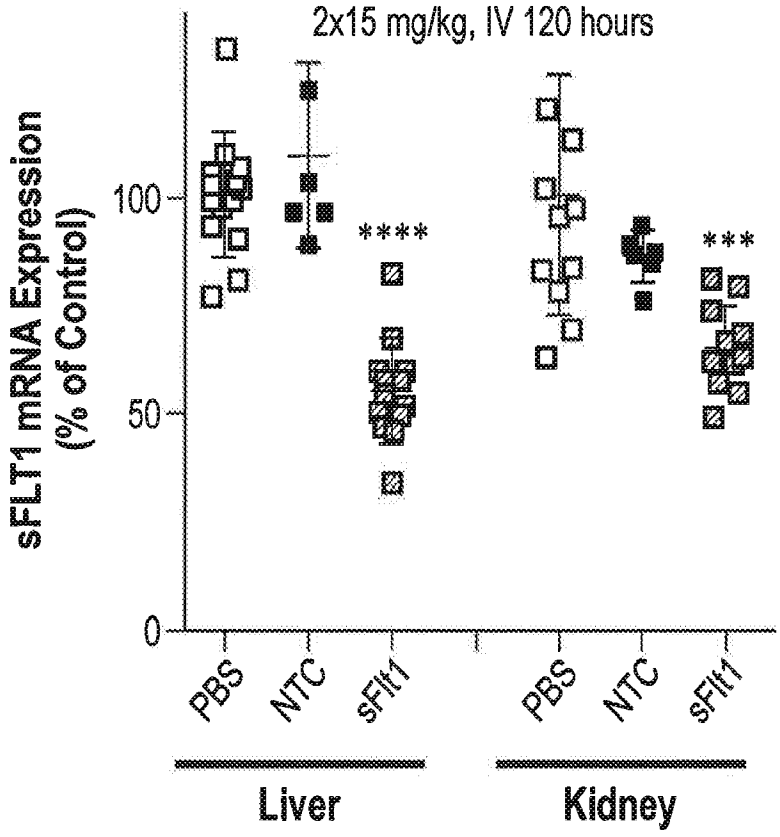


Fig. 110F

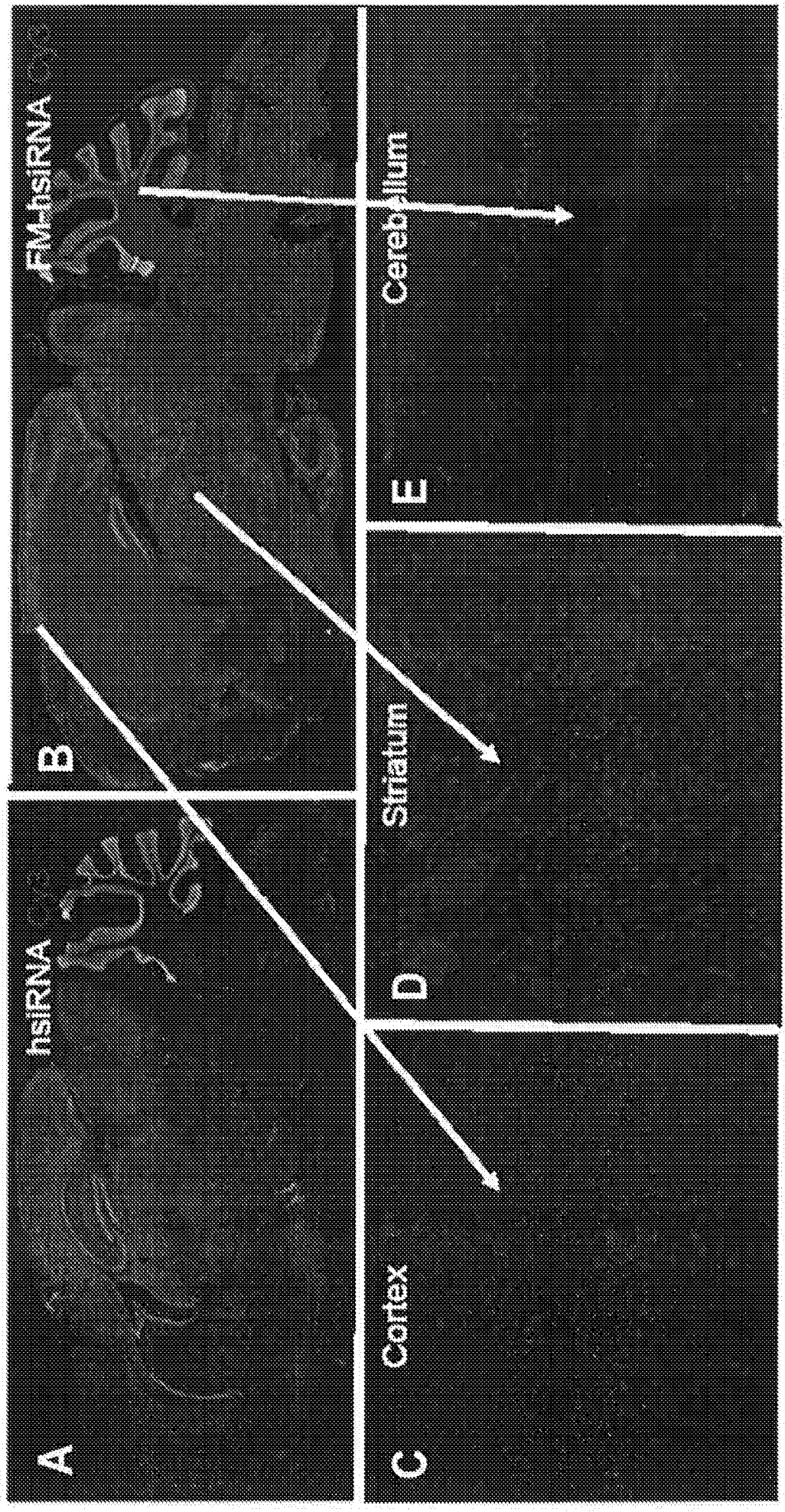


Fig. 111A-E

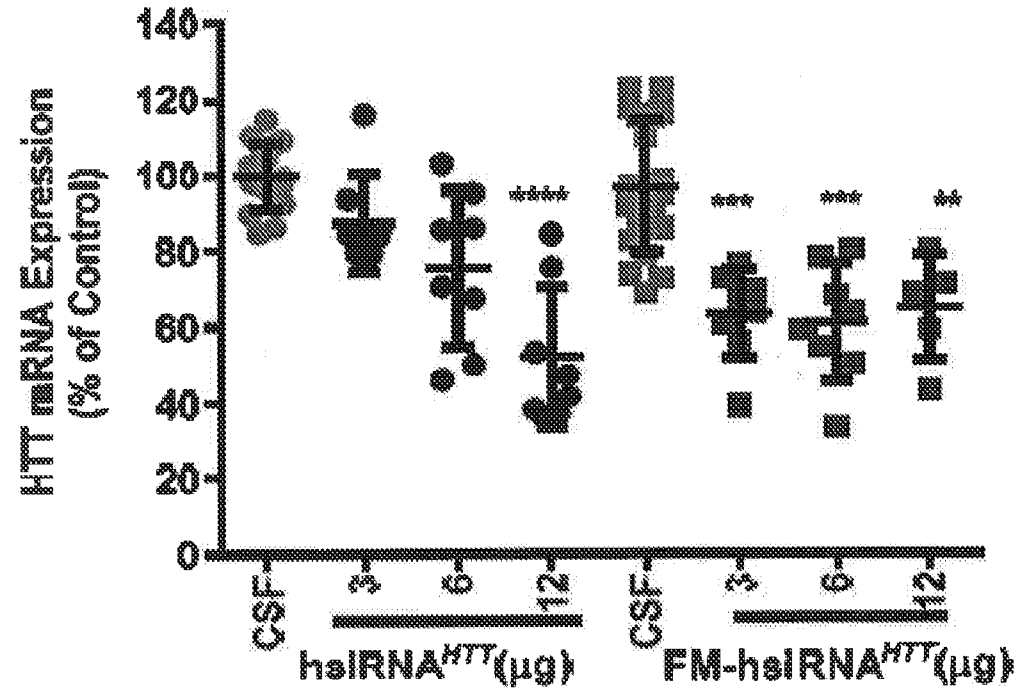


Fig. 111F

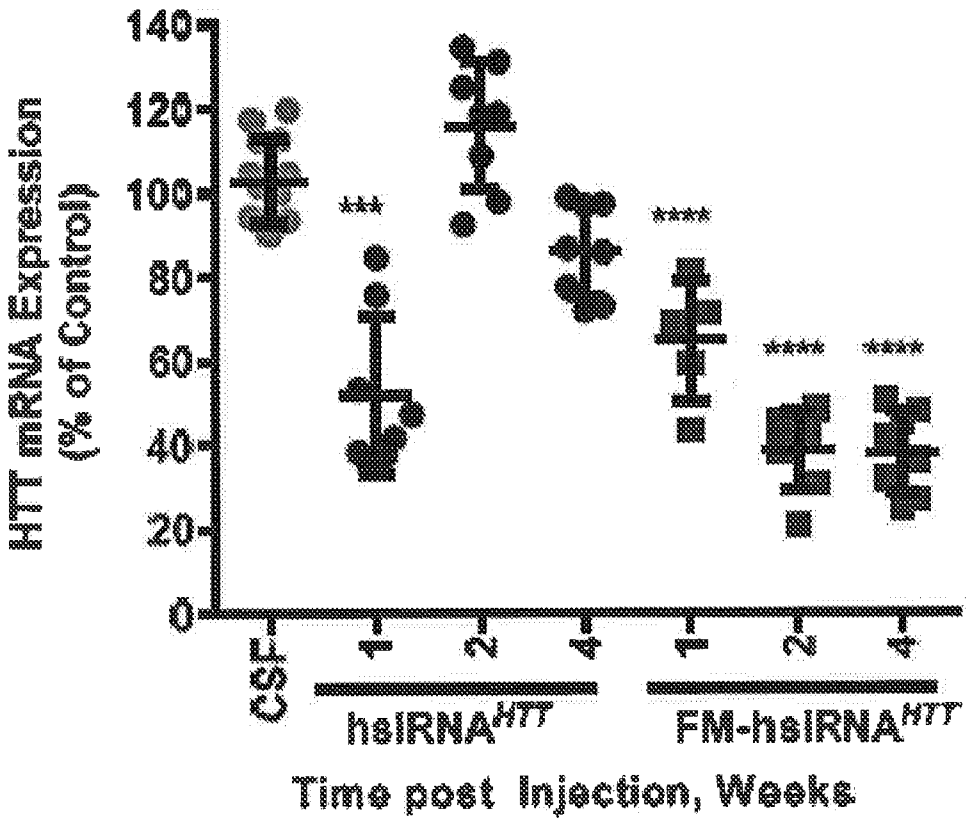


Fig. 111G

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2016/025722

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N15/113 A61K31/713 A61P25/14
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, BIOSIS, WPI Data, Sequence Search

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2012/005898 A2 (ALNYLAM PHARMACEUTICALS INC [US]; ROSSOMANDO ANTHONY [US]; POLLARD STU) 12 January 2012 (2012-01-12) page 1 - page 8; claims 14-30; sequence 606036 paragraph [0098] - paragraph [0150] -& Anonymous: "GS_NUC_ALERT:WO2012005898.606036", , 12 January 2012 (2012-01-12), XP055293757, Retrieved from the Internet: URL:http://ibis.internal.epo.org/exam/dbfetch.jsp?id=GS_NUC_ALERT:WO2012005898.606036 [retrieved on 2016-08-05] the whole document ----- -/--	1-16, 29-32, 66-69, 72-76

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
8 August 2016	12/08/2016

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Spindler, Mark-Peter

1

Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	LAU NELSON C ET AL: "Characterization of the piRNA complex from rat testes", SCIENCE, AMERICAN ASSOCIATION FOR THE ADVANCEMENT OF SCIENCE, US, vol. 313, no. 5785, 21 July 2006 (2006-07-21), pages 363-367, XP002500303, ISSN: 0036-8075, DOI: 10.1126/SCIENCE.1130164 the whole document -& Anonymous: "EM_STD:DQ766949", , 12 July 2006 (2006-07-12), XP055293752, Retrieved from the Internet: URL:http://ibis.internal.epo.org/exam/dbfetch.jsp?id=EM_STD:DQ766949 [retrieved on 2016-08-05] the whole document	33,34
A	WO 2007/051045 A2 (ALNYLAM PHARMACEUTICALS INC [US]; SAH DINAH WEN-YEE [US]; HADWIGER PHI) 3 May 2007 (2007-05-03) the whole document	1-76
A	DONGBO YU ET AL: "Single-Stranded RNAs Use RNAi to Potently and Allele-Selectively Inhibit Mutant Huntingtin Expression", CELL, vol. 150, no. 5, 31 August 2012 (2012-08-31), pages 895-908, XP055166558, ISSN: 0092-8674, DOI: 10.1016/j.cell.2012.08.002 the whole document	1-76
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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	DIFIGLIA M ET AL: "Therapeutic silencing of mutant huntingtin with siRNA attenuates striatal and cortical neuropathology and behavioral deficits", PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES, NATIONAL ACADEMY OF SCIENCES, US, vol. 104, no. 43, 23 October 2007 (2007-10-23), pages 17204-17209, XP002526486, ISSN: 0027-8424, DOI: 10.1073/PNAS.0708285104 the whole document	1-76
A	WO 2010/033247 A2 (RXI PHARMACEUTICALS CORP [US]; KHVOROVA ANASTASIA [US]; SALOMON WILLIA) 25 March 2010 (2010-03-25) the whole document	1-76
X,P	JULIA F ALTERMAN ET AL: "Hydrophobically Modified siRNAs Silence Huntingtin mRNA in Primary Neurons and Mouse Brain", MOLECULAR THERAPY-NUCLEIC ACIDS, vol. 4, no. 12, 1 December 2015 (2015-12-01), page e266, XP055293631, DOI: 10.1038/mtna.2015.38 the whole document	1-76
X,P	WO 2015/161184 A1 (UNIV MASSACHUSETTS [US]) 22 October 2015 (2015-10-22) the whole document	1-76
A,P	ANASTASIA KHVOROVA: "Advances in oligonucleotide chemistry for the treatment of neurodegenerative disorders and brain tumors ¦ Cancer Research", CANCER RESEARCH, vol. 76, no. 6 (suppl.), 15 March 2016 (2016-03-15), page IA27, XP055293840, the whole document	1-74

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2016/025722

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☒ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1-76

RNA molecule of 15-35 bases comprising a sequence substantially complementary to SEQ ID NO: 1, 2, or 3; implementations thereof

1.1. claims: 1-32, 72-76(completely); 66-71(partially)

RNA molecule of 15-35 bases comprising a sequence substantially complementary to SEQ ID NO: 1; implementations thereof

1.2. claims: 33-71(partially)

RNA molecule of 15-35 bases comprising a sequence substantially complementary to SEQ ID NO: 2; implementations thereof

1.3. claims: 33-71(partially)

RNA molecule of 15-35 bases comprising a sequence substantially complementary to SEQ ID NO: 3; implementations thereof

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/US2016/025722

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