(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
1 March 2012 (01.03.2012)

PCT

(10) International Publication Number
WO 2012/027713 A2

(51) **International Patent Classification:**
A61K 48/00 (2006.01)

(21) **International Application Number:**
PCT/US201 1/049427

(22) **International Filing Date:**
26 August 201 1 (26.08.201 1)

(25) **Filing Language:** English

(26) **Publication Language:** English

(30) **Priority Data:**
61/377,437 26 August 2010 (26.08.2010) US

(71) **Applicant** *(for all designated States except US):* **ALNYLAM PHARMACEUTICALS, INC.** [US/US]; 300 Third Street, Third Floor, Cambridge, MA 02 142 (US).

(72) **Inventors; and**
(75) **Inventors/ Applicants** *(for US only):* **HINKLE, Gregory** [US/US]; C/o Alnylam Pharmaceuticals, Inc., 300 Third Street, Third Floor, Cambridge, MA 02142 (US). **BUMCROT, David** [US/US]; C/o Alnylam Pharmaceuticals, Inc., 300 Third Street, Third Floor, Cambridge, MA 02142 (US).

(74) **Agents: SHUSTER, Michael, J.** et al; Fenwick & West LLP, Silicon Valley Center, 801 California Street, Mountain View, CA 94041 (US).

(81) **Designated States** *(unless otherwise indicated, for every kind of national protection available):* AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PE, PG, PH, PL, PT, QA, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) **Designated States** *(unless otherwise indicated, for every kind of regional protection available):* ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— *without international search report and to be republished upon receipt of that report (Rule 48.2(g))*

WO 2012/027713 A2

(54) Title: COMPOSITIONS AND METHODS FOR INHIBITION OF SNCA

(57) Abstract: The invention relates to a double-stranded ribonucleic acid (dsRNA) targeting alpha- synuclein (SNCA) gene expression and methods of using the dsRNA to inhibit expression of SNCA.

COMPOSITIONS AND METHODS FOR INHIBITION OF SNCA

Cross Reference to Related Applications

This application claims the benefit of U.S. Provisional Application No. 61/377,437, filed August 26, 2010, the disclosure of which is hereby incorporated by reference in its entirety for all purposes.

Field of the Invention

The invention relates to compositions and methods for treating neurodegenerative diseases, and more particularly to the downregulation of the alpha-synuclein gene for the treatment of synucleinopathies.

Background of the Invention

Expression of the alpha-synuclein (SNCA) gene produces the protein alpha-synuclein. Mutations in the SNCA gene and SNCA gene multiplications have been linked to familial Parkinson's disease. Parkinson's disease patients demonstrate alpha-synuclein protein aggregates in the brain. Similar aggregates are observed in patients diagnosed with sporadic Parkinson's disease, Alzheimer's disease, multiple system atrophy, and Lewy body dementia.

Double-stranded RNA molecules (dsRNA) have been shown to block gene expression in a highly conserved regulatory mechanism known as RNA interference (RNAi). WO 99/32619 (Fire *et al.)* disclosed the use of a dsRNA of at least 25 nucleotides in length to inhibit the expression of genes in C. *elegans.* dsRNA has also been shown to degrade target RNA in other organisms, including plants (see, *e.g.*, WO 99/53050, Waterhouse *et al;* and WO 99/6163 1, Heifetz *et al), Drosophila* (see, *e.g.*, Yang, D., *et al, Curr. Biol.* (2000) 10:1 191-1200), and mammals (see WO 00/44895, Limmer; and DE 101 00 586.5, Kreutzer *et al).*

Disclosure of dsRNA targeting SNCA can be found in the following published references, patents, and patent applications:

U.S. Pat. No. 7,579,458;

Lewis et al, In vivo silencing of alpha-synuclein using naked siRNA, Mol. Neurodegener. 2008 Nov 1; 3:19;

Sapru et al, Silencing of human alpha-synuclein in vitro and in rat brain using lentiviral-mediated RNAi, Exp Neurol. 2006 Apr;198(2):382-90.;

Manfredsson et al, RNA knockdown as a potential therapeutic strategy in Parkinson's disease, Gene Ther. 2006 Mar;13(6):517-24.;

U.S. Ser. No. 12/334,080;

U.S. Ser. No. 12/725,337;

U.S. Pat. No. 7,595,306;

U.S. Ser. No. 10/991,286;

U.S. Ser. No. 12/416,140;

PCT/US04/18271

Summary of the Invention

Disclosed herein are dsRNAs targeted to the alpha-synuclein (SNCA) gene for inhibiting expression of SNCA in a cell. Also disclosed are methods of using the SNCA dsRNA for siRNA inhibition of SNCA expression and treatment of disease associated with expression and/or over expression of SNCA, *e.g.,* neurodegenerative disorders.

In one embodiment, the invention provides a double-stranded ribonucleic acid (dsRNA) for inhibiting expression of SNCA, comprising a sense strand and an antisense strand comprising a region of complementarity to an mRNA encoding SNCA, wherein each strand is at least 15 nucleotides in length, and wherein the sense strand and antisense strand comprise at least 15 contiguous nucleotides of sequences selected from Tables 2, 3, 4, or 6. In a related embodiment, the sense strand comprises SEQ ID NO:l, 17, 27, 41, 63, 75, 79, 85, 107, 113, 119, 123, 127, 135, 147, 155, 157, 161, 165, or 173 and/or the antisense strand comprises SEQ ID NO:2, 18, 28, 42, 64, 76, 80, 86, 108, 114, 120, 124, 128, 136, 148, 156, 158, 162, 166, or 174. In another related embodiment, the antisense strand is complementary to at least 15 contiguous nucleotides of SEQ ID NO: 157. In yet another related embodiment, the antisense strand is complementary to at least the first 11 nucleotides of SEQ ID NO: 158. In yet another related embodiment, the sense strand sequence comprises SEQ ID NO: 157 and the antisense strand sequence comprises SEQ ID NO: 158.

In yet another embodiment, the invention provides a dsRNA for inhibiting expression of alpha-synuclein SNCA, comprising a sense strand and an antisense strand comprising a region of complementarity to an mRNA encoding SNCA, wherein each strand is at least 15 nucleotides in length, wherein the sense strand comprises SEQ ID NO:553, 569, 579, 593, 615, 627, 631, 637, 659, 665, 671, 675, 679, 687, 699, 707, 709, 713, 717, or 725; and/or the antisense strand comprises SEQ ID NO:554, 570, 580, 594, 616, 628, 632, 638, 660, 666, 672, 676, 680, 688, 700, 708, 710, 714, 718, or 726. In yet another embodiment, the sense strand sequence comprises SEQ ID NO: 707 and the antisense strand sequence comprises SEQ ID NO: 708.

In yet another embodiment, the invention provides a dsRNA selected from the group consisting of: AD-21752, AD-21760, AD-21765, AD-21772, AD-21781, AD-21786, AD-21788,

AD-21790, AD-21801, AD-21804, AD-21806, AD-21808, AD-21810, AD-21813, AD-21817, AD-21821, AD-21822, AD-21824, AD-21826, and AD-21830. In a related embodiment, the dsRNA is AD-21822.

In yet another embodiment, the invention provides a dsRNA for inhibiting expression of SNCA, comprising a sense strand and an antisense strand comprising a region of complementarity to an mRNA encoding SNCA, wherein each strand is at least 15 nucleotides in length, and wherein the sense strand and antisense strand comprise at least 15 contiguous nucleotides of sequences selected from Tables 2, 3, 4, or 6, and wherein the SNCA is either human SNCA encoded by NM_007308. 1 or monkey SNCA encoded by XM_001095402. 1. In a related embodiment, the region of complementarity is between 19 and 2 1 nucleotides in length. In another related related embodiment, the region of complementarity is 19 nucleotides in length.

In another embodiment of the dsRNA for inhibiting expression of SNCA described in the Summary above, at least one strand of the dsRNA comprises a 3' overhang of at least 1 nucleotide. In another embodiment, the dsRNA comprises a nucleotide overhang having 1 to 4 nucleotides. In yet another embodiment, each strand comprises a 3' overhang consisting of 2 nucleotides. In yet another embodiment, each strand comprises a 3' overhang consisting of dTsdT.

In yet another embodiment, the dsRNA for inhibiting expression of SNCA comprises at least one modified nucleotide. In a related embodiment, the modified nucleotide is selected from the group of: a 2'-0-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 another related embodiment, 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. In yet another related embodiment, the dsRNA comprises at least one 2'-0-methyl modified nucleotide and at least one 2'-deoxythymidine-3'-phosphate nucleotide comprising a 5'-phosphorothioate group. In yet another related embodiment, the sense strand of the modified dsRNA comprises all 2'-0-methyl modified pyrimidines and the antisense strand comprises 2'-0-methyl modified pyrimidines when the pyrimidine is adjacent to A; and wherein each strand comprises dTdT at the 3' end. In yet another related embodiment, the sense strand of the modified dsRNA comprises all 2'-0-methyl modified pyrimidines and the antisense strand comprises 2'-0-methyl modified pyrimidines when the pyrimidine is adjacent to A; and wherein each strand comprises dTsdT at the 3' end. In yet another related embodiment, the sense strand of the modified dsRNA

comprises all 2'-0-methyl modified pyrimidines and the antisense strand comprises 2'-0-methyl modified pyrimidines when a) the pyrimidine is adjacent to A, or b) the pyrimidine is a uracil adjacent to a U or a G; and wherein each strand comprises dTsdT at the 3' end.

In yet another embodiment, the dsRNA for inhibiting expression of SNCA further comprising a ligand. In a related embodiment, the ligand is conjugated to the 3'-end of the sense strand of the dsRNA.

In yet another embodiment, the invention provides a composition for inhibiting expression of a SNCA gene comprising one of the dsRNAs for inhibitting expression of SNCA described above and a pharmaceutical formulation. In a related embodiment, the pharmaceutical formulation is a lipid formulation. In another related embodiment, the pharmaceutical formulation is a LNP formulation, a LNP01 formulation, a XTC-SNALP formulation, a SNALP formulation, or a LNP 11 formulation.

In another embodiment, the invention provides a dsRNA composition described above for inhibiting expression of a SNCA gene, wherein upon contact with a cell expressing the SNCA gene, the dsRNA inhibits expression of the SNCA gene by at least 40% compared to a cell not so contacted. In another embodiment, the dsRNA has an IC50 of 10 pM or less. In another embodiment, the invention provides a dsRNA composition described above for inhibiting expression of a SNCA gene, wherein administration of 0.1 nM of the dsRNA to a NCI-H460 cell or a BE(2)M17 cell results in greater than 50% inhibition of SNCA mRNA expression as measured by a real time PCR assay or administration of 10 nM of the dsRNA to a NCI-H460 cell or a BE(2)M17 cell results in greater than 66% inhibition of SNCA mRNA expression as measured by a real time PCR assay.

In another embodiment, the invention provides an isolated cell containing one of the dsRNAs described above for inhibiting expression of a SCNA gene. In yet another embodiment, the invention provides a vector comprising a nucleotide sequence that encodes the nucleic acid sequence of at least one strand of a dsRNA described above. In a related embodiment, the invention provides a cell comprising this vector.

In yet another embodiment, the invention provides a method of inhibiting SNCA expression in a cell, the method comprising: (a) introducing into the cell a dsRNA comprising a sense strand and an antisense strand comprising a region of complementarity to an mRNA encoding SNCA, wherein each strand is at least 15 nucleotides in length, and wherein the sense strand and antisense strand comprise at least 15 contiguous nucleotides of sequences selected from Tables 2, 3, 4, or 6; and (b) maintaining the cell produced in step (a) for a time sufficient to obtain degradation of the mRNA transcript of a SNCA gene, thereby inhibiting expression of the

SNCA gene in the cell. In a related embodiment, expression is inhibited by at least 20%, 40%, 60%, or at least 80%. In another related embodiment, the dsRNA is a dsRNA for inhibiting SNCA expression described above. In yet another related embodiment, the invention provides a method treating a disorder mediated by SNCA expression, comprising administering to a human in need of such treatment a therapeutically effective amount of a dsRNA for inhibiting SNCA expression described above. In yet another embodiment, the invention provides a method of treating, preventing or managing a neurodegenerative disorder comprising administering to a patient in need of such treatment, prevention or management a therapeutically or prophylactically effective amount of a dsRNA for inhibiting SNCA described above. In a related embodiment, the neurodegenerative disorder is a synucleinopathy. In another related embodiment, the neurodegenerative disorder is Parkinson's disease. In yet another related embodiment, the neurodegenerative disorder is Alzheimer's disease, multiple system atrophy, or Lewy body dementia.

In yet another embodiment, the invention provides a method of treating a human comprising: identifying a human diagnosed as having a neurodegenerative disorder or at risk for developing the neurodegenerative disorder and administering a dsRNA for inhibiting SNCA described above. In a related embodiment, the method comprises administering an additional composition. In yet another related embodiment, the additional composition is a second dsRNA.

In a further embodiment, the invention provides a method of inhibiting SNCA expression in the brain comprising administering an effective amount of a dsRNA described above into the brain of a subject to obtain suppression of expression of SNCA mRNA, thereby inhibiting SNCA expression in the brain. In a related embodiment, the administration is by infusion. In other related embodiments, the dsRNA comprises a sense strand of SEQ ID NO: 709 or 679 and/or an antisense strand of SEQ ID NO: 710 or 680. In further related embodiments, the dsRNA is AD-21822 or AD-21810. In another related embodiment, the administration of the dsRNA results in greater than 40%, 50%, 60%, 70%, 80%, or 90% inhibition of SNCA mRNA expression. In additional related embodiments, the administration of the dsRNA results in greater than 65% inhibition of SNCA mRNA expression at the end of infusion, greater than 70% inhibition of SNCA mRNA expression one week after infusion, greater than 40% inhibition of SNCA mRNA expression two weeks after infusion, or greater than 50% inhibition of SNCA mRNA expression four weeks after infusion. In other related embodiments, the administration of the dsRNA results in greater than 50%, 60%, 70%, or 80% inhibition of SNCA mRNA expression after 7 or 28 days of infusion.

Additional embodiments of the invention are set forth in the following description and in the claims.

Description of the Drawings

Not applicable.

Detailed Description of the Invention

The invention provides dsRNAs and methods of using the dsRNAs for inhibiting the expression of alpha-synuclein (SNCA). The invention also provides compositions and methods for treating pathological conditions and diseases, such as neurodegenerative diseases in a mammal caused by the over-expression of SNCA. A dsRNA directs the sequence-specific degradation of mRNA through a process known as RNA interference (RNAi).

The dsRNAs of the compositions featured herein include an antisense strand having a region which is 30 nucleotides or less in length, generally 19-24 nucleotides in length, and is complementary to at least part of an mRNA transcript of a SNCA gene. The use of these dsRNAs enables the targeted degradation of mRNAs of genes that are implicated in pathologies associated with SNCA expression in mammals. Very low dosages of SNCA dsRNAs in particular can specifically and efficiently mediate RNAi, resulting in significant inhibition of expression of a SNCA gene. Using cell-based assays, the present inventors demonstrate that dsRNAs targeting SNCA can specifically and efficiently mediate RNAi, resulting in significant inhibition of expression of a SNCA gene. Thus, methods and compositions including these dsRNAs are useful for treating pathological processes that can be mediated by down regulating SNCA over-expression, such as, *e.g.,* treatment of neurodegenerative diseases.

The following detailed description discloses how to make and use the compositions containing dsRNAs to inhibit the expression of a SNCA gene, as well as compositions *(e.g.,* pharmaceutical compositions) and methods for treating diseases and disorders caused by the expression of this gene.

Accordingly, in some aspects, pharmaceutical compositions containing a SNCA dsRNA and a pharmaceutically acceptable carrier, methods of using the compositions to inhibit expression of a SNCA gene, and methods of using the pharmaceutical compositions to treat diseases caused by expression of a SNCA gene are featured in the invention.

As used herein, disorders associated with SNCA expression refer to any biological or pathological state that (1) is mediated in part by the presence of SNCA protein and (2) whose outcome can be affected by reducing the level of SNCA protein present. Specific disorders associated with SNCA expression are noted below.

Because dsRNA mediated silencing can persist for several days after administering the dsRNA composition, in many instances, it is possible to administer the composition with a frequency once per day or less, or, for some instances, only once for the entire therapeutic regimen.

Definitions

For convenience, the meaning of certain terms and phrases used in the specification, examples, and appended claims, are provided below. If there is an apparent discrepancy between the usage of a term in other parts of this specification and its definition provided in this section, the definition in this section shall prevail.

"G," "C," "A" and "U" each generally stand for a nucleotide that contains guanine, cytosine, adenine, and uracil as a base, respectively. "T" and "dT" are used interchangeably herein and refer to a deoxyribonucleotide wherein the nucleobase is thymine, *e.g.,* deoxyribothymine. However, it will be understood that the term "ribonucleotide" or "nucleotide" or "deoxyribonucleotide" can also refer to a modified nucleotide, as further detailed below, or a surrogate replacement moiety. The skilled person is well aware that guanine, cytosine, adenine, and uracil may be replaced by other moieties without substantially altering the base pairing properties of an oligonucleotide comprising a nucleotide bearing such replacement moiety. For example, without limitation, a nucleotide comprising inosine as its base may base pair with nucleotides containing adenine, cytosine, or uracil. Hence, nucleotides containing uracil, guanine, or adenine may be replaced in the nucleotide sequences of the invention by a nucleotide containing, for example, inosine. Sequences comprising such replacement moieties are embodiments of the invention.

As used herein "SNCA" refers to a SNCA mRNA, protein, peptide, or polypeptide. The term "SNCA" is also known in the art as alpha-synuclein.

The sequence of a human SNCA mRNA transcript can be found at NM_007308. 1. The sequence of rhesus monkey SNCA mRNA can be found at XM_00 1095402. 1.

As used herein "target" or "target gene" refers to a SNCA gene.

As used herein, "target sequence" refers to a contiguous portion of the nucleotide sequence of an mRNA molecule formed during the transcription of a SNCA gene, including mRNA that is a product of RNA processing of a primary transcription product.

As used herein, the term "strand comprising a sequence" refers to an oligonucleotide comprising a chain of nucleotides that is described by the sequence referred to using the standard nucleotide nomenclature.

As used herein, and unless otherwise indicated, the term "complementary," when used to describe a first nucleotide sequence in relation to a second nucleotide sequence, refers to the ability of an oligonucleotide or polynucleotide comprising the first nucleotide sequence to hybridize and form a duplex structure under certain conditions with an oligonucleotide or polynucleotide comprising the second nucleotide sequence, as will be understood by the skilled person. Such conditions can, for example, be stringent conditions, where stringent conditions may include: 400 mM NaCl, 40 mM PIPES pH 6.4, 1 mM EDTA, 50°C or 70°C for 12-16 hours followed by washing. Other conditions, such as physiologically relevant conditions as may be encountered inside an organism, can apply. The skilled person will be able to determine the set of conditions most appropriate for a test of complementarity of two sequences in accordance with the ultimate application of the hybridized nucleotides.

This includes base-pairing of the oligonucleotide or polynucleotide comprising the first nucleotide sequence to the oligonucleotide or polynucleotide comprising the second nucleotide sequence over the entire length of the first and second nucleotide sequence. Such sequences can be referred to as "fully complementary" with respect to each other herein. However, where a first sequence is referred to as "substantially complementary" with respect to a second sequence herein, the two sequences can be fully complementary, or they may form one or more, but generally not more than 4, 3 or 2 mismatched base pairs upon hybridization, while retaining the ability to hybridize under the conditions most relevant to their ultimate application. However, where two oligonucleotides are designed to form, upon hybridization, one or more single stranded overhangs, such overhangs shall not be regarded as mismatches with regard to the determination of complementarity. For example, a dsRNA comprising one oligonucleotide 21 nucleotides in length and another oligonucleotide 23 nucleotides in length, wherein the longer oligonucleotide comprises a sequence of 21 nucleotides that is fully complementary to the shorter oligonucleotide, may yet be referred to as "fully complementary" for the purposes described herein.

"Complementary" sequences, as used herein, may also include, or be formed entirely from, non-Watson-Crick base pairs and/or base pairs formed from non-natural and modified nucleotides, in as far as the above requirements with respect to their ability to hybridize are fulfilled. Such non-Watson-Crick base pairs includes, but not limited to, G:U Wobble or Hoogstein base pairing.

The terms "complementary," "fully complementary" and "substantially complementary" herein may be used with respect to the base matching between the sense strand and the antisense

strand of a dsRNA, or between the antisense strand of a dsRNA and a target sequence, as will be understood from the context of their use.

As used herein, a polynucleotide that is "substantially complementary to at least part of a messenger RNA (mRNA) refers to a polynucleotide that is substantially complementary to a contiguous portion of the mRNA of interest *(e.g.,* a target gene, *e.g.,* an mRNA encoding SNCA) including a 5' UTR, an open reading frame (ORF), or a 3' UTR. For example, a polynucleotide is complementary to at least a part of a SNCA mRNA if the sequence is substantially complementary to a non-interrupted portion of an mRNA encoding SNCA.

The term "double-stranded RNA" or "dsRNA," as used herein, refers to a complex of ribonucleic acid molecules, having a duplex structure comprising two anti-parallel and substantially complementary, as defined above, nucleic acid strands. In general, the majority of nucleotides of each strand are ribonucleotides, but as described in detail herein, each or both strands can also include at least one non-ribonucleotide, *e.g.,* a deoxyribonucleotide and/or a modified nucleotide. In addition, as used in this specification, "dsRNA" may include chemical modifications to ribonucleotides, including substantial modifications at multiple nucleotides and including all types of modifications disclosed herein or known in the art. Any such modifications, as used in an siRNA type molecule, are encompassed by "dsRNA" for the purposes of this specification and claims

The two strands forming the duplex structure may be different portions of one larger RNA molecule, or they may be separate RNA molecules. Where the two strands are part of one larger molecule, and therefore are connected by an uninterrupted chain of nucleotides between the 3'-end of one strand and the 5'-end of the respective other strand forming the duplex structure, the connecting RNA chain is referred to as a "hairpin loop." Where the two strands are connected covalently by means other than an uninterrupted chain of nucleotides between the 3'-end of one strand and the 5'-end of the respective other strand forming the duplex structure, the connecting structure is referred to as a "linker." The RNA strands may have the same or a different number of nucleotides. The maximum number of base pairs is the number of nucleotides in the shortest strand of the dsRNA minus any overhangs that are present in the duplex. In addition to the duplex structure, a dsRNA may comprise one or more nucleotide overhangs. The term "siRNA" is also used herein to refer to a dsRNA as described above.

As used herein, a "nucleotide overhang" refers to the unpaired nucleotide or nucleotides that protrude from the duplex structure of a dsRNA when a 3'-end of one strand of the dsRNA extends beyond the 5'-end of the other strand, or vice versa. "Blunt" or "blunt end" means that there are no unpaired nucleotides at that end of the dsRNA, *i.e.,* no nucleotide overhang. A

"blunt ended" dsRNA is a dsRNA that is double-stranded over its entire length, *i.e.*, no nucleotide overhang at either end of the molecule.

The term "antisense strand" refers to the strand of a dsRNA which includes a region that is substantially complementary to a target sequence. As used herein, the term "region of complementarity" refers to the region on the antisense strand that is substantially complementary to a sequence, for example a target sequence, as defined herein. Where the region of complementarity is not fully complementary to the target sequence, the mismatches are most tolerated in the terminal regions and, if present, are generally in a terminal region or regions, *e.g.*, within 6, 5, 4, 3, or 2 nucleotides of the 5' and/or 3' terminus.

The term "sense strand," as used herein, refers to the strand of a dsRNA that includes a region that is substantially complementary to a region of the antisense strand.

As used herein, the term "SNALP" refers to a stable nucleic acid-lipid particle. A SNALP represents a vesicle of lipids coating a reduced aqueous interior comprising a nucleic acid such as an iRNA agent or a plasmid from which an iRNA agent is transcribed. SNALP are described, *e.g.*, in U.S. Patent Application Publication Nos. 2006/0240093, 2007/0135372, and USSN 61/045,228 filed on April 15, 2008. These applications are hereby incorporated by reference.

"Introducing into a cell," when referring to a dsRNA, means facilitating uptake or absorption into the cell, as is understood by those skilled in the art. Absorption or uptake of dsRNA can occur through unaided diffusive or active cellular processes, or by auxiliary agents or devices. The meaning of this term is not limited to cells *in vitro;* a dsRNA may also be "introduced into a cell," wherein the cell is part of a living organism. In such instance, introduction into the cell will include the delivery to the organism. For example, for *in vivo* delivery, dsRNA can be injected into a tissue site or administered systemically. *In vitro* introduction into a cell includes methods known in the art such as electroporation and lipofection.

The terms "silence," "inhibit the expression of," "down-regulate the expression of," "suppress the expression of and the like, in as far as they refer to a target gene, herein refer to the at least partial suppression of the expression of a SNCA gene, as manifested by a reduction of the amount of mRNA which may be isolated or detected from a first cell or group of cells in which a SNCA gene is transcribed and which has or have been treated such that the expression of a SNCA gene is inhibited, as compared to a second cell or group of cells substantially identical to the first cell or group of cells but which has or have not been so treated (control cells). The degree of inhibition is usually expressed in terms of

$$\frac{(\text{mRNA in control cells}) - (\text{mRNA in treated cells})}{(\text{mRNA in control cells})} \cdot 100\%$$

Alternatively, the degree of inhibition may be given in terms of a reduction of a parameter that is functionally linked to SNCA gene transcription, *e.g.,* the amount of protein encoded by a SNCA gene which is secreted by a cell, or the number of cells displaying a certain phenotype, *e.g.,* apoptosis. In principle, SNCA gene silencing may be determined in any cell expressing the target, either constitutively or by genomic engineering, and by any appropriate assay. However, when a reference is needed in order to determine whether a given dsRNA inhibits the expression of a SNCA gene by a certain degree and therefore is encompassed by the instant invention, the assays provided in the Examples below shall serve as such reference.

For example, in certain instances, expression of a SNCA gene is suppressed by at least about 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, or 50% by administration of the double-stranded oligonucleotide featured in the invention. In some embodiments, a SNCA gene is suppressed by at least about 60%, 70%, or 80% by administration of the double-stranded oligonucleotide featured in the invention. In some embodiments, a SNCA gene is suppressed by at least about 85%, 90%, or 95% by administration of the double-stranded oligonucleotide featured in the invention.

As used herein in the context of SNCA expression, the terms "treat," "treatment," and the like, refer to relief from or alleviation of pathological processes mediated by SNCA expression. In the context of the present invention insofar as it relates to any of the other conditions recited herein below (other than pathological processes mediated by SNCA expression), the terms "treat," "treatment," and the like mean to relieve or alleviate at least one symptom associated with such condition, or to slow or reverse the progression of such condition.

As used herein, the term "SNCA-mediated condition or disease" and related terms and phrases refer to a condition or disorder characterized by inappropriate, *e.g.* , greater than normal, SNCA activity. Inappropriate SNCA functional activity might arise as the result of SNCA expression in cells which normally do not express SNCA, or increased SNCA expression (leading to, *e.g.,* neurodegenerative disease). A SNCA-mediated condition or disease may be completely or partially mediated by inappropriate SNCA functional activity. However, a SNCA-mediated condition or disease is one in which modulation of SNCA results in some effect on the underlying condition or disorder *(e.g.,* a SNCA inhibitor results in some improvement in patient well-being in at least some patients).

As used herein, the phrases "therapeutically effective amount" and "prophylactically effective amount" refer to an amount that provides a therapeutic benefit in the treatment,

prevention, or management of a pathological processes mediated by SNCA expression, such as a neurodegenerative disorder such as synucleinopathy *(e.g.,* Parkinson's disease) or an overt symptom of pathological processes mediated by SNCA expression. The specific amount that is therapeutically effective can be readily determined by an ordinary medical practitioner, and may vary depending on factors known in the art, such as, for example, the type of pathological processes mediated by SNCA expression, the patient's history and age, the stage of pathological processes mediated by SNCA expression, and the administration of other anti-pathological processes mediated by SNCA expression agents.

As used herein, a "pharmaceutical composition" comprises a pharmacologically effective amount of a dsRNA and a pharmaceutically acceptable carrier. As used herein, "pharmacologically effective amount," "therapeutically effective amount" or simply "effective amount" refers to that amount of an RNA effective to produce the intended pharmacological, therapeutic or preventive result. For example, if a given clinical treatment is considered effective when there is at least a 25% reduction in a measurable parameter associated with a disease or disorder, a therapeutically effective amount of a drug for the treatment of that disease or disorder is the amount necessary to effect at least a 25% reduction in that parameter.

The term "pharmaceutically acceptable carrier" refers to a carrier for administration of a therapeutic agent. Such carriers include, but are not limited to, saline, buffered saline, dextrose, water, glycerol, ethanol, and combinations thereof. The term specifically excludes cell culture medium. For drugs administered orally, pharmaceutically acceptable carriers include, but are not limited to pharmaceutically acceptable excipients such as inert diluents, disintegrating agents, binding agents, lubricating agents, sweetening agents, flavoring agents, coloring agents and preservatives. Suitable inert diluents include sodium and calcium carbonate, sodium and calcium phosphate, and lactose, while corn starch and alginic acid are suitable disintegrating agents. Binding agents may include starch and gelatin, while the lubricating agent, if present, will generally be magnesium stearate, stearic acid or talc. If desired, the tablets may be coated with a material such as glyceryl monostearate or glyceryl distearate, to delay absorption in the gastrointestinal tract.

As used herein, a "transformed cell" is a cell into which a vector has been introduced from which a dsRNA molecule may be expressed.

Alpha-synuclein

The alpha-synuclein protein (SNCA) is primarily found in the cytoplasm, but has also been localized to the nucleus. In dopaminergic neurons, alpha-synuclein is membrane bound. The protein is a soluble monomer normally localized at the presynaptic region of axons. The

protein can form filamentous aggregates that are the major component of intracellular inclusions in neurodegenerative synucleinopathies.

The alpha-synuclein protein is associated with a number of diseases characterized by synucleinopathies. Three point mutations (A53T, A30P and E46K), and SNCA duplication and triplication events are linked to autosomal dominant Parkinson's disease (familial Parkinson's disease, also called FPD). The A53T and A30P mutations cause configuration changes in the SNCA protein that promote *in vitro* protofibril formation. The triplication event results in a two-fold overexpression of SNCA protein. Alpha-synuclein is a major fibrillar component of Lewy bodies, the cytoplasmic inclusions that are characteristic of FPD and idiopathic Parkinson's disease, and the substantia nigra of a Parkinson's disease brain is characterized by fibrillar alpha-synuclein. In Alzheimer's patients, SNCA peptides are a major component of amyloid plaques in the brains of patients with Alzheimer's disease.

Aggregation of alpha-synuclein in the cytoplasm of cells can be caused by a number of mechanisms, including overexpression of the protein, inhibition of protein degradation, or a mutation that affects the structure of the protein, resulting in an increased tendency of the protein to self-associate.

An SNCA gene product can be a target for treatment methods of neurodegenerative diseases such as Parkinson's disease. The treatment methods can include targeting of an SNCA nucleic acid with a dsRNA. Alternatively, or additionally, an antisense RNA can be used to inhibit gene expression, or an antibody or small molecule can be used to target an SNCA nucleic acid. In general, an antisense RNA, anti-SNCA antibody, or small molecule can be used in place of a dsRNA, *e.g.*, by any of the methods or compositions described herein. A combination of therapies to downregulate SNCA expression and activity can also be used.

Sequencing of the SNCA gene has revealed common variants including a dinucleotide repeat sequence (REPl) within the promoter. REPl varies in length across populations, and certain allelic variants are associated with an increased risk for Parkinson's disease (Kruger *et ah*, Ann Neurol. 45:61 1-7, 1999). The SNCA gene REPl locus is necessary for normal gene expression (Touchman *et ah*, Genome Res. 11:78-86, 2001). SNCA gene expression levels among the different REPl alleles varied significantly over a 3-fold range, suggesting that the association of specific genotypes with an increased risk for Parkinson's disease may be a consequence of SNCA gene over-expression (Chiba-Falek and Nussbaum, Hum Mol. Genet. 10:3 101-9, 2001). Functional analysis of intra-allelic variation at the SNCA gene REPl locus implied that overall length of the allele plays the main role in transcriptional regulation; sequence heterogeneity is unlikely to confound genetic association studies based on alleles

defined by length (Chiba-Falek *et al,* Hum Genet. 113:426-3 1, 2003). The recent discovery of SNCA gene triplication as a rare cause of Parkinson's disease is consistent with the observation that polymorphism within the gene promoter confers susceptibility via the same mechanism of gene over-expression (Singleton *et al,* Science 302:841, 2003).

Three splice variants of SNCA have been identified. The full-length 140 amino acid protein is the most abundant form. A 128 amino acid form lacks exon 3, and a 112 amino acid form lacks exon 5. An iRNA featured in the invention can target any isoform of SNCA. An iRNA can target a common exon *(e.g.,* exon 2, 4, 6, or 7) to effectively target all known isoforms. A dsRNA can target a splice junction or an alternatively spliced exon to target specific isoforms. For example, to target the 112 amino acid isoform, a dsRNA can target an mRNA sequence that overlaps the exon 4/exon 6 splice junction. To target the 128 amino acid protein isoform, a dsRNA can target an mRNA sequence that overlaps the exon 2/exon 4 junction.

Double-stranded ribonucleic acid (dsRNA)

As described in more detail herein, the invention provides double-stranded ribonucleic acid (dsRNA) molecules for inhibiting the expression of a SNCA gene in a cell or mammal, where the dsRNA includes a sense strand having a first sequence and an antisense strand comprising a second sequence complementary to mRNA encoding SNCA, wherein said first sequence is complementary to said second sequence at a region of complementarity and wherein each strand is 15 to 30 base pairs in length. In some embodiments, the dsRNA of the invention inhibits the expression of said SNCA gene by at least 40% as assayed by, for example, a PCR or branched DNA (bDNA)-based method, or by a protein-based method, such as by Western blot. Expression of a SNCA gene can be reduced by at least 30% when measured by an assay as described in the Examples below. For example, expression of a SNCA gene in cell culture, such as in HepB3 cells, can be assayed by measuring SNCA mRNA levels, such as by bDNA or TaqMan assay, or by measuring protein levels, such as by ELISA assay.

The dsRNA can be synthesized by standard methods known in the art as further discussed below, *e.g.,* by use of an automated DNA synthesizer, such as are commercially available from, for example, Biosearch, Applied Biosystems, Inc. The dsRNA includes two RNA strands that are sufficiently complementary to hybridize to form a duplex structure.

One strand of the dsRNA (the antisense strand) includes a region of complementarity that is complementary, to a target sequence, derived from the sequence of an mRNA formed during the expression of a target gene, the other strand (the sense strand) includes a region that is complementary to the antisense strand, such that the two strands hybridize and form a duplex structure when combined under suitable conditions. The region of complementarity is generally

at least 15 nucleotides in length, or between 19 and 21 nucleotides in length, or 19, 20, or 21 nucleotides in length. In some embodiments the region of complementarity includes at least 15 contiguous nucleotides of one of the antisense sequences listed in Tables 2, 3,or 4. In other embodiments the region of complementarity includes one of the antisense sequences listed in Tables 2, 3,or 4.

Generally, the duplex structure is between 15 and 30, or between 25 and 30, or between 18 and 25, or between 19 and 24, or between 19 and 21, or 19, 20, or 21 base pairs in length. In one embodiment the duplex is 19 base pairs in length. In another embodiment the duplex is 21 base pairs in length. When two different dsRNAs are used in combination, the duplex lengths can be identical or can differ.

Each strand of the dsRNA of invention is generally between 15 and 30, or between 18 and 25, or 18, 19, 20, 21, 22, 23, 24, or 25 nucleotides in length. In other embodiments, each is strand is 25-30 nucleotides in length. Each strand of the duplex can be the same length or of different lengths. When two different siRNAs are used in combination, the lengths of each strand of each siRNA can be identical or can differ.

The dsRNA of the invention can include one or more single-stranded overhang(s) of one or more nucleotides. In one embodiment, at least one end of the dsRNA has a single-stranded nucleotide overhang of 1 to 4, or 1, 2, 3, or 4 nucleotides. In another embodiment, the overhang includes dTdT. In another embodiment, the antisense strand of the dsRNA has 1-10 nucleotides overhangs each at the 3' end and the 5' end over the sense strand. In further embodiments, the sense strand of the dsRNA has 1-10 nucleotides overhangs each at the 3' end and the 5' end over the antisense strand.

A dsRNAs having at least one nucleotide overhang can have unexpectedly superior inhibitory properties than the blunt-ended counterpart. In some embodiments the presence of only one nucleotide overhang strengthens the interference activity of the dsRNA, without affecting its overall stability. A dsRNA having only one overhang has proven particularly stable and effective *in vivo*, as well as in a variety of cells, cell culture mediums, blood, and serum. Generally, the single-stranded overhang is located at the 3'-terminal end of the antisense strand or, alternatively, at the 3'-terminal end of the sense strand. The dsRNA can also have a blunt end, generally located at the 5'-end of the antisense strand. Such dsRNAs can have improved stability and inhibitory activity, thus allowing administration at low dosages, *i.e.*, 5 mg/kg body weight of the recipient or less per day. Generally, the antisense strand of the dsRNA has a nucleotide overhang at the 3'-end, and the 5'-end is blunt. In another embodiment, one or more of the nucleotides in the overhang is replaced with a nucleoside thiophosphate.

In one embodiment, a SNCA gene is a human SNCA gene, *e.g.,* the sequence identified by GenBank accession number NM_007308.1. In another embodiment, a SNCA gene is a rhesus monkey SNCA gene, *e.g.,* the sequence identified by GenBank accession number XM_00 1095402.1.

In specific embodiments, the sense strand of the dsRNA is one of the sense sequences from Tables 3-6, and the antisense strand is one of the antisense sequences of Tables 3-6. Alternative antisense agents that target elsewhere in the target sequence provided in Tables 3-6 can readily be determined using the target sequence and the flanking SNCA sequence.

The skilled person is well aware that dsRNAs having a duplex structure of between 20 and 23, but specifically 21, base pairs have been hailed as particularly effective in inducing RNA interference (Elbashir *et al,* EMBO 2001, 20:6877-6888). However, others have found that shorter or longer dsRNAs can be effective as well. In the embodiments described above, by virtue of the nature of the oligonucleotide sequences provided in Tables 3-6, the dsRNAs featured in the invention can include at least one strand of a length described therein. It can be reasonably expected that shorter dsRNAs having one of the sequences of Tables 3-6 minus only a few nucleotides on one or both ends may be similarly effective as compared to the dsRNAs described above. Hence, dsRNAs having a partial sequence of at least 15, 16, 17, 18, 19, 20, 21, or 22, or more contiguous nucleotides from one of the sequences of Tables 3-6, and differing in their ability to inhibit the expression of a SNCA gene in an assay as described herein below by not more than 5, 10, 15, 20, 25, or 30 % inhibition from a dsRNA comprising the full sequence, are contemplated by the invention. Further, dsRNAs that cleave within a desired SNCA target sequence can readily be made using the corresponding SNCA antisense sequence and a complementary sense sequence.

In addition, the dsRNAs provided in Tables 3-6 identify a site in a SNCA that is susceptible to RNAi based cleavage. As such, the present invention further features dsRNAs that target within the sequence targeted by one of the agents of the present invention. As used herein, a second dsRNA is said to target within the sequence of a first dsRNA if the second dsRNA cleaves the message anywhere within the mRNA that is complementary to the antisense strand of the first dsRNA. Such a second dsRNA will generally consist of at least 15 contiguous nucleotides from one of the sequences provided in Tables 3-6 coupled to additional nucleotide sequences taken from the region contiguous to the selected sequence in a SNCA gene.

Additional dsRNA of the invention include those that cleave a target mRNA at the same location as a dsRNA described in any of the tables. In general, a RISC complex will cleave a target mRNA between the nucleotides complementary to nucleotides 10 and 11 of the antisense

strand of a dsRNA, *e.g.,* siRNA, of the invention. Cleavage sites can be assayed using, *e.g.,* a 5' RACE assay.

For example, the duplex AD-21752 includes the sense and antisense strands below. Treatment of a cell with this duplex results in cleavage of human SNCA mRNA at the nucleotides complementary to nucleotides 10 and 11 of the antisense strand, *e.g.,* nucleotides 333 and 334. Therefore, also included in the invention are those dsRNA that cleave at that location.

The dsRNA featured in the invention can contain one or more mismatches to the target sequence. In one embodiment, the dsRNA featured in the invention contains no more than 3 mismatches. If the antisense strand of the dsRNA contains mismatches to a target sequence, it is preferable that the area of mismatch not be located in the center of the region of complementarity. If the antisense strand of the dsRNA contains mismatches to the target sequence, it is preferable that the mismatch be restricted to 5 nucleotides from either end, for example 5, 4, 3, 2, or 1 nucleotide from either the 5' or 3' end of the region of complementarity. For example, for a 23 nucleotide dsRNA strand which is complementary to a region of a target gene, the dsRNA generally does not contain any mismatch within the central 13 nucleotides. The methods described within the invention can be used to determine whether a dsRNA containing a mismatch to a target sequence is effective in inhibiting the expression of a target gene. Consideration of the efficacy of dsRNAs with mismatches in inhibiting expression of a target gene is important, especially if the particular region of complementarity in a target gene is known to have polymorphic sequence variation within the population.

Modifications

In yet another embodiment, the dsRNA is chemically modified to enhance stability. The nucleic acids featured in the invention may be synthesized and/or modified by methods well established in the art, such as those described in "Current protocols in nucleic acid chemistry," Beaucage, S.L. *et al.* (Edrs.), John Wiley & Sons, Inc., New York, NY, USA, which is hereby incorporated herein by reference. Specific examples of dsRNA compounds useful in this invention include dsRNAs containing modified backbones or no natural internucleoside linkages. As defined in this specification, dsRNAs having modified backbones include those that retain a phosphorus atom in the backbone and those that do not have a phosphorus atom in the backbone. For the purposes of this specification, and as sometimes referenced in the art, modified dsRNAs that do not have a phosphorus atom in their internucleoside backbone can also be considered to be oligonucleosides.

Modified dsRNA backbones include, for example, phosphorothioates, chiral phosphorothioates, phosphorodithioates, phosphotriesters, aminoalkylphosphotriesters, methyl

and other alkyl phosphonates including 3'-alkylene phosphonates and chiral phosphonates, phosphinates, phosphoramidates including 3'-amino phosphoramidate and aminoalkylphosphoramidates, thionophosphoramidates, thionoalkylphosphonates, thionoalkylphosphotriesters, and boranophosphates having normal 3'-5' linkages, 2'-5' linked analogs of these, and those) having inverted polarity wherein the adjacent pairs of nucleoside units are linked 3'-5' to 5'-3' or 2'-5' to 5'-2'. Various salts, mixed salts and free acid forms are also included.

Representative U.S. patents that teach the preparation of the above phosphorus-containing linkages include, but are not limited to, U.S. Pat. Nos. 3,687,808; 4,469,863; 4,476,301; 5,023,243; 5,177,195; 5,188,897; 5,264,423; 5,276,019; 5,278,302; 5,286,717; 5,321,131; 5,399,676; 5,405,939; 5,453,496; 5,455,233; 5,466,677; 5,476,925; 5,519,126; 5,536,821; 5,541,316; 5,550,11 1; 5,563,253; 5,571,799; 5,587,361; and 5,625,050, each of which is herein incorporated by reference.

Modified dsRNA backbones that do not include a phosphorus atom therein have backbones that are formed by short chain alkyl or cycloalkyl intemucleoside linkages, mixed heteroatoms and alkyl or cycloalkyl intemucleoside linkages, or ore or more short chain heteroatomic or heterocyclic intemucleoside linkages. These include those having morpholino linkages (formed in part from the sugar portion of a nucleoside); siloxane backbones; sulfide, sulfoxide and sulfone backbones; formacetyl and thioformacetyl backbones; methylene formacetyl and thioformacetyl backbones; alkene containing backbones; sulfamate backbones; methyleneimino and methylenehydrazino backbones; sulfonate and sulfonamide backbones; amide backbones; and others having mixed N, O, S and CH_2 component parts.

Representative U.S. patents that teach the preparation of the above oligonucleosides include, but are not limited to, U.S. Pat. Nos. 5,034,506; 5,166,315; 5,185,444; 5,214,134; 5,216,141; 5,235,033; 5,64,562; 5,264,564; 5,405,938; 5,434,257; 5,466,677; 5,470,967; 5,489,677; 5,541,307; 5,561,225; 5,596,086; 5,602,240; 5,608,046; 5,610,289; 5,618,704; 5,623,070; 5,663,312; 5,633,360; 5,677,437; and, 5,677,439, each of which is herein incorporated by reference.

In other suitable dsRNA mimetics, both the sugar and the intemucleoside linkage, *i.e.*, the backbone, of the nucleotide units are replaced with novel groups. The base units are maintained for hybridization with an appropriate nucleic acid target compound. One such oligomeric compound, a dsRNA mimetic that has been shown to have excellent hybridization properties, is referred to as a peptide nucleic acid (PNA). In PNA compounds, the sugar backbone of a dsRNA is replaced with an amide containing backbone, in particular an

aminoethylglycine backbone. The nucleobases are retained and are bound directly or indirectly to aza nitrogen atoms of the amide portion of the backbone. Representative U.S. patents that teach the preparation of PNA compounds include, but are not limited to, U.S. Pat. Nos. 5,539,082; 5,714,33 1; and 5,719,262, each of which is herein incorporated by reference. Further teaching of PNA compounds can be found in Nielsen *et ah,* Science, 1991, 254, 1497-1500.

Other embodiments of the invention are dsRNAs with phosphorothioate backbones and oligonucleosides with heteroatom backbones, and in particular —CH$_2$~NH-CH$_2$—, -CH$_2$—N(CH$_3$)-0~CH$_2$~ [known as a methylene (methylimino) or MMI backbone], —CH$_2$—O—N(CH$_3$)-CH$_2$- , -CH$_2$-N(CH$_3$)--N(CH$_3$)--CH$_2$- and -N(CH$_3$)-CH$_2$-CH$_2$-[wherein the native phosphodiester backbone is represented as —O—P-O—CH$_2$—] of the above-referenced U.S. Pat. No. 5,489,677, and the amide backbones of the above-referenced U.S. Pat. No. 5,602,240. Also preferred are dsRNAs having morpholino backbone structures of the above-referenced U.S. Pat. No. 5,034,506.

Modified dsRNAs may also contain one or more substituted sugar moieties. Preferred dsRNAs comprise one of the following at the 2' position: OH; F; 0-, S-, or N-alkyl; 0-, S-, or N-alkenyl; 0-, S- or N-alkynyl; or O-alkyl-O-alkyl, wherein the alkyl, alkenyl and alkynyl may be substituted or unsubstituted CI to CIO alkyl or C2 to CIO alkenyl and alkynyl. Particularly preferred are 0[(CH$_2$)$_n$O]$_m$CH$_3$, 0(CH$_2$)$_n$O CH$_3$, 0(CH$_2$)$_n$NH$_2$, 0(CH$_2$)$_n$ CH$_3$, 0(CH$_2$)$_n$ONH$_2$, and 0(CH$_2$)$_n$ON[(CH$_2$)$_n$ CH$_3$)]$_2$, where n and m are from 1 to about 10. Other preferred dsRNAs comprise one of the following at the 2' position: CI to CIO lower alkyl, substituted lower alkyl, alkaryl, aralkyl, O-alkaryl or O-aralkyl, SH, S CH$_3$, OCN, CI, Br, CN, CF$_3$, OCF$_3$, SO CH$_3$, S0$_2$ CH$_3$, ON0$_2$, N0$_2$, N$_3$, NH$_2$, heterocycloalkyl, heterocycloalkaryl, aminoalkylamino, polyalkylamino, substituted silyl, an RNA cleaving group, a reporter group, an intercalator, a group for improving the pharmacokinetic properties of a dsRNA, or a group for improving the pharmacodynamic properties of a dsRNA, and other substituents having similar properties. A preferred modification includes 2'-methoxyethoxy (2'-0— CH$_2$ CH$_2$0 CH$_3$, also known as 2'-0-(2-methoxyethyl) or 2'-MOE) (Martin *et al,* Helv. Chim. Acta, 1995, 78, 486-504) *i.e.,* an alkoxy-alkoxy group. A further preferred modification includes 2'-dimethylaminooxyethoxy, *i.e.,* a 0(CH2)20N(CH3)2 group, also known as 2'-DMAOE, as described in examples herein below, and 2'-dimethylaminoethoxyethoxy (also known in the art as 2'-0-dimethylaminoethoxyethyl or 2'-DMAEOE), *i.e.,* 2'-0- CH$_2$-0- CH$_2$-N(CH$_2$)$_2$, also described in examples herein below.

Other preferred modifications include 2'-methoxy (2'-OCH$_3$), 2'-aminopropoxy (2'-OCH$_2$CH$_2$CH$_2$NH$_2$) and 2'-fluoro (2'-F). Similar modifications may also be made at other positions on the dsRNA, particularly the 3' position of the sugar on the 3' terminal nucleotide or

in 2'-5' linked dsRNAs and the 5' position of 5' terminal nucleotide. DsRNAs may also have sugar mimetics such as cyclobutyl moieties in place of the pentofuranosyl sugar. Representative U.S. patents that teach the preparation of such modified sugar structures include, but are not limited to, U.S. Pat. Nos. 4,981,957; 5,118,800; 5,319,080; 5,359,044; 5,393,878; 5,446,137; 5,466,786; 5,514,785; 5,519,134; 5,567,811; 5,576,427; 5,591,722; 5,597,909; 5,610,300; 5,627,053; 5,639,873; 5,646,265; 5,658,873; 5,670,633; and 5,700,920, certain of which are commonly owned with the instant application, and each of which is herein incorporated by reference in its entirety.

A dsRNA may also include nucleobase (often referred to in the art simply as "base") modifications or substitutions. As used herein, "unmodified" or "natural" nucleobases include the purine bases adenine (A) and guanine (G), and the pyrimidine bases thymine (T), cytosine (C) and uracil (U). Modified nucleobases include other synthetic and natural nucleobases such as 5-methylcytosine (5-me-C), 5-hydroxymethyl cytosine, xanthine, hypoxanthine, 2-aminoadenine, 6-methyl and other alkyl derivatives of adenine and guanine, 2-propyl and other alkyl derivatives of adenine and guanine, 2-thiouracil, 2-thiothymine and 2-thiocytosine, 5-halouracil and cytosine, 5-propynyl uracil and cytosine, 6-azo uracil, cytosine and thymine, 5-uracil (pseudouracil), 4-thiouracil, 8-halo, 8-amino, 8-thiol, 8-thioalkyl, 8-hydroxyl anal other 8-substituted adenines and guanines, 5-halo, particularly 5-bromo, 5-trifluoromethyl and other 5-substituted uracils and cytosines, 7-methylguanine and 7-methyladenine, 8-azaguanine and 8-azaadenine, 7-deazaguanine and 7-daazaadenine and 3-deazaguanine and 3-deazaadenine. Further nucleobases include those disclosed in U.S. Pat. No. 3,687,808, those disclosed in The Concise Encyclopedia Of Polymer Science And Engineering, pages 858-859, Kroschwitz, J. L, ed. John Wiley & Sons, 1990, these disclosed by Englisch *et al.*, Angewandte Chemie, International Edition, 1991, 30, 613, and those disclosed by Sanghvi, Y S., Chapter 15, DsRNA Research and Applications, pages 289-302, Crooke, S. T. and Lebleu, B., Ed., CRC Press, 1993. Certain of these nucleobases are particularly useful for increasing the binding affinity of the oligomeric compounds featured in the invention. These include 5-substituted pyrimidines, 6-azapyrimidines and N-2, N-6 and 0-6 substituted purines, including 2-aminopropyladenine, 5-propynyluracil and 5-propynylcytosine. 5-methylcytosine substitutions have been shown to increase nucleic acid duplex stability by 0.6-1.2°C. (Sanghvi, Y. S., Crooke, S. T. and Lebleu, B., Eds., DsRNA Research and Applications, CRC Press, Boca Raton, 1993, pp. 276-278) and are exemplary base substitutions, even more particularly when combined with 2'-0-methoxy ethyl sugar modifications.

Representative U.S. patents that teach the preparation of certain of the above noted modified nucleobases as well as other modified nucleobases include, but are not limited to, the above noted U.S. Pat. No. 3,687,808, as well as U.S. Pat. Nos. 4,845,205; 5,130,30; 5,134,066; 5,175,273; 5,367,066; 5,432,272; 5,457,187; 5,459,255; 5,484,908; 5,502,177; 5,525,711; 5,552,540; 5,587,469; 5,594,121, 5,596,091; 5,614,617; and 5,681,941, each of which is herein incorporated by reference, and U.S. Pat. No. 5,750,692, also herein incorporated by reference.

Conjugates

Another modification of the dsRNAs featured in the invention involves chemically linking to the dsRNA one or more moieties or conjugates which enhance the activity, cellular distribution or cellular uptake of the dsRNA. Such moieties include but are not limited to lipid moieties such as a cholesterol moiety (Letsinger *et al*, Proc. Natl. Acid. Sci. USA, 1989, 86: 6553-6556), cholic acid (Manoharan *et al*, Biorg. Med. Chem. Let, 1994, 4:1053-1060), a thioether, *e.g.*, beryl-S-tritylthiol (Manoharan *et al*, Ann. N.Y. Acad. Sci., 1992, 660:306-309; Manoharan *et al*, Biorg. Med. Chem. Let., 1993, 3:2765-2770), a thiocholesterol (Oberhauser *et al*, Nucl. Acids Res., 1992, 20:533-538), an aliphatic chain, *e.g.*, dodecandiol or undecyl residues (Saison-Behmoaras *et al*, EMBO J, 1991, 10:1 11 1-11 18; Kabanov *et al*, FEBS Lett, 1990, 259:327-330; Svinarchuk *et al*, Biochimie, 1993, 75:49-54), a phospholipid, *e.g.*, di-hexadecyl-rac-glycerol or triethyl-ammonium l,2-di-0-hexadecyl-rac-glycero-3-Hphosphonate (Manoharan *etal*, Tetrahedron Lett, 1995, 36:3651-3654; Shea *etal*, Nucl. Acids Res., 1990, 18:3777-3783), a polyamine or a polyethylene glycol chain (Manoharan *et al*, Nucleosides & Nucleotides, 1995, 14:969-973), or adamantane acetic acid (Manoharan *etal*, Tetrahedron Lett., 1995, 36:3651-3654), apalmityl moiety (Mishra *et al*, Biochim. Biophys. Acta, 1995, 1264:229-237), or an octadecylamine or hexylamino-carbonyloxycholesterol moiety (Crooke *et al*, J. Pharmacol. Exp. Ther., 1996, 277:923-937).

Representative U.S. patents that teach the preparation of such dsRNA conjugates include, but are not limited to, U.S. Pat. Nos. 4,828,979; 4,948,882; 5,218,105; 5,525,465; 5,541,313; 5,545,730; 5,552,538; 5,578,717, 5,580,731; 5,591,584; 5,109,124; 5,1 18,802; 5,138,045; 5,414,077; 5,486,603; 5,512,439; 5,578,718; 5,608,046; 4,587,044; 4,605,735; 4,667,025; 4,762,779; 4,789,737; 4,824,941; 4,835,263; 4,876,335; 4,904,582; 4,958,013; 5,082,830; 5,1 12,963; 5,214,136; 5,082,830; 5,1 12,963; 5,214,136; 5,245,022; 5,254,469; 5,258,506; 5,262,536; 5,272,250; 5,292,873; 5,317,098; 5,371,241, 5,391,723; 5,416,203, 5,451,463; 5,510,475; 5,512,667; 5,514,785; 5,565,552; 5,567,810; 5,574,142; 5,585,481; 5,587,371; 5,595,726; 5,597,696; 5,599,923; 5,599,928 and 5,688,941, each of which is herein incorporated by reference.

It is not necessary for all positions in a given compound to be uniformly modified, and in fact more than one of the aforementioned modifications may be incorporated in a single compound or even at a single nucleoside within a dsRNA. The present invention also includes dsRNA compounds which are chimeric compounds. "Chimeric" dsRNA compounds or "chimeras," in the context of this invention, are dsRNA compounds, particularly dsRNAs, which contain two or more chemically distinct regions, each made up of at least one monomer unit, *i.e.*, a nucleotide in the case of a dsRNA compound. These dsRNAs typically contain at least one region wherein the dsRNA is modified so as to confer upon the dsRNA increased resistance to nuclease degradation, increased cellular uptake, and/or increased binding affinity for the target nucleic acid. An additional region of the dsRNA may serve as a substrate for enzymes capable of cleaving RNA:DNA or RNA:RNA hybrids. By way of example, RNase H is a cellular endonuclease which cleaves the RNA strand of an RNA:DNA duplex. Activation of RNase H, therefore, results in cleavage of the RNA target, thereby greatly enhancing the efficiency of dsRNA inhibition of gene expression. Consequently, comparable results can often be obtained with shorter dsRNAs when chimeric dsRNAs are used, compared to phosphorothioate deoxy dsRNAs hybridizing to the same target region. Cleavage of the RNA target can be routinely detected by gel electrophoresis and, if necessary, associated nucleic acid hybridization techniques known in the art.

In certain instances, the dsRNA may be modified by a non-ligand group. A number of non-ligand molecules have been conjugated to dsRNAs in order to enhance the activity, cellular distribution or cellular uptake of the dsRNA, and procedures for performing such conjugations are available in the scientific literature. Such non-ligand moieties have included lipid moieties, such as cholesterol (Letsinger *et al*, Proc. Natl. Acad. Sci. USA, 1989, 86:6553), cholic acid (Manoharan *etal*, Bioorg. Med. Chem. Lett., 1994, 4:1053), athioether, *e.g.*, hexyl-S-tritylthiol (Manoharan *et al*, Ann. N.Y. Acad. Sci., 1992, 660:306; Manoharan *et al*, Bioorg. Med. Chem. Let, 1993, 3:2765), a thiocholesterol (Oberhauser *et al*, Nucl. Acids Res., 1992, 20:533), an aliphatic chain, *e.g.*, dodecandiol or undecyl residues (Saison-Behmoaras *et al*, EMBO J., 1991, 10:1 11; Kabanov *et al*, FEBS Lett, 1990, 259:327; Svinarchuk *et al.*, Biochimie, 1993, 75:49), a phospholipid, *e.g.*, di-hexadecyl-rac-glycerol or triethylammonium 1,2-di-O-hexadecyl-rac-glycero-3-H-phosphonate (Manoharan *et al*, Tetrahedron Lett., 1995, 36:3651; Shea *et al*, Nucl. Acids Res., 1990, 18:3777), a polyamine or a polyethylene glycol chain (Manoharan *et al*, Nucleosides & Nucleotides, 1995, 14:969), or adamantane acetic acid (Manoharan *et al*, Tetrahedron Lett., 1995, 36:3651), a palmityl moiety (Mishra *et al*, Biochim. Biophys. Acta, 1995, 1264:229), or an octadecylamine or hexylamino-carbonyl-oxycholesterol moiety (Crooke *et al*, J. Pharmacol. Exp. Ther., 1996, 277:923). Representative United States patents that teach

the preparation of such dsRNA conjugates have been listed above. Typical conjugation protocols involve the synthesis of dsRNAs bearing an aminolinker at one or more positions of the sequence. The amino group is then reacted with the molecule being conjugated using appropriate coupling or activating reagents. The conjugation reaction may be performed either with the dsRNA still bound to the solid support or following cleavage of the dsRNA in solution phase. Purification of the dsRNA conjugate by HPLC typically affords the pure conjugate.

Vector encoded dsRNAs

In another aspect, dsRNA molecules of the invention are expressed from transcription units inserted into DNA or RNA vectors (see, *e.g.*, Couture, A, *et al, TIG.* (1996), 12:5-10; Skillern, A., *et al.*, International PCT Publication No. WO 00/22 113, Conrad, International PCT Publication No. WO 00/221 14, and Conrad, U.S. Pat. No. 6,054,299). These transgenes can be introduced as a linear construct, a circular plasmid, or a viral vector, which can be incorporated and inherited as a transgene integrated into the host genome. The transgene can also be constructed to permit it to be inherited as an extrachromosomal plasmid (Gassmann, *et al, Proc. Natl. Acad. Sci. USA* (1995) 92: 1292).

The individual strands of a dsRNA can be transcribed by promoters on two separate expression vectors and co-transfected into a target cell. Alternatively each individual strand of the dsRNA can be transcribed by promoters both of which are located on the same expression plasmid. In one embodiment, a dsRNA is expressed as an inverted repeat joined by a linker polynucleotide sequence such that the dsRNA has a stem and loop structure.

The recombinant dsRNA expression vectors are generally DNA plasmids or viral vectors. dsRNA expressing viral vectors can be constructed based on, but not limited to, adeno-associated virus (for a review, see Muzyczka, *et al, Curr. Topics Micro. Immunol.* (1992) 158:97-129)); adenovirus (see, for example, Berkner, *et al,* BioTechniques (1998) 6:616), Rosenfeld *et al.* (1991, Science 252:431-434), and Rosenfeld *et al.* (1992), *Cell* 68:143-155)); or alphavirus as well as others known in the art. Retroviruses have been used to introduce a variety of genes into many different cell types, including epithelial cells, *in vitro* and/or *in vivo* (see, *e.g.*, Eglitis, *et al, Science* (1985) 230:1395-1398; Danos and Mulligan, *Proc. Natl. Acad. Sci. USA* (1998) 85:6460-6464; Wilson *et al,* 1988, Proc. Natl. Acad. Sci. USA 85:3014-3018; Armentano *et al,* 1990, Proc. Natl. Acad. Sci. USA 87:61416145; Huber *et al,* 1991, Proc. Natl. Acad. Sci. USA 88:8039-8043; Ferry *etal,* 1991, Proc. Natl. Acad. Sci. USA 88:8377-8381; Chowdhury *etal,* 1991, Science 254:1802-1805; van Beusechem. *et al,* 1992, Proc. Nad. Acad. Sci. USA 89:7640-19 ; Kay *et al,* 1992, Human Gene Therapy 3:641-647; Dai *et al,* 1992, Proc. Natl.Acad. Sci. USA 89:10892-10895; Hwu *et al,* 1993, J. Immunol. 150:4104-41 15; U.S.

Patent No. 4,868,116; U.S. Patent No. 4,980,286; PCT Application WO 89/07136; PCT Application WO 89/02468; PCT Application WO 89/05345; and PCT Application WO 92/07573). Recombinant retroviral vectors capable of transducing and expressing genes inserted into the genome of a cell can be produced by transfecting the recombinant retroviral genome into suitable packaging cell lines such as PA317 and Psi-CRIP (Comette *et al,* 1991, Human Gene Therapy 2:5-10; Cone *et al,* 1984, Proc. Natl. Acad. Sci. USA 81:6349). Recombinant adenoviral vectors can be used to infect a wide variety of cells and tissues in susceptible hosts *(e.g.,* rat, hamster, dog, and chimpanzee) (Hsu *et al,* 1992, J. Infectious Disease, 166:769), and also have the advantage of not requiring mitotically active cells for infection.

Any viral vector capable of accepting the coding sequences for the dsRNA molecule(s) to be expressed can be used, for example vectors derived from adenovirus (AV); adeno-associated virus (AAV); retroviruses *(e.g.,* lentiviruses (LV), Rhabdoviruses, murine leukemia virus); herpes virus, and the like. The tropism of viral vectors can be modified by pseudotyping the vectors with envelope proteins or other surface antigens from other viruses, or by substituting different viral capsid proteins, as appropriate.

For example, lentiviral vectors featured in the invention can be pseudotyped with surface proteins from vesicular stomatitis virus (VSV), rabies, Ebola, Mokola, and the like. AAV vectors featured in the invention can be made to target different cells by engineering the vectors to express different capsid protein serotypes. For example, an AAV vector expressing a serotype 2 capsid on a serotype 2 genome is called AAV 2/2. This serotype 2 capsid gene in the AAV 2/2 vector can be replaced by a serotype 5 capsid gene to produce an AAV 2/5 vector. Techniques for constructing AAV vectors which express different capsid protein serotypes are within the skill in the art; see, *e.g.,* Rabinowitz J E *et al.* (2002), J Virol 76:791-801, the entire disclosure of which is herein incorporated by reference.

Selection of recombinant viral vectors suitable for use in the invention, methods for inserting nucleic acid sequences for expressing the dsRNA into the vector, and methods of delivering the viral vector to the cells of interest are within the skill in the art. See, for example, Dornburg R (1995), Gene Therap. 2: 301-310; Eglitis M A (1988), Biotechniques 6: 608-614; Miller A D (1990), Hum Gene Therap. 1: 5-14; Anderson W F (1998), Nature 392: 25-30; and Rubinson D A *et al,* Nat. Genet. 33: 401-406, the entire disclosures of which are herein incorporated by reference.

Viral vectors can be derived from AV and AAV. In one embodiment, the dsRNA featured in the invention is expressed as two separate, complementary single-stranded RNA

molecules from a recombinant AAV vector having, for example, either the U6 or H1 RNA promoters, or the cytomegalovirus (CMV) promoter.

A suitable AV vector for expressing the dsRNA featured in the invention, a method for constructing the recombinant AV vector, and a method for delivering the vector into target cells, are described in Xia H *et al.* (2002), *Nat. Biotech.* 20: 1006-1010.

Suitable AAV vectors for expressing the dsRNA featured in the invention, methods for constructing the recombinant AV vector, and methods for delivering the vectors into target cells are described in Samulski R *et al.* (1987), J. Virol. 61: 3096-3101; Fisher K J *et al.* (1996), J. Virol, 70: 520-532; Samulski R *et al.* (1989), J. Virol. 63: 3822-3826; U.S. Pat. No. 5,252,479; U.S. Pat. No. 5,139,941; International Patent Application No. WO 94/13788; and International Patent Application No. WO 93/24641, the entire disclosures of which are herein incorporated by reference.

The promoter driving dsRNA expression in either a DNA plasmid or viral vector featured in the invention may be a eukaryotic RNA polymerase I *(e.g.,* ribosomal RNA promoter), RNA polymerase II *(e.g.,* CMV early promoter or actin promoter or U1 snRNA promoter) or generally RNA polymerase III promoter *(e.g.,* U6 snRNA or 7SK RNA promoter) or a prokaryotic promoter, for example the T7 promoter, provided the expression plasmid also encodes T7 RNA polymerase required for transcription from a T7 promoter. The promoter can also direct transgene expression to the pancreas (see, *e.g.,* the insulin regulatory sequence for pancreas (Bucchini *et al,* 1986, Proc. Natl. Acad. Sci. USA 83:2511-2515)).

In addition, expression of the transgene can be precisely regulated, for example, by using an inducible regulatory sequence and expression systems such as a regulatory sequence that is sensitive to certain physiological regulators, *e.g.,* circulating glucose levels, or hormones (Docherty *et al,* 1994, FASEB J. 8:20-24). Such inducible expression systems, suitable for the control of transgene expression in cells or in mammals include regulation by ecdysone, by estrogen, progesterone, tetracycline, chemical inducers of dimerization, and isopropyl-beta-D 1-thiogalactopyranoside (EPTG). A person skilled in the art would be able to choose the appropriate regulatory/promoter sequence based on the intended use of the dsRNA transgene.

Generally, recombinant vectors capable of expressing dsRNA molecules are delivered as described below, and persist in target cells. Alternatively, viral vectors can be used that provide for transient expression of dsRNA molecules. Such vectors can be repeatedly administered as necessary. Once expressed, the dsRNAs bind to target RNA and modulate its function or expression. Delivery of dsRNA expressing vectors can be systemic, such as by intravenous or intramuscular administration, by administration to target cells ex-planted from the patient

followed by reintroduction into the patient, or by any other means that allows for introduction into a desired target cell.

dsRNA expression DNA plasmids are typically transfected into target cells as a complex with cationic lipid carriers *(e.g.,* Oligofectamine) or non-cationic lipid-based carriers *(e.g.,* Transit-TKO™). Multiple lipid transfections for dsRNA-mediated knockdowns targeting different regions of a single target gene or multiple target genes over a period of a week or more are also contemplated by the invention. Successful introduction of vectors into host cells can be monitored using various known methods. For example, transient transfection can be signaled with a reporter, such as a fluorescent marker, such as Green Fluorescent Protein (GFP). Stable transfection of cells *ex vivo* can be ensured using markers that provide the transfected cell with resistance to specific environmental factors *(e.g.,* antibiotics and drugs), such as hygromycin B resistance.

Target gene specific dsRNA molecules can also be inserted into vectors and used as gene therapy vectors for human patients. Gene therapy vectors can be delivered to a subject by, for example, intravenous injection, local administration (see U.S. Patent 5,328,470) or by stereotactic injection (see *e.g.,* Chen *etal.* (1994) Proc. Natl. Acad. Sci. USA 91:3054-3057). The pharmaceutical preparation of the gene therapy vector can include the gene therapy vector in an acceptable diluent, or can include a slow release matrix in which the gene delivery vehicle is imbedded. Alternatively, where the complete gene 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.

Pharmaceutical compositions containing dsRNA

In one embodiment, the invention provides pharmaceutical compositions containing a dsRNA, as described herein, and a pharmaceutically acceptable carrier. The pharmaceutical composition containing the dsRNA is useful for treating a disease or disorder associated with the expression or activity of a SNCA gene, such as pathological processes mediated by SNCA expression. Such pharmaceutical compositions are formulated based on the mode of delivery. One example is compositions that are formulated for systemic administration via parenteral delivery, *e.g.,* by intravenous (IV) delivery. Another example is compositions that are formulated for direct delivery into the brain parenchyma, *e.g.,* by infusion into the brain, such as by continuous pump infusion.

The pharmaceutical compositions featured herein are administered in dosages sufficient to inhibit expression of SNCA genes.

In general, a suitable dose of dsRNA will be in the range of 0.01 to 200.0 milligrams siRNA per kilogram body weight of the recipient per day, generally in the range of 1 to 50 mg per kilogram body weight per day. For example, the dsRNA can be administered at 0.01 mg/kg, 0.05 mg/kg, , 0.2 mg/kg, 0.3 mg/kg, 0.4 mg/kg, 0.5 mg/kg, 0.6 mg/kg, 0.7 mg/kg, 0.8 mg/kg, 0.9 mg/kg, 1 mg/kg, 1.1 mg/kg, 1.2 mg/kg, 1.3 mg/kg, 1.4 mg/kg, 1.5 mg/kg, 2 mg/kg, 3.0 mg/kg, 5.0 mg/kg, 10 mg/kg, 20 mg/kg, 30 mg/kg, 40 mg/kg, or 50 mg/kg per single dose.

In one embodiment, the dosage is between 0.01 and 0.2 mg/kg. For example, the dsRNA can be administered at a dose of 0.01 mg/kg, 0.02 mg/kg, 0.03 mg/kg, 0.04 mg/kg, 0.05 mg/kg, 0.06 mg/kg, 0.07 mg/kg 0.08 mg/kg 0.09 mg/kg , 0.10 mg/kg, 0.11 mg/kg, 0.12 mg/kg, 0.13 mg/kg, 0.14 mg/kg, 0.15 mg/kg, 0.16 mg/kg, 0.17 mg/kg, 0.18 mg/kg, 0.19 mg/kg, or 0.20 mg/kg.

The dsRNA can be administered at a dose of 0.03 mg/kg.

The dsRNA can be administered at a dose of 0 to 1 mg/kg, 1 to 2 mg/kg, 2 to 3 mg/kg, 3 to 4 mg/kg. 4 to 5 mg/kg, 5 to 6 mg/kg, 6 to 7 mg/kg, 7 to 8 mg/kg, 8 to 9 mg/kg, or 9 to 10 mg/kg.

The pharmaceutical composition may be administered once daily, or the dsRNA may be administered as two, three, or more sub-doses at appropriate intervals throughout the day or even using continuous infusion or delivery through a controlled release formulation. In that case, the dsRNA contained in each sub-dose must be correspondingly smaller in order to achieve the total daily dosage. The dosage unit can also be compounded for delivery over several days, *e.g.*, using a conventional sustained release formulation which provides sustained release of the dsRNA over a several day period. Sustained release formulations are well known in the art and are particularly useful for delivery of agents at a particular site, such as could be used with the agents of the present invention. In this embodiment, the dosage unit contains a corresponding multiple of the daily dose.

The effect of a single dose on SNCA levels is long lasting, such that subsequent doses are administered at not more than 3, 4, or 5 day intervals, or at not more than 1, 2, 3, or 4 week intervals, or at not more than 5, 6, 7, 8, 9, or 10 week intervals.

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 composition can include a single treatment or a series of treatments. Estimates of effective dosages and *in vivo* half-lives for the individual dsRNAs encompassed by the invention can be

made using conventional methodologies or on the basis of *in vivo* testing using an appropriate animal model, as described elsewhere herein.

Advances in mouse genetics have generated a number of mouse models for the study of various human diseases, such as pathological processes mediated by SNCA expression. Such models are used for *in vivo* testing of dsRNA, as well as for determining a therapeutically effective dose. A suitable mouse model is, for example, a mouse containing a plasmid expressing human SNCA. Another suitable mouse model is a transgenic mouse carrying a transgene that expresses human SNCA.

The data obtained from cell culture assays and animal studies can be used in formulating a range of dosage for use in humans. The dosage of compositions featured in the invention lies generally 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 methods featured in 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 of the compound or, when appropriate, of the polypeptide product of a target sequence *(e.g.,* achieving a decreased concentration of the polypeptide) that includes the IC50 *(i.e.,* the concentration of the test compound which achieves a half-maximal inhibition of symptoms) 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.

The dsRNAs featured in the invention can be administered in combination with other known agents effective in treatment of pathological processes mediated by target gene expression. In any event, the administering physician can adjust the amount and timing of dsRNA administration on the basis of results observed using standard measures of efficacy known in the art or described herein.

Administration

The present invention also includes pharmaceutical compositions and formulations which include the dsRNA compounds featured in the invention. 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, pulmonary, *e.g.,* by inhalation or insufflation of powders or aerosols, including by nebulizer; intratracheal, intranasal, epidermal and transdermal, oral or parenteral. Parenteral administration

includes intravenous, intraarterial, subcutaneous, intraperitoneal or intramuscular injection or infusion; or intracranial, *e.g.,* intraparenchymal, intrathecal or intraventricular, administration.

The dsRNA can be delivered in a manner to target a particular tissue, such as the liver *(e.g.,* the hepatocytes of the liver).

The present invention includes pharmaceutical compositions that 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), or the dsRNA 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 dsRNA can also be delivered into diffuse regions of the brain *(e.g.,* diffuse delivery to the cortex of the brain).

In one embodiment, a dsRNA targeting SNCA 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, corpus callosum or globus pallidus of the brain. The cannula can be connected to a reservoir of the dsRNA composition. 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. Infusion of the dsRNA composition into the brain can be over several hours or for several days, *e.g.,* for 1, 2, 3, 5, or 7 days or more. Devices for delivery to the brain are described, for example, in U.S. Patent Nos. 6,093,180, and 5,814,014.

Pharmaceutical compositions and formulations for topical administration may include transdermal patches, ointments, lotions, creams, gels, drops, suppositories, sprays, liquids and powders. Conventional pharmaceutical carriers, aqueous, powder or oily bases, thickeners and the like may be necessary or desirable. Coated condoms, gloves and the like may also be useful. Suitable topical formulations include those in which the dsRNAs featured in the invention are in admixture with a topical delivery agent such as lipids, liposomes, fatty acids, fatty acid esters, steroids, chelating agents and surfactants. Suitable lipids and liposomes include neutral *(e.g.,* dioleoylphosphatidyl DOPE ethanolamine, dimyristoylphosphatidyl choline DMPC, distearoylphosphatidyl choline) negative *(e.g.,* dimyristoylphosphatidyl glycerol DMPG) and cationic *(e.g.,* dioleoyltetramethylaminopropyl DOTAP and dioleoylphosphatidyl ethanolamine DOTMA). DsRNAs featured in the invention may be encapsulated within liposomes or may form complexes thereto, in particular to cationic liposomes. Alternatively, dsRNAs may be complexed to lipids, in particular to cationic lipids. Suitable fatty acids and esters include but are not limited to arachidonic acid, oleic acid, eicosanoic acid, lauric acid, caprylic acid, capric acid,

myristic acid, palmitic acid, stearic acid, linoleic acid, linolenic acid, dicaprate, tricaprate, monoolein, dilaurin, glyceryl 1-monocaprate, 1-dodecylazacycloheptan-2-one, an acylcarnitine, an acylcholine, or a C_{1-10} alkyl ester *(e.g.,* isopropylmyristate IPM), monoglyceride, diglyceride or pharmaceutically acceptable salt thereof. Topical formulations are described in detail in U.S. Patent No. 6,747,014, which is incorporated herein by reference.

Liposomal formulations

There are many organized surfactant structures besides microemulsions that have been studied and used for the formulation of drugs. These include monolayers, micelles, bilayers and vesicles. Vesicles, such as liposomes, have attracted great interest because of their specificity and the duration of action they offer from the standpoint of drug delivery. As used in the present invention, the term "liposome" means a vesicle composed of amphiphilic lipids arranged in a spherical bilayer or bilayers.

Liposomes are unilamellar or multilamellar vesicles which have a membrane formed from a lipophilic material and an aqueous interior. The aqueous portion contains the composition to be delivered. Cationic liposomes possess the advantage of being able to fuse to the cell wall. Non-cationic liposomes, although not able to fuse as efficiently with the cell wall, are taken up by macrophages *in vivo.*

In order to cross intact mammalian skin, lipid vesicles must pass through a series of fine pores, each with a diameter 50 nm or less, under the influence of a suitable transdermal gradient. Therefore, it is desirable to use a liposome which is highly deformable and able to pass through such fine pores.

Further advantages of liposomes include; liposomes obtained from natural phospholipids are biocompatible and biodegradable; liposomes can incorporate a wide range of water and lipid soluble drugs; liposomes can protect encapsulated drugs in their internal compartments from metabolism and degradation (Rosoff, in Pharmaceutical Dosage Forms, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 245). Important considerations in the preparation of liposome formulations are the lipid surface charge, vesicle size and the aqueous volume of the liposomes.

Liposomes are useful for the transfer and delivery of active ingredients to the site of action. Because the liposomal membrane is structurally similar to biological membranes, when liposomes are applied to a tissue, the liposomes start to merge with the cellular membranes and as the merging of the liposome and cell progresses, the liposomal contents are emptied into the cell where the active agent may act.

Liposomal formulations have been the focus of extensive investigation as the mode of delivery for many drugs. There is growing evidence that for topical administration, liposomes present several advantages over other formulations. Such advantages include reduced side-effects related to high systemic absorption of the administered drug, increased accumulation of the administered drug at the desired target, and the ability to administer a wide variety of drugs, both hydrophilic and hydrophobic, into the skin.

Several reports have detailed the ability of liposomes to deliver agents including high-molecular weight DNA into the skin. Compounds including analgesics, antibodies, hormones and high-molecular weight DNAs have been administered to the skin. The majority of applications resulted in the targeting of the upper epidermis

Liposomes fall into two broad classes. Cationic liposomes are positively charged liposomes which interact with the negatively charged DNA molecules to form a stable complex. The positively charged DNA/liposome complex binds to the negatively charged cell surface and is internalized in an endosome. Due to the acidic pH within the endosome, the liposomes are ruptured, releasing their contents into the cell cytoplasm (Wang *et al,* Biochem. Biophys. Res. Commun, 1987, 147, 980-985).

Liposomes which are pH-sensitive or negatively-charged, entrap DNA rather than complex with it. Since both the DNA and the lipid are similarly charged, repulsion rather than complex formation occurs. Nevertheless, some DNA is entrapped within the aqueous interior of these liposomes. pH-sensitive liposomes have been used to deliver DNA encoding the thymidine kinase gene to cell monolayers in culture. Expression of the exogenous gene was detected in the target cells (Zhou *et al,* Journal of Controlled Release, 1992, 19, 269-274).

One major type of liposomal composition includes phospholipids other than naturally-derived phosphatidylcholine. Neutral liposome compositions, for example, can be formed from dimyristoyl phosphatidylcholine (DMPC) or dipalmitoyl phosphatidylcholine (DPPC). Anionic liposome compositions generally are formed from dimyristoyl phosphatidylglycerol, while anionic fusogenic liposomes are formed primarily from dioleoyl phosphatidylethanolamine (DOPE). Another type of liposomal composition is formed from phosphatidylcholine (PC) such as, for example, soybean PC, and egg PC. Another type is formed from mixtures of phospholipid and/or phosphatidylcholine and/or cholesterol.

Several studies have assessed the topical delivery of liposomal drug formulations to the skin. Application of liposomes containing interferon to guinea pig skin resulted in a reduction of skin herpes sores while delivery of interferon via other means *(e.g.,* as a solution or as an emulsion) were ineffective (Weiner *et al,* Journal of Drug Targeting, 1992, 2, 405-410). Further,

an additional study tested the efficacy of interferon administered as part of a liposomal formulation to the administration of interferon using an aqueous system, and concluded that the liposomal formulation was superior to aqueous administration (du Plessis *et al,* Antiviral Research, 1992, 18, 259-265).

Non-ionic liposomal systems have also been examined to determine their utility in the delivery of drugs to the skin, in particular systems comprising non-ionic surfactant and cholesterol. Non-ionic liposomal formulations comprising Novasome™ I (glyceryl dilaurate/cholesterol/polyoxyethylene-10-stearyl ether) and Novasome™ II (glyceryl distearate/cholesterol/polyoxyethylene-10-stearyl ether) were used to deliver cyclosporin-A into the dermis of mouse skin. Results indicated that such non-ionic liposomal systems were effective in facilitating the deposition of cyclosporin-A into different layers of the skin (Hu *et al.* S.T.P.Pharma. Sci., 1994, 4, 6, 466).

Liposomes also include "sterically stabilized" liposomes, a term which, as used herein, refers to liposomes comprising one or more specialized lipids that, when incorporated into liposomes, result in enhanced circulation lifetimes relative to liposomes lacking such specialized lipids. Examples of sterically stabilized liposomes are those in which part of the vesicle-forming lipid portion of the liposome (A) comprises one or more glycolipids, such as monosialoganglioside GMI, or (B) is derivatized with one or more hydrophilic polymers, such as a polyethylene glycol (PEG) moiety. While not wishing to be bound by any particular theory, it is thought in the art that, at least for sterically stabilized liposomes containing gangliosides, sphingomyelin, or PEG-derivatized lipids, the enhanced circulation half-life of these sterically stabilized liposomes derives from a reduced uptake into cells of the reticuloendothelial system (RES) (Allen *et al,* FEBS Letters, 1987, 223, 42; Wu *et al,* Cancer Research, 1993, 53, 3765).

Various liposomes comprising one or more glycolipids are known in the art. Papahadjopoulos *et al* (Ann. N.Y. Acad. Sci., 1987, 507, 64) reported the ability of monosialoganglioside GMI, galactocerebroside sulfate and phosphatidylinositol to improve blood half-lives of liposomes. These findings were expounded upon by Gabizon *et al* (Proc. Natl. Acad. Sci. U.S.A., 1988, 85, 6949). U.S. Pat. No. 4,837,028 and WO 88/04924, both to Allen *et al,* disclose liposomes comprising (1) sphingomyelin and (2) the ganglioside GMI or a galactocerebroside sulfate ester. U.S. Pat. No. 5,543,152 (Webb *et al.)* discloses liposomes comprising sphingomyelin. Liposomes comprising 1,2-sn-dimyristoylphosphatidylcholine are disclosed in WO 97/13499 (Lim *et al).*

Many liposomes comprising lipids derivatized with one or more hydrophilic polymers, and methods of preparation thereof, are known in the art. Sunamoto *et al.* (Bull. Chem. Soc. Jpn.,

1980, 53, 2778) described liposomes comprising a nonionic detergent, $2C_{1215_G}$, that contains a PEG moiety. Ilium *et al.* (FEBS Lett., 1984, 167, 79) noted that hydrophilic coating of polystyrene particles with polymeric glycols results in significantly enhanced blood half-lives. Synthetic phospholipids modified by the attachment of carboxylic groups of polyalkylene glycols *(e.g.,* PEG) are described by Sears (U.S. Pat. Nos. 4,426,330 and 4,534,899). Klibanov *et al.* (FEBS Lett., 1990, 268, 235) described experiments demonstrating that liposomes comprising phosphatidylethanolamine (PE) derivatized with PEG or PEG stearate have significant increases in blood circulation half-lives. Blume *et al.* (Biochimica et Biophysica Acta, 1990, 1029, 91) extended such observations to other PEG-derivatized phospholipids, *e.g.,* DSPE-PEG, formed from the combination of distearoylphosphatidylethanolamine (DSPE) and PEG. Liposomes having covalently bound PEG moieties on their external surface are described in European Patent No. EP 0 445 131 Bl and WO 90/04384 to Fisher. Liposome compositions containing 1-20 mole percent of PE derivatized with PEG, and methods of use thereof, are described by Woodle *et al.* (U.S. Pat. Nos. 5,013,556 and 5,356,633) and Martin *et al.* (U.S. Pat. No. 5,213,804 and European Patent No. EP 0 496 813 Bl). Liposomes comprising a number of other lipid-polymer conjugates are disclosed in WO 91/05545 and U.S. Pat. No. 5,225,212 (both to Martin *et al.)* and in WO 94/20073 (Zalipsky *et al.)* Liposomes comprising PEG-modified ceramide lipids are described in WO 96/10391 (Choi *et al).* U.S. Pat. No. 5,540,935 (Miyazaki *et al.)* and U.S. Pat. No. 5,556,948 (Tagawa *et al.)* describe PEG-containing liposomes that can be further derivatized with functional moieties on their surfaces.

A number of liposomes comprising nucleic acids are known in the art. WO 96/40062 to Thierry *et al.* discloses methods for encapsulating high molecular weight nucleic acids in liposomes. U.S. Pat. No. 5,264,221 to Tagawa *etal.* discloses protein-bonded liposomes and asserts that the contents of such liposomes may include a dsRNA. U.S. Pat. No. 5,665,710 to Rahman *et al.* describes certain methods of encapsulating oligodeoxynucleotides in liposomes. WO 97/04787 to Love *et al.* discloses liposomes comprising dsRNAs targeted to the raf gene.

Transfersomes are yet another type of liposomes, and are highly deformable lipid aggregates which are candidates for drug delivery vehicles. Transfersomes may be described as lipid droplets which are so highly deformable that they are easily able to penetrate through pores which are smaller than the droplet. Transfersomes are adaptable to the environment in which they are used, *e.g.,* they are self-optimizing (adaptive to the shape of pores in the skin), self-repairing, frequently reach their targets without fragmenting, and often self-loading. To make transfersomes it is possible to add surface edge-activators, usually surfactants, to a standard liposomal composition. Transfersomes have been used to deliver serum albumin to the skin. The

transfersome-mediated delivery of serum albumin has been shown to be as effective as subcutaneous injection of a solution containing serum albumin.

Surfactants find wide application in formulations such as emulsions (including microemulsions) and liposomes. The most common way of classifying and ranking the properties of the many different types of surfactants, both natural and synthetic, is by the use of the hydrophile/lipophile balance (HLB). The nature of the hydrophilic group (also known as the "head") provides the most useful means for categorizing the different surfactants used in formulations (Rieger, in Pharmaceutical Dosage Forms, Marcel Dekker, Inc., New York, N.Y., 1988, p. 285).

If the surfactant molecule is not ionized, it is classified as a nonionic surfactant. Nonionic surfactants find wide application in pharmaceutical and cosmetic products and are usable over a wide range of pH values. In general their HLB values range from 2 to about 18 depending on their structure. Nonionic surfactants include nonionic esters such as ethylene glycol esters, propylene glycol esters, glyceryl esters, polyglyceryl esters, sorbitan esters, sucrose esters, and ethoxylated esters. Nonionic alkanolamides and ethers such as fatty alcohol ethoxylates, propoxylated alcohols, and ethoxylated/propoxylated block polymers are also included in this class. The polyoxyethylene surfactants are the most popular members of the nonionic surfactant class.

If the surfactant molecule carries a negative charge when it is dissolved or dispersed in water, the surfactant is classified as anionic. Anionic surfactants include carboxylates such as soaps, acyl lactylates, acyl amides of amino acids, esters of sulfuric acid such as alkyl sulfates and ethoxylated alkyl sulfates, sulfonates such as alkyl benzene sulfonates, acyl isethionates, acyl taurates and sulfosuccinates, and phosphates. The most important members of the anionic surfactant class are the alkyl sulfates and the soaps.

If the surfactant molecule carries a positive charge when it is dissolved or dispersed in water, the surfactant is classified as cationic. Cationic surfactants include quaternary ammonium salts and ethoxylated amines. The quaternary ammonium salts are the most used members of this class.

If the surfactant molecule has the ability to carry either a positive or negative charge, the surfactant is classified as amphoteric. Amphoteric surfactants include acrylic acid derivatives, substituted alkylamides, N-alkylbetaines and phosphatides.

The use of surfactants in drug products, formulations and in emulsions has been reviewed (Rieger, in Pharmaceutical Dosage Forms, Marcel Dekker, Inc., New York, N.Y., 1988, p. 285).

Nucleic acid lipid particles

In one embodiment, a SNCA dsRNA featured in the invention is fully encapsulated in the lipid formulation, *e.g.,* to form a SPLP, pSPLP, SNALP, or other nucleic acid-lipid particle. As used herein, the term "SNALP" refers to a stable nucleic acid-lipid particle, including SPLP. As used herein, the term "SPLP" refers to a nucleic acid-lipid particle comprising plasmid DNA encapsulated within a lipid vesicle. SNALPs and SPLPs typically contain a cationic lipid, a non-cationic lipid, and a lipid that prevents aggregation of the particle *(e.g.,* a PEG-lipid conjugate). SNALPs and SPLPs are extremely useful for systemic applications, as they exhibit extended circulation lifetimes following intravenous (i.v.) injection and accumulate at distal sites *(e.g.,* sites physically separated from the administration site). SPLPs include "pSPLP," which include an encapsulated condensing agent-nucleic acid complex as set forth in PCT Publication No. WO 00/03683. The particles of the present invention typically have a mean diameter of about 50 nm to about 150 nm, more typically about 60 nm to about 130 nm, more typically about 70 nm to about 110 nm, most typically about 70 nm to about 90 nm, and are substantially nontoxic. In addition, the nucleic acids when present in the nucleic acid- lipid particles of the present invention are resistant in aqueous solution to degradation with a nuclease. Nucleic acid-lipid particles and their method of preparation are disclosed in, *e.g.,* U.S. Patent Nos. 5,976,567; 5,981,501; 6,534,484; 6,586,410; 6,815,432; and PCT Publication No. WO 96/40964.

In one embodiment, the lipid to drug ratio (mass/mass ratio) *(e.g.,* lipid to dsRNA ratio) will be in the range of from about 1:1 to about 50:1, from about 1:1 to about 25:1, from about 3:1 to about 15:1, from about 4:1 to about 10:1, from about 5:1 to about 9:1, or about 6:1 to about 9:1. In some embodiments the lipid to dsRNA ratio can be about 1:1, 2:1, 3:1, 4:1, 5:1, 6:1, 7:1, 8:1, 9:1, 10:1, or 11:1.

In general, the lipid-nucleic acid particle is suspended in a buffer, e.g., PBS, for administration. In one embodiment, the pH of the lipid formulated siRNA is between 6.8 and 7.8, e.g., 7.3 or 7.4. The osmolality can be, e.g., between 250 and 350 mOsm/kg, e.g., around 300, e.g., 298, 299, 300, 301, 302, 303, 304, or 305.

The cationic lipid may be, for example, N,N-dioleyl-N,N-dimethylammonium chloride (DODAC), N,N-distearyl-N,N-dimethylammonium bromide (DDAB), N-(I -(2,3-dioleoyloxy)propyl)-N,N,N-trimethylammonium chloride (DOTAP), N-(I -(2,3-dioleyloxy)propyl)-N,N,N-trimethylammonium chloride (DOTMA), N,N-dimethyl-2,3-dioleyloxy)propylamine (DODMA), 1,2-DiLinoleyloxy-N,N-dimethylaminopropane (DLinDMA), l,2-Dilinolenyloxy-N,N-dimethylaminopropane (DLenDMA), 1,2-Dilinoleylcarbamoyloxy-3-dimethylaminopropane (DLin-C-DAP), l,2-Dilinoleyoxy-3-

(dimethylamino)acetoxypropane (DLin-DAC), l,2-Dilinoleyoxy-3-morpholinopropane (DLin-MA), l,2-Dilinoleoyl-3-dimethylaminopropane (DLinDAP), l,2-Dilinoleylthio-3-dimethylaminopropane (DLin-S-DMA), l-Linoleoyl-2-linoleyloxy-3-dimethylaminopropane (DLin-2-DMAP), l,2-Dilinoleyloxy-3-trimethylaminopropane chloride salt (DLin-TMA.Cl), l,2-Dilinoleoyl-3-trimethylaminopropane chloride salt (DLin-TAP.Cl), l,2-Dilinoleyloxy-3-(N-methylpiperazino)propane (DLin-MPZ), or 3-(N,N-Dilinoleylamino)-l,2-propanediol (DLinAP), 3-(N,N-Dioleylamino)-l,2-propanedio (DOAP), l,2-Dilinoleyloxo-3-(2-N,N-dimethylamino)ethoxypropane (DLin-EG-DMA), l,2-Dilinolenyloxy-N,N-dimethylaminopropane (DLinDMA), 2,2-Dilinoleyl-4-dimethylaminomethyl-[l,3]-dioxolane (DLin-K-DMA) or analogs thereof, (3aR,5s,6aS)-N,N-dimethyl-2,2-di((9Z, 12Z)-octadeca-9, 12-dienyl)tetrahydro-3aH-cyclopenta[d][l,3]dioxol-5-amine (ALN100), (6Z,9Z,28Z,3 1Z)-heptatriaconta-6,9,28,31-tetraen-19-yl 4-(dimethylamino)butanoate (MC3), l,l'-(2-(4-(2-((2-(bis(2-hydroxydodecyl)amino)ethyl)(2-hydroxydodecyl)amino)ethyl)piperazin-l-yl)ethylazanediyl)didodecan-2-ol (Tech Gl), or a mixture thereof. The cationic lipid may comprise from about 20 mol % to about 50 mol % or about 40 mol % of the total lipid present in the particle.

The non-cationic lipid may be an anionic lipid or a neutral lipid including, but not limited to, distearoylphosphatidylcholine (DSPC), dioleoylphosphatidylcholine (DOPC), dipalmitoylphosphatidylcholine (DPPC), dioleoylphosphatidylglycerol (DOPG), dipalmitoylphosphatidylglycerol (DPPG), dioleoyl-phosphatidylethanolamine (DOPE), palmitoyloleoylphosphatidylcholine (POPC), palmitoyloleoylphosphatidylethanolamine (POPE), dioleoyl- phosphatidylethanolamine 4-(N-maleimidomethyl)-cyclohexane-l- carboxylate (DOPE-mal), dipalmitoyl phosphatidyl ethanolamine (DPPE), dimyristoylphosphoethanolamine (DMPE), distearoyl-phosphatidyl-ethanolamine (DSPE), 16-O-monomethyl PE, 16-O-dimethyl PE, 18-1 -trans PE, 1 -stearoyl-2-oleoyl- phosphatidyethanolamine (SOPE), cholesterol, or a mixture thereof. The non-cationic lipid may be from about 5 mol % to about 90 mol %, about 10 mol %, or about 58 mol % if cholesterol is included, of the total lipid present in the particle.

The conjugated lipid that inhibits aggregation of particles may be, for example, a polyethyleneglycol (PEG)-lipid including, without limitation, a PEG-diacylglycerol (DAG), a PEG-dialkyloxypropyl (DAA), a PEG-phospholipid, a PEG-ceramide (Cer), or a mixture thereof. The PEG-DAA conjugate may be, for example, a PEG-dilauryloxypropyl (Ci_2), a PEG-dimyristyloxypropyl (Ci_4), a PEG-dipalmityloxypropyl (CI $_{6),}$ or a PEG- distearyloxypropyl (Cis). Other examples of PEG conjugates include PEG-cDMA (N-[(methoxy poly(ethylene glycol)2000)carbamyl]-l,2-dimyristyloxlpropyl-3 -amine), mPEG2000-DMG (mPEG-dimyrystylglycerol (with an average molecular weight of 2,000) and PEG-C-DOMG (R-3-[(co-

methoxy-poly(ethylene glycol)2000)carbamoyl)]-l,2-dimyristyloxlpropyl-3-amine). The conjugated lipid that prevents aggregation of particles may be from 0 mol % to about 20 mol % or about 1.0, 1.1., 1.2, .13, 1.4, 1.5, 1.6, 1.7, 1.8, 1.9, or 2 mol % of the total lipid present in the particle.

In some embodiments, the nucleic acid-lipid particle further includes cholesterol at, *e.g.,* about 10 mol % to about 60 mol % or about 48 mol % of the total lipid present in the particle.

In one embodiment, the compound 2,2-dilinoleyl-4-dimethylaminoethyl-[l,3]-dioxolane can be used to prepare lipid-siRNA nanoparticles. Synthesis of 2,2-dilinoleyl-4-dimethylaminoethyl-[l,3]-dioxolane is described in United States provisional patent application number 61/107,998 filed on October 23, 2008, which is herein incorporated by reference.

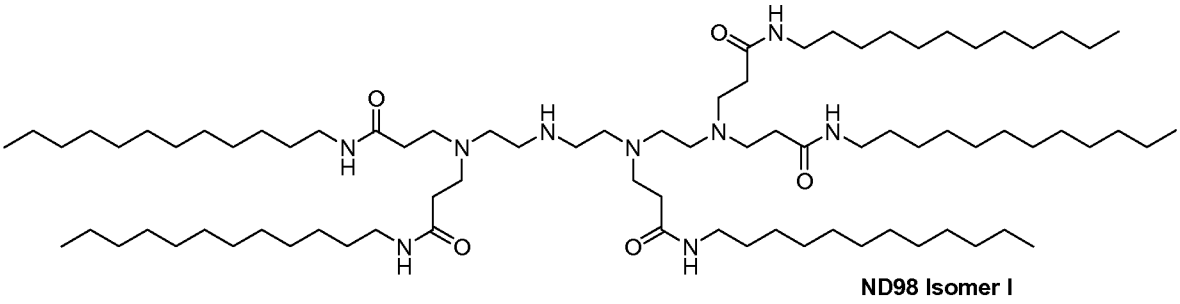
For example, the lipid-siRNA particle can include 40% 2, 2-dilinoleyl-4-dimethylaminoethyl-[l,3]-dioxolane: 10% DSPC: 40% Cholesterol: 10% PEG-C-DOMG (mole percent) with a particle size of 63.0 ± 20 nm and a 0.027 siRNA/Lipid Ratio.

In still another embodiment, the compound l,l'-(2-(4-(2-((2-(bis(2-hydroxydodecyl)amino)ethyl)(2-hydroxydodecyl)amino)ethyl)piperazin- 1-yl)ethylazanediyl)didodecan-2-ol (Tech Gl) can be used to prepare lipid-siRNA particles. For example, the dsRNA can be formulated in a lipid formulation comprising Tech-Gl, distearoyl phosphatidylcholine (DSPC), cholesterol and mPEG2000-DMG at a molar ratio of 50:10:38.5:1.5 at atotal lipid to siRNA ratio of 7:1 (wtwt).

LNP01

In one embodiment, the lipidoid ND98-4HC1 (MW 1487) (Formula 1), Cholesterol (Sigma-Aldrich), and PEG-Ceramide C16 (Avanti Polar Lipids) can be used to prepare lipid-siRNA nanoparticles *(i.e.,* LNP01 particles). Stock solutions of each in ethanol can be prepared as follows: ND98, 133 mg/ml; Cholesterol, 25 mg/ml, PEG-Ceramide C16, 100 mg/ml. The ND98, Cholesterol, and PEG-Ceramide C16 stock solutions can then be combined in a, *e.g.,* 42:48:10 molar ratio. The combined lipid solution can be mixed with aqueous siRNA *(e.g.,* in sodium acetate pH 5) such that the final ethanol concentration is about 35-45% and the final sodium acetate concentration is about 100-300 mM. Lipid-siRNA nanoparticles typically form spontaneously upon mixing. Depending on the desired particle size distribution, the resultant nanoparticle mixture can be extruded through a polycarbonate membrane *(e.g.,* 100 nm cut-off) using, for example, a thermobarrel extruder, such as Lipex Extruder (Northern Lipids, Inc). In some cases, the extrusion step can be omitted. Ethanol removal and simultaneous buffer exchange can be accomplished by, for example, dialysis or tangential flow filtration. Buffer can

be exchanged with, for example, phosphate buffered saline (PBS) at about pH 7, *e.g.,* about pH 6.9, about pH 7.0, about pH 7.1, about pH 7.2, about pH 7.3, or about pH 7.4.



Formula 1

LNPOl formulations are described, *e.g.,* in International Application Publication No. WO 2008/042973, which is hereby incorporated by reference.

Additional exemplary lipid-siRNA formulations are as follows:

	Cationic Lipid	**cationic lipid/non-cationic lipid/cholesterol/PEG-lipid conjugate Lipid:siRNA ratio**
SNALP	l,2-Dilinolenyloxy-N,N-dimethylaminopropane (DLinDMA)	DLinDMA/DPPC/Cholesterol/PEG-cDMA (57.1/7.1/34.4/1.4) lipid:siRNA ~ 7:1
S-XTC	2,2-Dilinoleyl-4-dimethylaminoethyl-[1,3]-dioxolane (XTC)	XTC/DPPC/Cholesterol/PEG-cDMA 57.1/7.1/34.4/1.4 lipid:siRNA ~ 7:1
LNP05	2,2-Dilinoleyl-4-dimethylaminoethyl-[1,3]-dioxolane (XTC)	XTC/DSPC/Cholesterol/PEG-DMG 57.5/7.5/31.5/3.5 lipid:siRNA ~ 6:1
LNP06	2,2-Dilinoleyl-4-dimethylaminoethyl-[1,3]-dioxolane (XTC)	XTC/DSPC/Cholesterol/PEG-DMG 57.5/7.5/31.5/3.5 lipid:siRNA ~ 11:1
LNP07	2,2-Dilinoleyl-4-dimethylaminoethyl-[1,3]-dioxolane (XTC)	XTC/DSPC/Cholesterol/PEG-DMG 60/7.5/31/1.5, lipid:siRNA ~ 6:1
LNP08	2,2-Dilinoleyl-4-dimethylaminoethyl-[1,3]-dioxolane (XTC)	XTC/DSPC/Cholesterol/PEG-DMG 60/7.5/31/1.5, lipid:siRNA ~ 11:1
LNP09	2,2-Dilinoleyl-4-dimethylaminoethyl-[1,3]-dioxolane (XTC)	XTC/DSPC/Cholesterol/PEG-DMG 50/10/38.5/1.5 Lipid:siRNA 10:1

LNP10	(3aR,5s,6aS)-N,N-dimethyl-2,2-di((9Z,12Z)-octadeca-9, 12-dienyl)tetrahydro-3 aH-cyclopentafd][1,3]dioxol-5-amine (ALN 100)	ALNlOO/DSPC/Cholesterol/PEG-DMG 50/10/38.5/1 .5 Lipid: siRNA 10: 1
LNP1 1	(6Z,9Z,28Z,3 1Z)-heptatriaconta-6,9,28,3 1-tetraen- 19-yl 4-(dimethylamino)butanoate (MC3)	MC-3/DSPC/Cholesterol/PEG-DMG 50/10/38.5/1 .5 Lipid: siRNA 10: 1
LNP12	l,l'-(2-(4-(2-((2-(bis(2-hydroxydodecyl)amino)ethyl)(2-hydroxydodecyl)amino)ethyl)piperazin- 1-yl)ethylazanediyl)didodecan-2-ol (C12-200)	C12-200/DSPC/Cholesterol/PEG-DMG 50/10/38.5/1 .5 Lipid: siRNA 10: 1
LNP13	XTC	XTC/DSPC/Chol/PEG-DMG 50/10/38.5/1 .5 Lipid:siRNA: 33: 1
LNP14	MC3	MC3/DSPC/Chol/PEG-DMG 40/15/40/5 Lipid:siRNA: 11:1
LNP15	MC3	MC3/DSPC/Chol/PEG-DSG/GalNAc-PEG-DSG 50/10/35/4.5/0.5 Lipid:siRNA: 11:1
LNP16	MC3	MC3/DSPC/Chol/PEG-DMG 50/10/38.5/1 .5 Lipid:siRNA: 7:1
LNP17	MC3	MC3/DSPC/Chol/PEG-DSG 50/10/38.5/1 .5 Lipid:siRNA: 10: 1
LNP18	MC3	MC3/DSPC/Chol/PEG-DMG 50/10/38.5/1 .5 Lipid:siRNA: 12: 1
LNP19	MC3	MC3/DSPC/Chol/PEG-DMG 50/10/35/5 Lipid:siRNA: 8:1
LNP20	MC3	MC3/DSPC/Chol/PEG-DPG 50/10/38.5/1 .5 Lipid:siRNA: 10: 1
LNP21	C12-200	C12-200/DSPC/Chol/PEG-DSG 50/10/38.5/1 .5 Lipid:siRNA: 7:1
LNP22	XTC	XTC/DSPC/Chol/PEG-DSG 50/10/38.5/1 .5 Lipid:siRNA: 10: 1

DSPC: distearoy lphosphatidy lcholine

DPPC: dipalmitoylphosphatidylcholine

PEG-DMG: PEG-didimyristoyl glycerol (C14-PEG, or PEG-C14) (PEG with avg mol wt of 2000)

PEG-DSG: PEG-distyryl glycerol (CI 8-PEG, or PEG-C1 8) (PEG with avg mol wt of 2000)

PEG-cDMA: PEG-carbamoyl-l,2-dimyristyloxypropylamine (PEG with avg mol wt of 2000)

SNALP (l,2-Dilinolenyloxy-N,N-dimethylaminopropane (DLinDMA)) comprising formulations are described in International Publication No. WO2009/127060, filed April 15, 2009, which is hereby incorporated by reference.

LNP09 formulations and XTC comprising formulations are described, e.g., in U.S. Provisional Serial No. 61/239,686, filed September 3, 2009, and International patent application no. PCT/US 10/226 14, filed January 29, 2010, which are hereby incorporated by reference.

LNP11 formulations and MC3 comprising formulations are described, e.g., in U.S. Provisional Serial No. 61/244,834, filed September 22, 2009, and U.S. Provisional Serial No. 61/185,800, filed June 10, 2009, which are hereby incorporated by reference.

ALN100, i.e., ALNY-100 comprising formulations are described, e.g., International patent application number PCT/US09/63933, filed on November 10, 2009, which is hereby incorporated by reference.

LNP12 formulations and C12-200 comprising formulations are described, *e.g.,* in U.S. Provisional Serial No. 61/175,770, filed May 5, 2009, and International Application No. PCT/USlO/33777, filed May 5, 2010, which are hereby incorporated by reference.

Formulations prepared by either the standard or extrusion-free method can be characterized in similar manners. For example, formulations are typically characterized by visual inspection. They should be whitish translucent solutions free from aggregates or sediment. Particle size and particle size distribution of lipid-nanoparticles can be measured by light scattering using, for example, a Malvern Zetasizer Nano ZS (Malvern, USA). Particles should be about 20-300 nm, such as 40-100 nm in size. The particle size distribution should be unimodal. The total siRNA concentration in the formulation, as well as the entrapped fraction, is estimated using a dye exclusion assay. A sample of the formulated siRNA can be incubated with an RNA-binding dye, such as Ribogreen (Molecular Probes) in the presence or absence of a formulation disrupting surfactant, *e.g.,* 0.5% Triton-X100. The total siRNA in the formulation can be determined by the signal from the sample containing the surfactant, relative to a standard curve. The entrapped fraction is determined by subtracting the "free" siRNA content (as measured by the signal in the absence of surfactant) from the total siRNA content. Percent entrapped siRNA is typically >85%. For SNALP formulation, the particle size is at least 30 nm, at least 40 nm, at least 50 nm, at least 60 nm, at least 70 nm, at least 80 nm, at least 90 nm, at least 100 nm, at least 110 nm, and at least 120 nm. The suitable range is typically about at least 50 nm to about at least 110 nm, about at least 60 nm to about at least 100 nm, or about at least 80 nm to about at least 90 nm.

Compositions and formulations for oral administration include powders or granules, microparticulates, nanoparticulates, suspensions or solutions in water or non-aqueous media, capsules, gel capsules, sachets, tablets or minitablets. Thickeners, flavoring agents, diluents, emulsifiers, dispersing aids or binders may be desirable. In some embodiments, oral formulations are those in which dsRNAs featured in the invention are administered in conjunction with one or more penetration enhancers surfactants and chelators. Suitable surfactants include fatty acids and/or esters or salts thereof, bile acids and/or salts thereof. Suitable bile acids/salts include chenodeoxycholic acid (CDCA) and ursodeoxychenodeoxycholic acid (UDCA), cholic acid, dehydrocholic acid, deoxycholic acid, glucholic acid, glycholic acid, glycodeoxycholic acid, taurocholic acid, taurodeoxy cholic acid, sodium tauro-24,25-dihydro-fusidate and sodium glycodihydrofusidate. Suitable fatty acids include arachidonic acid, undecanoic acid, oleic acid, lauric acid, caprylic acid, capric acid, myristic acid, palmitic acid, stearic acid, linoleic acid, linolenic acid, dicaprate, tricaprate, monoolein, dilaurin, glyceryl 1-monocaprate, 1-dodecylazacycloheptan-2-one, an acylcarnitine, an acylcholine, or a monoglyceride, a diglyceride or a pharmaceutically acceptable salt thereof (e.g., sodium). In some embodiments, combinations of penetration enhancers are used, for example, fatty acids/salts in combination with bile acids/salts. One exemplary combination is the sodium salt of lauric acid, capric acid and UDCA. Further penetration enhancers include polyoxyethylene-9-lauryl ether, polyoxyethylene-20-cetyl ether. DsRNAs featured in the invention may be delivered orally, in granular form including sprayed dried particles, or complexed to form micro or nanoparticles. DsRNA complexing agents include poly-amino acids; polyimines; polyacrylates; polyalkylacrylates, polyoxethanes, polyalkylcyanoacrylates; cationized gelatins, albumins, starches, acrylates, polyethyleneglycols (PEG) and starches; polyalkylcyanoacrylates; DEAE-derivatized polyimines, pollulans, celluloses and starches. Suitable complexing agents include chitosan, N-trimethylchitosan, poly-L-lysine, polyhistidine, polyornithine, polyspermines, protamine, polyvinylpyridine, polythiodiethylaminomethylethylene P(TDAE), polyaminostyrene *(e.g.,* p-amino), poly(methylcyanoacrylate), poly(ethylcyanoacrylate), poly(butylcyanoacrylate), poly(isobutylcyanoacrylate), poly(isohexylcynaoacrylate), DEAE-methacrylate, DEAE-hexylacrylate, DEAE-acrylamide, DEAE-albumin and DEAE-dextran, polymethylacrylate, polyhexylacrylate, poly(D,L-lactic acid), poly(DL-lactic-co-glycolic acid (PLGA), alginate, and polyethyleneglycol (PEG). Oral formulations for dsRNAs and their preparation are described in detail in U.S. Patent 6,887,906, U.S. Publication No. 20030027780, and U.S. Patent No. 6,747,014, each of which is incorporated herein by reference.

Compositions and formulations for parenteral, intraparenchymal (into the brain), intrathecal, intraventricular or intrahepatic administration may include sterile aqueous solutions

which may also contain buffers, diluents and other suitable additives such as, but not limited to, penetration enhancers, carrier compounds and other pharmaceutically acceptable carriers or excipients.

Pharmaceutical compositions of the present invention include, but are not limited to, solutions, emulsions, and liposome-containing formulations. These compositions may be generated from a variety of components that include, but are not limited to, preformed liquids, self-emulsifying solids and self-emulsifying semisolids. Particularly preferred are formulations that target the liver when treating hepatic disorders such as hepatic carcinoma.

The pharmaceutical formulations of the present invention, which may conveniently be presented in unit dosage form, may be prepared according to conventional techniques well known in the pharmaceutical industry. Such techniques include the step of bringing into association the active ingredients with the pharmaceutical carrier(s) or excipient(s). In general, the formulations are prepared by uniformly and intimately bringing into association the active ingredients with liquid carriers or finely divided solid carriers or both, and then, if necessary, shaping the product.

The compositions of the present invention may be formulated into any of many possible dosage forms such as, but not limited to, tablets, capsules, gel capsules, liquid syrups, soft gels, suppositories, and enemas. The compositions of the present invention may also be formulated as suspensions in aqueous, non-aqueous or mixed media. Aqueous suspensions may further contain substances which increase the viscosity of the suspension including, for example, sodium carboxymethylcellulose, sorbitol and/or dextran. The suspension may also contain stabilizers.

Emulsions

The compositions of the present invention may be prepared and formulated as emulsions. Emulsions are typically heterogeneous systems of one liquid dispersed in another in the form of droplets usually exceeding 0.1 μιη in diameter (Idson, in Pharmaceutical Dosage Forms, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 199; Rosoff, in Pharmaceutical Dosage Forms, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., Volume 1, p. 245; Block in Pharmaceutical Dosage Forms, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 2, p. 335; Higuchi *et ah,* in Remington's Pharmaceutical Sciences, Mack Publishing Co., Easton, Pa., 1985, p. 301). Emulsions are often biphasic systems comprising two immiscible liquid phases intimately mixed and dispersed with each other. In general, emulsions may be of either the water-in-oil (w/o) or the oil-in-water (o/w) variety. When an aqueous phase is finely divided into and dispersed as minute droplets into a bulk oily phase, the resulting composition is

called a water-in-oil (w/o) emulsion. Alternatively, when an oily phase is finely divided into and dispersed as minute droplets into a bulk aqueous phase, the resulting composition is called an oil-in-water (o/w) emulsion. Emulsions may contain additional components in addition to the dispersed phases, and the active drug which may be present as a solution in either the aqueous phase, oily phase or itself as a separate phase. Pharmaceutical excipients such as emulsifiers, stabilizers, dyes, and anti-oxidants may also be present in emulsions as needed. Pharmaceutical emulsions may also be multiple emulsions that are comprised of more than two phases such as, for example, in the case of oil-in-water-in-oil (o/w/o) and water-in-oil-in-water (w/o/w) emulsions. Such complex formulations often provide certain advantages that simple binary emulsions do not. Multiple emulsions in which individual oil droplets of an o/w emulsion enclose small water droplets constitute a w/o/w emulsion. Likewise a system of oil droplets enclosed in globules of water stabilized in an oily continuous phase provides an o/w/o emulsion.

Emulsions are characterized by little or no thermodynamic stability. Often, the dispersed or discontinuous phase of the emulsion is well dispersed into the external or continuous phase and maintained in this form through the means of emulsifiers or the viscosity of the formulation. Either of the phases of the emulsion may be a semisolid or a solid, as is the case of emulsion-style ointment bases and creams. Other means of stabilizing emulsions entail the use of emulsifiers that may be incorporated into either phase of the emulsion. Emulsifiers may broadly be classified into four categories: synthetic surfactants, naturally occurring emulsifiers, absorption bases, and finely dispersed solids (Idson, in Pharmaceutical Dosage Forms, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 199).

Synthetic surfactants, also known as surface active agents, have found wide applicability in the formulation of emulsions and have been reviewed in the literature (Rieger, in Pharmaceutical Dosage Forms, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 285; Idson, in Pharmaceutical Dosage Forms, Lieberman, Rieger and Banker (Eds.), Marcel Dekker, Inc., New York, N.Y., 1988, volume 1, p. 199). Surfactants are typically amphiphilic and comprise a hydrophilic and a hydrophobic portion. The ratio of the hydrophilic to the hydrophobic nature of the surfactant has been termed the hydrophile/lipophile balance (HLB) and is a valuable tool in categorizing and selecting surfactants in the preparation of formulations. Surfactants may be classified into different classes based on the nature of the hydrophilic group: nonionic, anionic, cationic and amphoteric (Rieger, in Pharmaceutical Dosage Forms, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 285).

Naturally occurring emulsifiers used in emulsion formulations include lanolin, beeswax, phosphatides, lecithin and acacia. Absorption bases possess hydrophilic properties such that they can soak up water to form w/o emulsions yet retain their semisolid consistencies, such as anhydrous lanolin and hydrophilic petrolatum. Finely divided solids have also been used as good emulsifiers especially in combination with surfactants and in viscous preparations. These include polar inorganic solids, such as heavy metal hydroxides, nonswelling clays such as bentonite, attapulgite, hectorite, kaolin, montmorillonite, colloidal aluminum silicate and colloidal magnesium aluminum silicate, pigments and nonpolar solids such as carbon or glyceryl tristearate.

A large variety of non-emulsifying materials are also included in emulsion formulations and contribute to the properties of emulsions. These include fats, oils, waxes, fatty acids, fatty alcohols, fatty esters, humectants, hydrophilic colloids, preservatives and antioxidants (Block, in Pharmaceutical Dosage Forms, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 335; Idson, in Pharmaceutical Dosage Forms, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 199).

Hydrophilic colloids or hydrocolloids include naturally occurring gums and synthetic polymers such as polysaccharides (for example, acacia, agar, alginic acid, carrageenan, guar gum, karaya gum, and tragacanth), cellulose derivatives (for example, carboxymethylcellulose and carboxypropylcellulose), and synthetic polymers (for example, carbomers, cellulose ethers, and carboxyvinyl polymers). These disperse or swell in water to form colloidal solutions that stabilize emulsions by forming strong interfacial films around the dispersed-phase droplets and by increasing the viscosity of the external phase.

Since emulsions often contain a number of ingredients such as carbohydrates, proteins, sterols and phosphatides that may readily support the growth of microbes, these formulations often incorporate preservatives. Commonly used preservatives included in emulsion formulations include methyl paraben, propyl paraben, quaternary ammonium salts, benzalkonium chloride, esters of p-hydroxybenzoic acid, and boric acid. Antioxidants are also commonly added to emulsion formulations to prevent deterioration of the formulation. Antioxidants used may be free radical scavengers such as tocopherols, alkyl gallates, butylated hydroxyanisole, butylated hydroxytoluene, or reducing agents such as ascorbic acid and sodium metabisulfite, and antioxidant synergists such as citric acid, tartaric acid, and lecithin.

The application of emulsion formulations via dermatological, oral, and parenteral routes and methods for their manufacture have been reviewed in the scientific literature (Idson, in Pharmaceutical Dosage Forms, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker,

Inc., New York, N.Y., volume 1, p. 199). Emulsion formulations for oral delivery have been very widely used because of ease of formulation, as well as efficacy from an absorption and bioavailability standpoint (Rosoff, in Pharmaceutical Dosage Forms, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 245; Idson, in Pharmaceutical Dosage Forms, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 199). Mineral-oil base laxatives, oil-soluble vitamins and high fat nutritive preparations are among the materials that have commonly been administered orally as o/w emulsions.

In one embodiment of the present invention, the compositions of dsRNAs and nucleic acids are formulated as microemulsions. A microemulsion may be defined as a system of water, oil and amphiphile which is a single optically isotropic and thermodynamically stable liquid solution (Rosoff, in Pharmaceutical Dosage Forms, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 245). Typically microemulsions are systems that are prepared by first dispersing an oil in an aqueous surfactant solution and then adding a sufficient amount of a fourth component, generally an intermediate chain-length alcohol to form a transparent system. Therefore, microemulsions have also been described as thermodynamically stable, isotropically clear dispersions of two immiscible liquids that are stabilized by interfacial films of surface-active molecules (Leung and Shah, in: Controlled Release of Drugs: Polymers and Aggregate Systems, Rosoff, M., Ed., 1989, VCH Publishers, New York, pages 185-215). Microemulsions commonly are prepared via a combination of three to five components that include oil, water, surfactant, cosurfactant and electrolyte. Whether the microemulsion is of the water-in-oil (w/o) or an oil-in-water (o/w) type is dependent on the properties of the oil and surfactant used and on the structure and geometric packing of the polar heads and hydrocarbon tails of the surfactant molecules (Schott, in Remington's Pharmaceutical Sciences, Mack Publishing Co., Easton, Pa., 1985, p. 271).

The phenomenological approach utilizing phase diagrams has been extensively studied and has yielded a comprehensive knowledge, to one skilled in the art, of how to formulate microemulsions (Rosoff, in Pharmaceutical Dosage Forms, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 245; Block, in Pharmaceutical Dosage Forms, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 335). Compared to conventional emulsions, microemulsions offer the advantage of solubilizing water-insoluble drugs in a formulation of thermodynamically stable droplets that are formed spontaneously.

Surfactants used in the preparation of microemulsions include, but are not limited to, ionic surfactants, non-ionic surfactants, Brij 96, polyoxyethylene oleyl ethers, polyglycerol fatty acid esters, tetraglycerol monolaurate (ML3 10), tetraglycerol monooleate (M03 10), hexaglycerol monooleate (PO310), hexaglycerol pentaoleate (PO500), decaglycerol monocaprate (MCA750), decaglycerol monooleate (MO750), decaglycerol sequioleate (SO750), decaglycerol decaoleate (DAO750), alone or in combination with cosurfactants. The cosurfactant, usually a short-chain alcohol such as ethanol, 1-propanol, and 1-butanol, serves to increase the interfacial fluidity by penetrating into the surfactant film and consequently creating a disordered film because of the void space generated among surfactant molecules. Microemulsions may, however, be prepared without the use of cosurfactants and alcohol-free self-emulsifying microemulsion systems are known in the art. The aqueous phase may typically be, but is not limited to, water, an aqueous solution of the drug, glycerol, PEG300, PEG400, polyglycerols, propylene glycols, and derivatives of ethylene glycol. The oil phase may include, but is not limited to, materials such as Captex 300, Captex 355, Capmul MCM, fatty acid esters, medium chain (C8-C12) mono, di, and tri-glycerides, polyoxyethylated glyceryl fatty acid esters, fatty alcohols, polyglycolized glycerides, saturated polyglycolized C8-C10 glycerides, vegetable oils and silicone oil.

Microemulsions are particularly of interest from the standpoint of drug solubilization and the enhanced absorption of drugs. Lipid based microemulsions (both o/w and w/o) have been proposed to enhance the oral bioavailability of drugs, including peptides (Constantinides *et ah,* Pharmaceutical Research, 1994, 11, 1385-1390; Ritschel, Meth. Find. Exp. Clin. Pharmacol, 1993, 13, 205). Microemulsions afford advantages of improved drug solubilization, protection of drug from enzymatic hydrolysis, possible enhancement of drug absorption due to surfactant-induced alterations in membrane fluidity and permeability, ease of preparation, ease of oral administration over solid dosage forms, improved clinical potency, and decreased toxicity (Constantinides *et al,* Pharmaceutical Research, 1994, 11, 1385; Ho *etal,* J. Pharm. Sci., 1996, 85, 138-143). Often microemulsions may form spontaneously when their components are brought together at ambient temperature. This may be particularly advantageous when formulating thermolabile drugs, peptides or dsRNAs. Microemulsions have also been effective in the transdermal delivery of active components in both cosmetic and pharmaceutical applications. It is expected that the microemulsion compositions and formulations of the present invention will facilitate the increased systemic absorption of dsRNAs and nucleic acids from the gastrointestinal tract, as well as improve the local cellular uptake of dsRNAs and nucleic acids.

Microemulsions of the present invention may also contain additional components and additives such as sorbitan monostearate (Grill 3), Labrasol, and penetration enhancers to improve

the properties of the formulation and to enhance the absorption of the dsRNAs and nucleic acids of the present invention. Penetration enhancers used in the microemulsions of the present invention may be classified as belonging to one of five broad categories—surfactants, fatty acids, bile salts, chelating agents, and non-chelating non-surfactants (Lee *et al,* Critical Reviews in Therapeutic Drug Carrier Systems, 1991, p. 92). Each of these classes has been discussed above.

Penetration Enhancers

In one embodiment, the present invention employs various penetration enhancers to effect the efficient delivery of nucleic acids, particularly dsRNAs, to the skin of animals. Most drugs are present in solution in both ionized and nonionized forms. However, usually only lipid soluble or lipophilic drugs readily cross cell membranes. It has been discovered that even non-lipophilic drugs may cross cell membranes if the membrane to be crossed is treated with a penetration enhancer. In addition to aiding the diffusion of non-lipophilic drugs across cell membranes, penetration enhancers also enhance the permeability of lipophilic drugs.

Penetration enhancers may be classified as belonging to one of five broad categories, *i.e.*, surfactants, fatty acids, bile salts, chelating agents, and non-chelating non-surfactants (Lee *et al*, Critical Reviews in Therapeutic Drug Carrier Systems, 1991, p.92). Each of the above mentioned classes of penetration enhancers are described below in greater detail.

Surfactants: In connection with the present invention, surfactants (or "surface-active agents") are chemical entities which, when dissolved in an aqueous solution, reduce the surface tension of the solution or the interfacial tension between the aqueous solution and another liquid, with the result that absorption of dsRNAs through the mucosa is enhanced. In addition to bile salts and fatty acids, these penetration enhancers include, for example, sodium lauryl sulfate, polyoxyethylene-9-lauryl ether and polyoxyethylene-20-cetyl ether) (Lee *et al,* Critical Reviews in Therapeutic Drug Carrier Systems, 1991, p.92); and perfluorochemical emulsions, such as FC-43. Takahashi *et al*, J. Pharm. Pharmacol, 1988, 40, 252).

Fatty acids: Various fatty acids and their derivatives which act as penetration enhancers include, for example, oleic acid, lauric acid, capric acid (n-decanoic acid), myristic acid, palmitic acid, stearic acid, linoleic acid, linolenic acid, dicaprate, tricaprate, monoolein (1-monooleoyl-rac-glycerol), dilaurin, caprylic acid, arachidonic acid, glycerol 1-monocaprate, 1-dodecylazacycloheptan-2-one, acylcarnitines, acylcholines, C_{1-10} alkyl esters thereof *(e.g.*, methyl, isopropyl and t-butyl), and mono- and di-glycerides thereof (i.e., oleate, laurate, caprate, myristate, palmitate, stearate, linoleate, *etc.)* (Lee *et al,* Critical Reviews in Therapeutic Drug Carrier Systems, 1991, p.92; Muranishi, Critical Reviews in Therapeutic Drug Carrier Systems, 1990, 7, 1-33; El Hariri *et al*, J. Pharm. Pharmacol, 1992, 44, 651-654).

Bile salts: The physiological role of bile includes the facilitation of dispersion and absorption of lipids and fat-soluble vitamins (Brunton, Chapter 38 in: Goodman & Gilman's The Pharmacological Basis of Therapeutics, 9th Ed., Hardman *et al.* Eds., McGraw-Hill, New York, 1996, pp. 934-935). Various natural bile salts, and their synthetic derivatives, act as penetration enhancers. Thus the term "bile salts" includes any of the naturally occurring components of bile as well as any of their synthetic derivatives. Suitable bile salts include, for example, cholic acid (or its pharmaceutically acceptable sodium salt, sodium cholate), dehydrocholic acid (sodium dehydrocholate), deoxycholic acid (sodium deoxycholate), glucholic acid (sodium glucholate), glycholic acid (sodium glycocholate), glycodeoxycholic acid (sodium glycodeoxycholate), taurocholic acid (sodium taurocholate), taurodeoxycholic acid (sodium taurodeoxycholate), chenodeoxycholic acid (sodium chenodeoxycholate), ursodeoxycholic acid (UDCA), sodium tauro-24,25-dihydro-fusidate (STDHF), sodium glycodihydrofusidate and polyoxyethylene-9-lauryl ether (POE) (Lee *et al.,* Critical Reviews in Therapeutic Drug Carrier Systems, 1991, page 92; Swinyard, Chapter 39 In: Remington's Pharmaceutical Sciences, 18th Ed., Gennaro, ed., Mack Publishing Co., Easton, Pa., 1990, pages 782-783; Muranishi, Critical Reviews in Therapeutic Drug Carrier Systems, 1990, 7, 1-33; Yamamoto *etal,* J. Pharm. Exp. Ther., 1992, 263, 25; Yamashita *et al,* J. Pharm. Sci., 1990, 79, 579-583).

Chelating agents: Chelating agents, as used in connection with the present invention, can be defined as compounds that remove metallic ions from solution by forming complexes therewith, with the result that absorption of dsRNAs through the mucosa is enhanced. With regards to their use as penetration enhancers in the present invention, chelating agents have the added advantage of also serving as DNase inhibitors, as most characterized DNA nucleases require a divalent metal ion for catalysis and are thus inhibited by chelating agents (Jarrett, J. Chromatogr., 1993, 618, 315-339). Suitable chelating agents include but are not limited to disodium ethylenediaminetetraacetate (EDTA), citric acid, salicylates *(e.g.,* sodium salicylate, 5-methoxysalicylate and homovanilate), N-acyl derivatives of collagen, laureth-9 and N-amino acyl derivatives of beta-diketones (enamines)(Lee *et al,* Critical Reviews in Therapeutic Drug Carrier Systems, 1991, page 92; Muranishi, Critical Reviews in Therapeutic Drug Carrier Systems, 1990, 7, 1-33; Buur *et al,* J. Control Rel, 1990, 14, 43-51).

Non-chelating non-surfactants: As used herein, non-chelating non-surfactant penetration enhancing compounds can be defined as compounds that demonstrate insignificant activity as chelating agents or as surfactants but that nonetheless enhance absorption of dsRNAs through the alimentary mucosa (Muranishi, Critical Reviews in Therapeutic Drug Carrier Systems, 1990, 7, 1-33). This class of penetration enhancers include, for example, unsaturated cyclic ureas, 1-alkyl- and 1-alkenylazacyclo-alkanone derivatives (Lee *et al,* Critical Reviews in Therapeutic

Drug Carrier Systems, 1991, page 92); and non-steroidal anti-inflammatory agents such as diclofenac sodium, indomethacin and phenylbutazone (Yamashita *et al,* J. Pharm. Pharmacol, 1987, 39, 621-626).

Carriers

Certain compositions of the present invention also incorporate carrier compounds in the formulation. As used herein, "carrier compound" or "carrier" can refer to a nucleic acid, or analog thereof, which is inert *(i.e.,* does not possess biological activity per se) but is recognized as a nucleic acid by *in vivo* processes that reduce the bioavailability of a nucleic acid having biological activity by, for example, degrading the biologically active nucleic acid or promoting its removal from circulation. The coadministration of a nucleic acid and a carrier compound, typically with an excess of the latter substance, can result in a substantial reduction of the amount of nucleic acid recovered in the liver, kidney or other extracirculatory reservoirs, presumably due to competition between the carrier compound and the nucleic acid for a common receptor. For example, the recovery of a partially phosphorothioate dsRNA in hepatic tissue can be reduced when it is coadministered with polyinosinic acid, dextran sulfate, polycytidic acid or 4-acetamido-4'isothiocyano-stilbene-2,2'-disulfonic acid (Miyao *et al,* DsRNA Res. Dev., 1995, 5, 115-121; Takakura *et* fl/., DsRNA & Nucl. Acid Drug Dev., 1996, 6, 177-183.

Excipients

In contrast to a carrier compound, a "pharmaceutical carrier" or "excipient" is a pharmaceutically acceptable solvent, suspending agent or any other pharmacologically inert vehicle for delivering one or more nucleic acids to an animal. The excipient may be liquid or solid and is selected, with the planned manner of administration in mind, so as to provide for the desired bulk, consistency, *etc.,* when combined with a nucleic acid and the other components of a given pharmaceutical composition. Typical pharmaceutical carriers include, but are not limited to, binding agents *(e.g.,* pregelatinized maize starch, polyvinylpyrrolidone or hydroxypropyl methylcellulose, *etc.);* fillers *(e.g.,* lactose and other sugars, microcrystalline cellulose, pectin, gelatin, calcium sulfate, ethyl cellulose, polyacrylates or calcium hydrogen phosphate, *etc.);* lubricants *(e.g.,* magnesium stearate, talc, silica, colloidal silicon dioxide, stearic acid, metallic stearates, hydrogenated vegetable oils, corn starch, polyethylene glycols, sodium benzoate, sodium acetate, *etc.);* disintegrants *(e.g.,* starch, sodium starch glycolate, *etc.);* and wetting agents *(e.g.,* sodium lauryl sulphate, *etc).*

Pharmaceutically acceptable organic or inorganic excipients suitable for non-parenteral administration which do not deleteriously react with nucleic acids can also be used to formulate the compositions of the present invention. Suitable pharmaceutically acceptable carriers include,

but are not limited to, water, salt solutions, alcohols, polyethylene glycols, gelatin, lactose, amylose, magnesium stearate, talc, silicic acid, viscous paraffin, hydroxymethylcellulose, polyvinylpyrrolidone and the like.

Formulations for topical administration of nucleic acids may include sterile and non-sterile aqueous solutions, non-aqueous solutions in common solvents such as alcohols, or solutions of the nucleic acids in liquid or solid oil bases. The solutions may also contain buffers, diluents and other suitable additives. Pharmaceutically acceptable organic or inorganic excipients suitable for non-parenteral administration which do not deleteriously react with nucleic acids can be used.

Suitable pharmaceutically acceptable excipients include, but are not limited to, water, salt solutions, alcohol, polyethylene glycols, gelatin, lactose, amylose, magnesium stearate, talc, silicic acid, viscous paraffin, hydroxymethylcellulose, polyvinylpyrrolidone and the like.

Other Components

The compositions of the present invention may additionally contain other adjunct components conventionally found in pharmaceutical compositions, at their art-established usage levels. Thus, for example, the compositions may contain additional, compatible, pharmaceutically-active materials such as, for example, antipruritics, astringents, local anesthetics or anti-inflammatory agents, or may contain additional materials useful in physically formulating various dosage forms of the compositions of the present invention, such as dyes, flavoring agents, preservatives, antioxidants, opacifiers, thickening agents and stabilizers. However, such materials, when added, should not unduly interfere with the biological activities of the components of the compositions of the present invention. The formulations can be sterilized and, if desired, mixed with auxiliary agents, *e.g.,* lubricants, preservatives, stabilizers, wetting agents, emulsifiers, salts for influencing osmotic pressure, buffers, colorings, flavorings and/or aromatic substances and the like which do not deleteriously interact with the nucleic acid(s) of the formulation.

Aqueous suspensions may contain substances which increase the viscosity of the suspension including, for example, sodium carboxymethylcellulose, sorbitol and/or dextran. The suspension may also contain stabilizers.

In some embodiments, pharmaceutical compositions featured in the invention include (a) one or more dsRNA compounds and (b) one or more anti-cytokine biologic agents which function by a non-RNAi mechanism. Examples of such biologies include, biologies that target IL 1β *(e.g.* , anakinra), IL6 (tocilizumab), or TNF (etanercept, infliximab, adlimumab, or certolizumab).

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 high therapeutic indices are preferred.

The data obtained from cell culture assays and animal studies can be used in formulating a range of dosage for use in humans. The dosage of compositions featured in the invention lies generally 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 methods featured in 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 of the compound or, when appropriate, of the polypeptide product of a target sequence *(e.g.,* achieving a decreased concentration of the polypeptide) that includes the IC50 *(i.e.,* the concentration of the test compound which achieves a half-maximal inhibition of symptoms) 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.

In addition to their administration, as discussed above, the dsRNAs featured in the invention can be administered in combination with other known agents effective in treatment of pathological processes mediated by SNCA expression. In any event, the administering physician can adjust the amount and timing of dsRNA administration on the basis of results observed using standard measures of efficacy known in the art or described herein.

Evaluation of candidate dsRNA agents

A candidate dsRNA agent can be evaluated for its ability to down-regulate SNCA gene expression. For example, a candidate dsRNA agent can be provided, and contacted with a cell that expresses the SNCA gene. The level of SNCA gene expression prior to and following contact with the candidate dsRNA agent can then be compared. The SNCA target gene can be an endogenous or exogenous gene within the cell. If it is determined that the amount of RNA or protein expressed from the SNCA gene is lower following contact with the dsRNA agent, then it can be concluded that the dsRNA agent downregulates SNCA gene expression. The level of SNCA RNA or protein in the cell can be determined by any method desired. For example, the level of SNCA RNA can be determined by Northern blot analysis, reverse transcription coupled

with polymerase chain reaction (RT-PCR), or RNAse protection assay. The level of protein can be determined by, for example, Western blot analysis.

The dsRNA agent can be tested in an *in vitro* or/and in an *in vivo* system. For example, the target gene or a fragment thereof can be fused to a reporter gene on a plasmid. The plasmid can be transfected into a cell with a candidate dsRNA agent. The efficacy of the dsRNA agent can be evaluated by monitoring expression of the reporter gene. The reporter gene can be monitored *in vivo,* such as by fluorescence or *in situ* hybridization. Exemplary fluorescent reporter genes include but are not limited to green fluorescent protein and luciferase. Expression of the reporter gene can also be monitored by Northern blot, RT-PCR, RNAse-protection assay, or Western blot analysis as described above.

Efficacy of a dsRNA agent can be tested in a mammalian cell line *(e.g.,* a mammalian neural cell line), such as a human neuroblastoma cell line. For example, cell lines useful for testing efficacy of a dsRNA agent are those with a neuronal phenotype (neuroblastomas, neuronally differentiated phaeochromocytomas and primary neuronal cultures) or non-neuronal cell lines *(e.g.,* kidney, muscle or ovarian cells). Neuroblastoma cell lines include BE(2)-M17, SH-SY5Y (both human) and N2a (mouse). BE(2)-M17 cells biochemically mimic dopaminergic neurons of the human brain affected by alpha-synucleinopathies.

Controls include:

(1) testing the efficacy and specificity of a dsRNA by assaying for a decrease in expression of the target gene by, for example, comparison to expression of an endogenous or exogenous off-target RNA or protein; and

(2) testing specificity of the effect on target gene expression by administering a "nonfunctional" dsRNA agent.

Nonfunctional control dsRNA agents can:

(a) target a gene not expressed in the cell;

(b) be of nonsensical sequence *(e.g.,* a scrambled version of the test dsRNA); or

(c) have a sequence complementary to the target gene, but be known by previous experiments to lack an ability to silence gene expression.

Assays include time course experiments to monitor stability and duration of silencing effect by a dsRNA agent and monitoring in dividing versus nondividing cells. Presumably in dividing cells, the dsRNA is diluted out over time, thus decreasing the duration of the silencing effect. The implication is that dosage will have to be adjusted *in vivo,* and/or a dsRNA agent will have to be administered more frequently to maintain the silencing effect. To monitor nondividing

cells, cells can be arrested by serum withdrawal. Neurons are post-mitotic cells, and thus neural cells are aptly suited for assaying the stability of dsRNA agents, such as an anti-SNCA dsRNA agent, for use in therapeutic compositions for the treatment of disorders of the nervous system, *e.g.*, neurodegenerative disorders.

A candidate dsRNA agent can also be evaluated for cross-species reactivity. For example, cell lines derived from different species *(e.g.*, mouse vs. human) or in biological samples *(e.g.*, serum or tissue extracts) isolated from different species can be transfected with a target dsRNA agent and a candidate dsRNA agent. The efficacy of the dsRNA agent can be determined for the cell from the different species.

In another aspect, the invention features a method of evaluating an agent, *e.g.*, an agent of a type described herein, such as a dsRNA agent having an antisense strand shown in Tables 2, 3,or 4, and a sense strand shown in Tables 2, 3,or 4, dsRNA for the ability to inhibit SNCA expression, *e.g.*, an agent that targets an SNCA or SNCA nucleic acid. The method includes: providing a candidate agent and determining, *e.g.*, by the use of one or more of the test systems described herein, if said candidate agent modulates, *e.g.*, inhibits, SNCA expression.

In one embodiment the method includes evaluating the agent in a first test system; and, if a predetermined level of modulation is seen, evaluating the candidate in a second, *e.g.*, a different, test system. In one embodiment the second test system includes administering the candidate agent to an animal and evaluating the effect of the candidate agent on SNCA expression in the animal. In certain embodiments, two test systems are used and the first is a high-throughput system. For example, in such embodiments the first or initial test is used to screen at least 100, 1,000, or 10,000 times more agents than is the second test, *e.g.*, an animal system.

A test system can include: contacting the candidate agent with a target molecule, *e.g.*, an SNCA nucleic acid, *e.g.*, an RNA, such as *in vitro*, and determining if there is an interaction, *e.g.*, binding of the candidate agent to the target, or modifying the target, *e.g.*, by making or breaking a covalent bond in the target. Modification is correlated with the ability to modulate SNCA expression. The test system can include contacting the candidate agent with a cell and evaluating modulation of SNCA expression. For example, this can include contacting the candidate agent with a cell capable of expressing SNCA or SNCA RNA (from an endogenous gene or from an exogenous construct) and evaluating the level of SNCA or SNCA RNA. In another embodiment, the test system can include contacting the candidate agent with a cell that expresses an RNA or protein from an SNCA control region *(e.g.*, an SNCA control region) linked to a heterologous sequence, *e.g.*, a marker protein, *e.g.*, a fluorescent protein such as GFP,

which construct can be either chromosomal or episomal, and determining the effect on RNA or protein levels. The test system can also include contacting the candidate agent, *in vitro,* with a tissue sample, *e.g.,* a brain tissue sample, *e.g.,* a slice or section, an optical tissue sample, or other sample which includes neural tissue, and evaluating the level of SNCA or SNCA RNA. The test system can include administering the candidate agent, *in vivo,* to an animal, and evaluating the level of SNCA or SNCA RNA. In any of these the effect of the candidate agent on SNCA expression can include comparing SNCA gene expression with a predetermined standard, such as a control, *e.g.,* an untreated cell, tissue or animal. SNCA gene expression can be compared, *e.g.,* before and after contacting with the candidate agent. The method allows determining whether the dsRNA is useful for inhibiting SNCA gene expression.

In one embodiment, SNCA gene expression can be evaluated by a method to examine SNCA RNA levels *(e.g.,* Northern blot analysis, RT-PCR, or RNAse protection assay) or SNCA protein levels *(e.g.,* Western blot).

In one embodiment, a second test is performed by administering the agent to an animal, *e.g.,* a mammal, such as a mouse, rat, rabbit, human, or non-human primate, and the animal is monitored for an effect of the agent. For example, a tissue of the animal, such as, a brain tissue or ocular tissue, is examined for an effect of the agent on SNCA expression. The tissue can be examined for the presence of SNCA RNA and/or protein, for example. In one embodiment, the animal is observed to monitor an improvement or stabilization of a cognitive symptom. The agent can be administered to the animal by any method, *e.g.,* orally, or by intrathecal or parenchymal injection, such as by stereoscopic injection into the brain. In some embodiments, the agent is administered to the substantia nigra, hippocampus or cortex of the brain.

In one embodiment, the invention features a method of evaluating a dsRNA, *e.g.,* a dsRNA described herein, that targets an SNCA nucleic acid. The method includes providing a dsRNA that targets an SNCA nucleic acid *(e.g.,* an SNCA RNA); contacting the dsRNA with a cell containing, and capable of expressing, an SNCA gene; and evaluating the effect of the dsRNA on SNCA expression, *e.g.,* by comparing SNCA gene expression with a control, *e.g.,* in the cell. SNCA gene expression can be compared, *e.g.,* before and after contacting the dsRNA with the cell. The method allows determining whether the dsRNA is useful for inhibiting SNCA gene expression. For example, the dsRNA can be determined to be useful for inhibiting SNCA gene expression if the dsRNA reduces expression by a predetermined amount, *e.g.,* by 10, 25, 50, 75, or 90%, *e.g.,* as compared with a predetermined reference value, *e.g.,* as compared with the amount of SNCA RNA or protein prior to contacting the dsRNA with the cell. The SNCA gene can be endogenously or exogenously expressed.

In vivo Testing

A dsRNA agent identified as being capable of inhibiting SNCA gene expression can be tested for functionality *in vivo* in an animal model *(e.g.,* in a mammal, such as in mouse or rat). For example, the dsRNA agent can be administered to an animal, and the dsRNA agent evaluated with respect to its biodistribution, stability, and its ability to inhibit SNCA gene expression.

The dsRNA agent can be administered directly to the target tissue, such as by injection, or the dsRNA agent can be administered to the animal model in the same manner that it would be administered to a human. For example, the dsRNA agent can be injected directly into a target region of the brain *(e.g.,* into the cortex, the substantia nigra, the globus pallidus, or the hippocampus), and after a period of time, the brain can be harvested and tissue slices examined for distribution of the agent.

The dsRNA agent can also be evaluated for its intracellular distribution. The evaluation can include determining whether the dsRNA agent was taken up into the cell. The evaluation can also include determining the stability *(e.g.,* the half-life) of the dsRNA agent. Evaluation of a dsRNA agent *in vivo* can be facilitated by use of a dsRNA agent conjugated to a traceable marker (e.g., a fluorescent marker such as fluorescein; a radioactive label, such as ^{32}P, ^{33}P, or ^{3}H; gold particles; or antigen particles for immunohistochemistry).

A dsRNA agent useful for monitoring biodistribution can lack gene silencing activity *in vivo.* For example, the dsRNA agent can target a gene not present in the animal *(e.g.,* a dsRNA agent injected into mouse can target luciferase), or a dsRNA agent can have a non-sense sequence, which does not target any gene, *e.g.,* any endogenous gene). Localization/biodistribution of the dsRNA can be monitored by a traceable label attached to the dsRNA agent, such as a traceable agent described above.

The dsRNA agent can be evaluated with respect to its ability to down regulate SNCA expression. Levels of SNCA expression *in vivo* can be measured, for example, by *in situ* hybridization, or by the isolation of RNA from tissue prior to and following exposure to the dsRNA agent. SNCA RNA can be detected by any desired method, including but not limited to RT-PCR, Northern blot, or RNAase protection assay. Alternatively, or additionally, SNCA gene expression can be monitored by performing Western blot analysis on tissue extracts treated with the anti-SNCA dsRNA agent.

An anti-SNCA dsRNA agent can be tested in a mouse model for Parkinson's disease, such as a mouse carrying a wildtype copy of the human SNCA gene (Masliah et al, Science 287: 1265-1269, 2000) or in mouse carrying a mutant human SNCA (Richfield et al, Exp. Neurol.

175: 35-48, 2002; Giasson et al, Neuron 34: 521-533, 2002; Lee et al, Proc Natl Acad. Sci. 99: 8968-8973, 2002). The mutant mouse can carry a human SNCA gene that expresses an A53T, A3OP, or E46K mutation. A treated mouse model can be observed for a decrease in symptoms associated with Parkinson's disease.

Methods for treating diseases caused by expression of a SNCA gene

The invention relates in particular to the use of a dsRNA targeting SNCA and compositions containing at least one such dsRNA for the treatment of a SNCA-mediated disorder or disease. For example, a dsRNA targeting a SNCA gene can be useful for the treatment of neurodegenerative diseases, such as a synucleinopathy *(e.g.,* Parkinson's disease), that have either an activating mutation of SNCA and/or are the result of overexpression of SNCA. The method includes administering a pharmaceutical composition featured in the invention to the patient *(e.g.,* human), such that expression of the SNCA gene is silenced. Because of their high specificity, the dsRNAs described herein specifically target mRNAs of the SNCA gene.

As used herein, the term "SNCA-mediated condition or disease" and related terms and phrases refer to a condition or disorder characterized by unwanted or inappropriate, *e.g.,* abnormal SNCA activity. Inappropriate SNCA functional activity might arise as the result of SNCA expression in cells which normally do not express SNCA, increased SNCA expression and/or activity (leading to, *e.g.,* neurodegenerative disease, or increased susceptibility to disease). A SNCA-mediated condition or disease may be completely or partially mediated by inappropriate SNCA functional activity which may result by way of inappropriate activation of SNCA. Regardless, a SNCA-mediated condition or disease is one in which modulation of SNCA via RNA interference results in some effect on the underlying condition or disorder *(e.g.,* a SNCA inhibitor results in some improvement in patient well-being in at least some patients).

The dsRNA molecules featured herein may, therefore, be used to treat neurodegenerative diseases, such as Parkinson's disease, Alzheimer's disease, multiple system atrophy, and Lewy body dementia. The dsRNA molecules featured herein are also useful for the treatment of a retinal disorder, *e.g.,* a retinopathy.

In one aspect of the invention, methods are provided for treating a disorder mediated by SNCA expression by administering to a human in need of such treatment a therapeutically effective amount of any of the dsRNA of the invention. Also included is a method of treating, preventing or managing a neurodegenerative disorder in which a patient in need of such treatment, prevention or management is administered a therapeutically or prophylactically

effective amount of a dsRNA of the invention. The method of treatment can include administering an additional composition, *e.g.,* a second dsRNA.

It has been discovered that a single administration can provide prolonged silencing. Thus, in another embodiment, a dose of SNCA dsRNA is administered to a patient and the dose is sufficient to downregulate SNCA mRNA or protein levels to a state that is less than or equal to 20% of pretreatment levels (or levels that would be seen in the absence of treatment) for at least 5, 10, or 15 days post-treatment; less than or equal to 40% of pretreatment levels (or levels that would be seen in the absence of treatment) for at least 5, 10, or 15 days post-treatment; less than or equal to 60% of pretreatment levels (or levels that would be seen in the absence of treatment) for at least 5, 10, 15, or 20 days post-treatment; or less than or equal to 80% of pretreatment levels (or levels that would be seen in the absence of treatment) for at least 5, 10, 15, 20, or 25 days post-treatment.

In one embodiment, a first dose of SNCA dsRNA is administered, and no subsequent dose of SNCA dsRNA is administered for at least 5, 10, 15, 20 or 30 days after the first dose. In another embodiment, a subsequent dose is administered but not until at least 5, 10, 15, 20, or 30 days have elapsed since the first dose.

In another embodiment, a patient continues to receive at least one other therapeutic treatment for the synucleinopathy while receiving treatment with SNCA dsRNA. For example, a patient with Parkinson's disease can continue to receive administration of agent for alleviating symptoms, a neuroprotective agent *(e.g.,* for slowing or halting disease progression), or a restorative agent *(e.g.,* for reversing the disease process). Symptomatic therapies include the drugs carbidopa/levodopa, entacapone, tolcapone, pramipexole, ropinerole, pergolide, bromocriptine, selegeline, amantadine, and several anticholingergic agents. Deep brain stimulation surgery as well as stereotactic brain lesioning may also provide symptomatic relief. Neuroprotective therapies include, for example, carbidopa/levodopa, selegeline, vitamin E, amantadine, pramipexole, ropinerole, coenzyme Q10, and GDNF. Restorative therapies can include, for example, surgical transplantation of stem cells.

In another aspect, the invention features a method of providing instructions, *e.g.,* to a healthcare provider or a patient on the administration of SNCA dsRNA. The method includes: providing instructions to administer to the patient a dose of SNCA dsRNA in a treatment regimen described herein, *e.g.,* a dose followed by at least 2 1 days within a subsequent dose of SNCA dsRNA.

In another aspect, the invention features a method of selecting or treating a patient in need of SNCA dsRNA to treat a disorder described herein. The method includes selecting a

patient on the basis of the patient being in need of decreased SNCA RNA for at least 5, 10, 15, 20 or 30 days, and optionally administering the drug to the patient.

In another aspect, the invention features a method of reducing the amount of SNCA or SNCA RNA in a cell of a subject *(e.g.,* a mammalian subject, such as a human). The method includes contacting the cell with an agent that inhibits the expression of SNCA, *e.g.,* a dsRNA described herein, *e.g.,* in Tables 2, 3,or 4. The inhibition can be effected at any level, *e.g.,* at the level of transcription, the level of translation, or post-translationally.

The methods and compositions featured in the invention, *e.g.,* the methods and dsRNA compositions to treat the neurodegenerative disorders described herein, can be used with any dosage and/or formulation described herein, as well as with any route of administration described herein.

Treatment of Parkinson's disease

Any patient having Parkinson's disease (or any other alpha-synuclein related disorder), is a candidate for treatment with a method or composition described herein. Typically, the patient is not terminally ill *(e.g.,* the patient has life expectancy of two years or more), and has not reached end-stage Parkinson's disease *(i.e.,* Hoehn and Yahr stage 5).

Presymptomatic subjects can also be candidates for treatment with an anti-SNCA agent, *e.g.,* an anti-SNCA dsRNA described herein, *e.g.,* in Tables 2, 3,or 4. In one embodiment, a presymptomatic candidate is identified by either or both of risk-factor profiling and functional neuroimaging *(e.g.,* by fluorodopa and positron emission tomography). For example, the candidate can be identified by risk-factor profiling followed by functional neuroimaging.

Individuals having any genotype are candidates for treatment. In some embodiments the patient will carry a particular genetic mutation that places the patient at increased risk for developing Parkinson's disease. For example, an individual carrying an SNCA gene multiplication, *e.g.,* an SNCA gene duplication or triplication is at increased risk for developing Parkinson's disease and is a candidate for treatment with the dsRNA. In addition, a gain-of-function mutation in SNCA can increase an individual's risk for developing Parkinson's disease. An individual carrying an SNCA REP1 genotype *(e.g.,* a REP1 "+1 allele" heterozygous or homozygous genotype) can be a candidate for such treatment. An individual homozygous for the REPl+1 allele overexpresses SNCA. An individual carrying a mutation in the UCHL-1, parkin, or SNCA gene is at increased risk for Parkinson's disease and can be a candidate for treatment with an anti-SNCA dsRNA. Particularly, a mutation in the UCHL-1 or parkin gene will cause a decrease in gene or protein activity. An individual carrying a Tau genotype *(e.g.* , a mutation in the Tau gene) or a Tau haplotype, such as the HI haplotype is also at risk for developing

Parkinson's disease. Other genetic risk factors include mutations in the MAPT, DJl, PTNKl, and NURR1 genes, and polymorphism in several genes including the SNCA, parkin, MAPT, and NAT2 genes.

Non-genetic *(e.g.,* environmental) risk factors for Parkinson's disease include age *(e.g.,* over age 30, 35, 40, 45, or 50 years), gender (men are generally have a higher risk than women), pesticide exposure, heavy metal exposure, and head trauma. In general, exogenous and endogenous factors that disrupt the ubiquitin proteasomal pathway or more specifically inhibit the proteasome, or which disrupt mitochondrial function, or which yield oxidative stress, or which promote the aggregation and fibrillization of alpha-synuclein, can increase the risk of an individual for developing Parkinson's disease, and can contribute to the pathogenesis of Parkinson's disease.

In one embodiment, a dsRNA can be used to target wildtype SNCA in subjects with Parkinson's disease.

Treatment of other SNCA-mediated disorders

Any disease characterized by a synucleinopathy can be treated with an inhibitory agent described herein *(e.g.,* an agent that targets SNCA), including Lewy body dementia, Multiple System Atrophy, and Alzheimer's disease. Individuals having any genotype are candidates for treatment. In some embodiments, the patient will carry a particular genetic mutation that places them at increased risk for developing a synucleinopathy.

In one embodiment, a dsRNA, *e.g.,* a dsRNA described in herein, *e.g.,* in Tables 2, 3,or 4 can be used to target wildtype SNCA in subjects with a neurodegenerative disorder.

An individual can develop a synucleinopathy as a result of certain environmental factors. For example, oxidative stress, certain pesticides *(e.g.,* 24D and agent orange), bacterial infection, and head trauma have been linked to an increase in the risk of developing Parkinson's disease, and can be determining factors for determining the risk of an individual for synucleinopathies. These factors (and others disclosed herein) can be considered when evaluating the risk profile of a candidate subject for anti-SNCA therapy.

In addition to their presence in the brain, alpha-synuclein polypeptides have been found in ocular tissues, including the retina and optic nerve. Accordingly, the compositions and methods described herein are suitable for treating synucleinopathies of the eye or ocular tissues, including but not limited to retinopathies.

Thus, in another aspect, the invention features a method of treating a subject by administering an agent which inhibits the expression of SNCA in the eye or in ocular tissue, *e.g.,* a dsRNA described herein, *e.g.,* in Tables 2, 3,or 4. In one embodiment, the subject is a mammal,

such as a human, *e.g.,* a subject diagnosed as having, or at risk for developing a synucleinopathy of the eye, *e.g.,* a retinopathy. The inhibition can be effected at any level, *e.g.,* at the level of transcription, the level of translation, or post-translationally.

Owing to the inhibitory effects on SNCA expression, a composition according to the invention or a pharmaceutical composition prepared therefrom can enhance the quality of life.

The invention further relates to the use of a dsRNA or a pharmaceutical composition thereof, *e.g.,* for treating a SNCA mediated disorder or disease, in combination with other pharmaceuticals and/or other therapeutic methods, *e.g.,* with known pharmaceuticals and/or known therapeutic methods, such as, for example, those which are currently employed for treating these disorders. In certain examples, a dsRNA targeting SNCA can be administered in combination with an agent for alleviating symptoms, a neuroprotective agent *(e.g.,* for slowing or halting disease progression), or a restorative agent *(e.g.,* for reversing the disease process). Symptomatic therapies include the drugs carbidopa/levodopa, entacapone, tolcapone, pramipexole, ropinerole, pergolide, bromocriptine, selegeline, amantadine, and several anticholingergic agents. Deep brain stimulation surgery as well as stereotactic brain lesioning may also provide symptomatic relief. Neuroprotective therapies include, for example, carbidopa/levodopa, selegeline, vitamin E, amantadine, pramipexole, ropinerole, coenzyme Q10, and GDNF. Restorative therapies can include, for example, surgical transplantation of stem cells.

The dsRNA and an additional therapeutic agent can be administered in the same combination, *e.g.,* parenterally, or the additional therapeutic agent can be administered as part of a separate composition or by another method described herein.

The invention features a method of administering a dsRNA targeting SNCA to a patient having a disease or disorder mediated by SNCA expression, such as a neurodegenerative disease, *e.g.,* Parkinson's disease. Patients can be administered a therapeutic amount of dsRNA, such as 0.5 mg/kg, 1.0 mg/kg, 1.5 mg/kg, 2.0 mg/kg, or 2.5 mg/kg dsRNA. The dsRNA can be administered by intravenous infusion over a period of time, such as over a 5 minute, 10 minute, 15 minute, 20 minute, or 25 minute period. The administration is repeated, for example, on a regular basis, such as biweekly *(i.e.,* every two weeks) for one month, two months, three months, four months or longer. After an initial treatment regimen, the treatments can be administered on a less frequent basis. For example, after administration biweekly for three months, administration can be repeated once per month, for six months or a year or longer. Administration of the dsRNA can reduce SNCA levels in the blood or urine of the patient by at least 20%, 25%, 30%, 40%, 50%, 60%, 70%, 80 % or 90% or more.

Before administration of a full dose of the dsRNA, patients can be administered a smaller dose, such as a 5% infusion reaction, and monitored for adverse effects, such as an allergic reaction.

Many SNCA-associated diseases and disorders are hereditary. Therefore, a patient in need of a SNCA dsRNA can be identified by taking a family history. A healthcare provider, such as a doctor, nurse, or family member, can take a family history before prescribing or administering a SNCA dsRNA. A DNA test may also be performed on the patient to identify a mutation in the SNCA gene, before a SNCA dsRNA is administered to the patient.

Methods for inhibiting expression of a SNCA gene

In yet another aspect, the invention provides a method for inhibiting the expression of a SNCA gene in a mammal. The method includes administering a composition featured in the invention to the mammal such that expression of the target SNCA gene is reduced or silenced. Because of their high specificity, the dsRNAs featured in the invention specifically target RNAs (primary or processed) of the target SNCA gene. Compositions and methods for inhibiting the

A dsRNA of the invention, upon contact with a cell expressing said SNCA, inhibits expression of said SNCA gene by at least 40% compared to a cell not so contacted. In some embodiments, a dsRNA of the invention has a pM IC50, *e.g.,* an IC50 of 10 pM or less.

Another aspect of the invention is method of inhibiting SNCA expression in a cell, the method including introducing into the cell any of the dsRNA of the invention and maintaining the cell for a time sufficient to obtain degradation of the mRNA transcript of a SNCA gene, thereby inhibiting expression of the SNCA gene in the cell. In some embodiments, expression is inhibited by at least 20%, 40%, 60%, or at least 80%.

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 dsRNAs and methods featured in the 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 examples are illustrative only and not intended to be limiting.

EXAMPLES

Below are examples of specific embodiments for carrying out the present invention. The examples are offered for illustrative purposes only, and are not intended to limit the scope of the

present invention in any way. Efforts have been made to ensure accuracy with respect to numbers used *(e.g.,* amounts, temperatures, etc.), but some experimental error and deviation should, of course, be allowed for.

Other embodiments are, for example, in the claims.

The practice of the present invention will employ, unless otherwise indicated, conventional methods of protein chemistry, biochemistry, recombinant DNA techniques and pharmacology, within the skill of the art. Such techniques are explained fully in the literature. See, *e.g.,* T.E. Creighton, Proteins: Structures and Molecular Properties (W.H. Freeman and Company, 1993); A.L. Lehninger, Biochemistry (Worth Publishers, Inc., current addition); Sambrook, *et ah,* Molecular Cloning: A Laboratory Manual (2nd Edition, 1989); Methods In Enzymology (S. Colowick and N. Kaplan eds., Academic Press, Inc.); Remington's Pharmaceutical Sciences, 18th Edition (Easton, Pennsylvania: Mack Publishing Company, 1990); Carey and Sundberg Advanced Organic Chemistry 3rd Ed. (Plenum Press) Vols A and B(1992).

Example 1. *dsRNA synthesis*

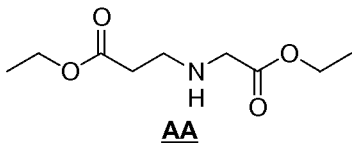
Source of reagents

Where the source of a reagent is not specifically given herein, such reagent may be obtained from any supplier of reagents for molecular biology at a quality/purity standard for application in molecular biology.

Conjugates

For the synthesis of 3'-cholesterol-conjugated siRNAs (herein referred to as -Chol-3'), an appropriately modified solid support is used for RNA synthesis. The modified solid support is prepared as follows:

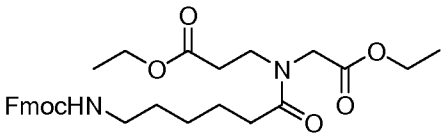
Diethyl-2-azabutane- 1,4-dicarboxylate AA



AA

A 4.7 M aqueous solution of sodium hydroxide (50 mL) is added into a stirred, ice-cooled solution of ethyl glycinate hydrochloride (32.19 g, 0.23 mole) in water (50 mL). Then, ethyl acrylate (23. 1 g, 0.23 mole) isis added and the mixture is stirred at room temperature until completion of the reaction is ascertained by TLC. After 19 h the solution is partitioned with dichloromethane (3 x 100 mL). The organic layer is dried with anhydrous sodium sulfate, filtered and evaporated. The residue is distilled to afford AA (28.8 g, 61%).

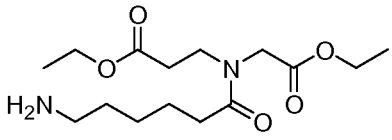
3-{Ethoxycarbonylmethyl-[6-(9H-fluoren-9-ylmethoxycarbonyl-amino)-hexanoyl]-amino}-propionic acid ethyl ester **AB**



AB

Fmoc-6-amino-hexanoic acid (9.12 g, 25.83 mmol) is dissolved in dichloromethane (50 mL) and cooled with ice. Diisopropylcarbodiimde (3.25 g, 3.99 mL, 25.83 mmol) is added to the solution at 0°C. It is then followed by the addition of diethyl-azabutane-l,4-dicarboxylate (5 g, 24.6 mmol) and dimethylamino pyridine (0.305 g, 2.5 mmol). The solution is brought to room temperature and stirred further for 6 h. Completion of the reaction is ascertained by TLC. The reaction mixture is concentrated under vacuum and ethyl acetate is added to precipitate diisopropyl urea. The suspension is filtered. The filtrate is washed with 5% aqueous hydrochloric acid, 5% sodium bicarbonate and water. The combined organic layer is dried over sodium sulfate and concentrated to give the crude product which is purified by column chromatography (50 % EtOAC/hexanes) to yield 11.87 g (88%) of AB.

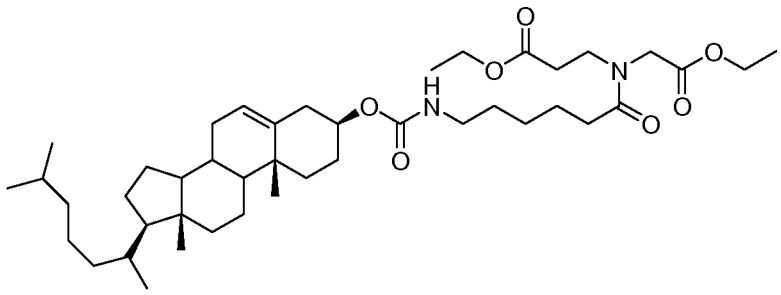
3-[(6-Amino-hexanoyl)-ethoxycarbonylmethyl-amino]-propionic acid ethyl ester **AC**



AC

3-{Ethoxycarbonylmethyl-[6-(9H-fluoren-9-ylmethoxycarbonylamino)-hexanoyl]-amino}-propionic acid ethyl ester AB (11.5 g, 21.3 mmol) is dissolved in 20% piperidine in dimethylformamide at 0°C. The solution is continued stirring for 1 h. The reaction mixture is concentrated under vacuum, water is added to the residue, and the product is extracted with ethyl acetate. The crude product is purified by conversion into its hydrochloride salt.

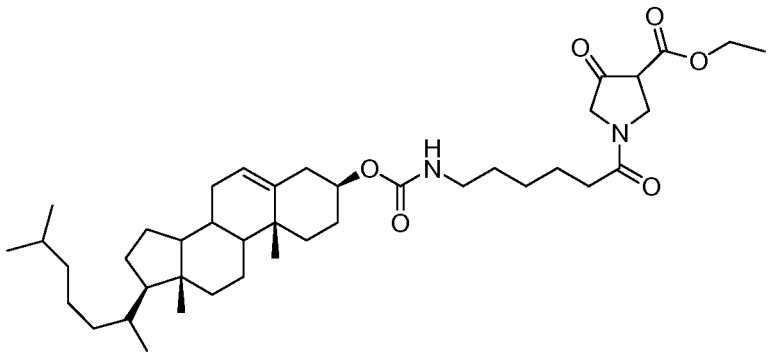
3-({6-[17-(l,5-Dimethyl-hexyl)-10,13-dimethyl-2,3,4,7,8,9,10,l 1,12,13, 14,15,16,17-tetradecahydro-lH-cyclopenta[a]phenanthren-3-yloxycarbonylamino]-hexanoyl} ethoxycarbonylmethyl-amino)-propionic acid ethyl ester **AD**



AD

The hydrochloride salt of 3-[(6-Amino-hexanoyl)-ethoxycarbonylmethyl-amino]-propionic acid ethyl ester AC (4.7 g, 14.8 mmol) is taken up in dichloromethane. The suspension is cooled to 0°C on ice. To the suspension diisopropylethylamine (3.87 g, 5.2 mL, 30 mmol) is added. To the resulting solution cholesteryl chloroformate (6.675 g, 14.8 mmol) is added. The reaction mixture is stirred overnight. The reaction mixture is diluted with dichloromethane and washed with 10% hydrochloric acid. The product is purified by flash chromatography (10.3 g, 92%).

l-{6-[17-(l,5-Dimethyl-hexyl)-10,13-dimethyl-2,3,4,7,8,9,10,ll, 12,13, 14,15,16,17-tetradecahydro- 1H-cyclopenta[a] phenanthren-3 -yloxycarbonylamino]-hexanoyl} -4-oxo-pyrrolidine-3-carboxylic acid ethyl ester AE

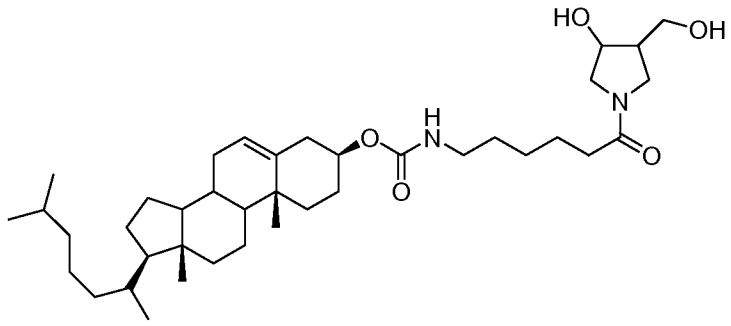


AE

Potassium t-butoxide (1.1 g, 9.8 mmol) is slurried in 30 mL of dry toluene. The mixture is cooled to 0°C on ice and 5 g (6.6 mmol) of diester AD is added slowly with stirring within 20 mins. The temperature is kept below 5°C during the addition. The stirring is continued for 30 mins at 0°C and 1 mL of glacial acetic acid is added, immediately followed by 4 g of NaH_2P0_4-H_20 in 40 mL of water The resultant mixture is extracted twice with 100 mL of dichloromethane each and the combined organic extracts are washed twice with 10 mL of phosphate buffer each, dried, and evaporated to dryness. The residue is dissolved in 60 mL of toluene, cooled to 0°C and extracted with three 50 mL portions of cold pH 9.5 carbonate buffer. The aqueous extracts are adjusted to pH 3 with phosphoric acid, and extracted with five 40 mL

portions of chloroform which are combined, dried and evaporated to dryness. The residue is purified by column chromatography using 25% ethylacetate/hexane to afford 1.9 g of b-ketoester (39%).

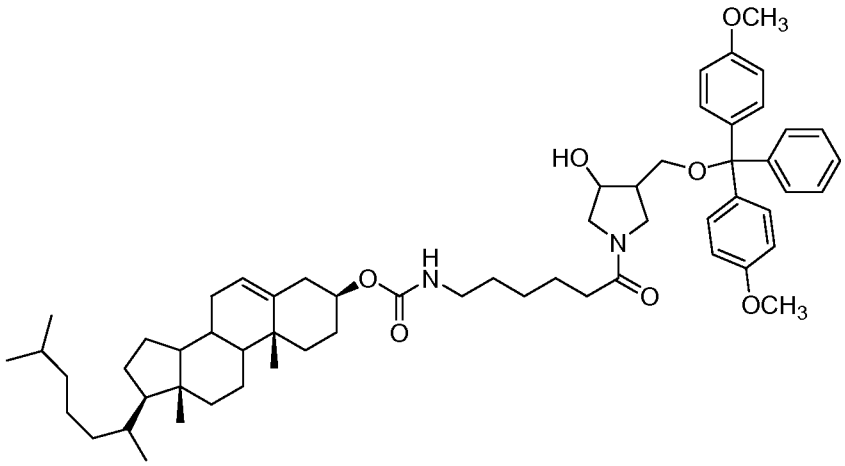
[6-(3-Hydroxy-4-hydroxymethyl-pyrrolidin-l-yl)-6-oxo-hexyl]-carbamic acid 17-(1,5-dimethyl-hexyl)-10,13-dimethyl-2,3,4,7,8,9,10,ll,12,13,14,15,16,17-tetradecahydro-lH-cyclopenta[a]phenanthren-3-yl ester AF



AF

Methanol (2 mL) is added dropwise over a period of 1 h to a refluxing mixture of b-ketoester AE (1.5 g, 2.2 mmol) and sodium borohydride (0.226 g, 6 mmol) in tetrahydrofuran (10 mL). Stirring is continued at reflux temperature for 1 h. After cooling to room temperature, 1 N HC1 (12.5 mL) is added, the mixture is extracted with ethylacetate (3 x 40 mL). The combined ethylacetate layer is dried over anhydrous sodium sulfate and concentrated under vacuum to yield the product which is purified by column chromatography (10% MeOH/CHCL;) (89%).

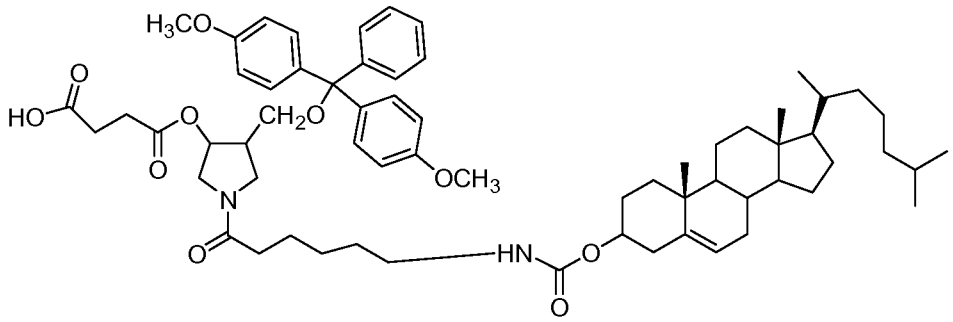
(6-{3-[Bis-(4-methoxy-phenyl)-phenyl-methoxymethyl]-4-hydroxy-pyrrolidin-l-yl}-6-oxo-hexyl)-carbamic acid 17-(1,5-dimethyl-hexyl)-10,13-dimethyl-2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-lH-cyclopenta[a]phenanthren-3-yl ester AG



AG

Diol AF (1.25 gm 1.994 mmol) is dried by evaporating with pyridine (2 x 5 mL) *in vacuo*. Anhydrous pyridine (10 mL) and 4,4'-dimethoxytritylchloride (0.724 g, 2.13 mmol) are added with stirring. The reaction is carried out at room temperature overnight. The reaction is quenched by the addition of methanol. The reaction mixture is concentrated under vacuum and to the residue dichloromethane (50 mL) is added. The organic layer is washed with 1M aqueous sodium bicarbonate. The organic layer is dried over anhydrous sodium sulfate, filtered and concentrated. The residual pyridine is removed by evaporating with toluene. The crude product is purified by column chromatography (2% MeOH/chloroform, Rf = 0.5 in 5% MeOH/$CHCl_3$) (1.75 g, 95%).

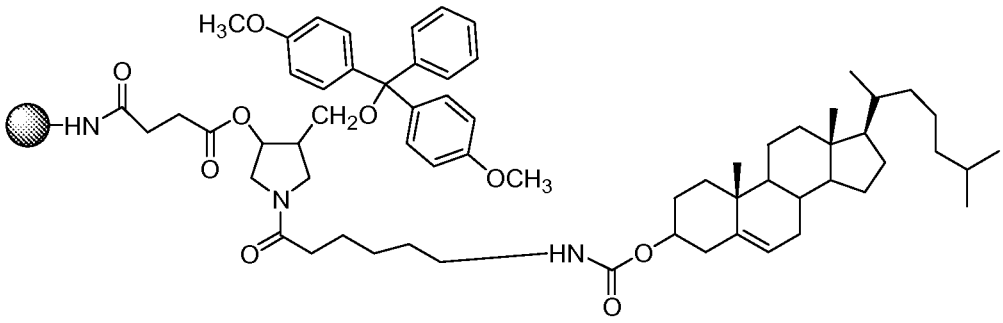
Succinic acid mono-(4-[bis-(4-methoxy-phenyl)-phenyl-methoxymethyl]-l-{6-[17-(l,5-dimethyl-hexyl)-l 0,13-dimethyl 2,3,4,7,8,9,10,11,12,13,14,15,16,17-tetradecahydro-lH cyclopenta[a]phenanthren-3-yloxycarbonylamino]-hexanoyl} -pyrrolidin-3-yl) ester **AH**



AH

Compound AG (1.0 g, 1.05 mmol) is mixed with succinic anhydride (0.150 g, 1.5 mmol) and DMAP (0.073 g, 0.6 mmol) and dried in a vacuum at 40°C overnight. The mixture is dissolved in anhydrous dichloroethane (3 mL), triethylamine (0.318 g, 0.440 mL, 3.15 mmol) is added and the solution is stirred at room temperature under argon atmosphere for 16 h. It is then diluted with dichloromethane (40 mL) and washed with ice cold aqueous citric acid (5 wt%, 30 mL) and water (2 X 20 mL). The organic phase is dried over anhydrous sodium sulfate and concentrated to dryness. The residue is used as such for the next step.

Cholesterol derivatized CPG **AI**



AI

Succinate AH (0.254 g, 0.242 mmol) is dissolved in a mixture of dichloromethane/acetonitrile (3:2, 3 mL). To that solution DMAP (0.0296 g, 0.242 mmol) in acetonitrile (1.25 mL), 2,2'-Dithio-bis(5-nitropyridine) (0.075 g, 0.242 mmol) in acetonitrile/dichloroethane (3:1, 1.25 mL) are added successively. To the resulting solution triphenylphosphine (0.064 g, 0.242 mmol) in acetonitrile (0.6 ml) is added. The reaction mixture turned bright orange in color. The solution is agitated briefly using a wrist-action shaker (5 mins). Long chain alkyl amine-CPG (LCAA-CPG) (1.5 g, 61 mM) is added. The suspension is agitated for 2 h. The CPG is filtered through a sintered funnel and washed with acetonitrile, dichloromethane and ether successively. Unreacted amino groups are masked using acetic anhydride/pyridine. The achieved loading of the CPG is measured by taking UV measurement (37 mM/g).

The synthesis of siRNAs bearing a 5'-12-dodecanoic acid bisdecylamide group (herein referred to as "5'-C32-") or a 5'-cholesteryl derivative group (herein referred to as "5'-Chol-") is performed as described in WO 2004/065601, except that, for the cholesteryl derivative, the oxidation step is performed using the Beaucage reagent in order to introduce a phosphorothioate linkage at the 5'-end of the nucleic acid oligomer.

Nucleic acid sequences are represented herein using standard nomenclature, and specifically the abbreviations of Table 1. It will be understood that these monomers, when present in an oligonucleotide, are mutually linked by 5'-3'-phosphodiester bonds.

Table 1: Abbreviations of nucleoside monomers used in nucleic acid sequence representation.

Abbreviation	**Nucleoside(s)**
A	Adenosine
C	Cytidine
G	Guanosine
U	Uridine
N	any nucleotide (G, A, C, U, or dT)

Abbreviation	Nucleoside(s)
a	2'-0-methyladenosine
c	2'-0-methylcytidine
G	2'-0-methylguanosine
U	2'-0-methyluridine
dT	2'-deoxythymidine
sT; sdT	2'-deoxy-thymidine-5'phosphate-phosphorothioate
s	a phosphorothioate linkage

Example 2. *siRNA Design and Synthesis*

Transcripts

Oligonucleotide design was carried out to identify siRNAs targeting the gene "synuclein-alpha (non A4 component of amyloid precursor)" from human (NCBI symbol SNCA) and the orthologous sequence from rhesus monkey *(Macaca mulatto)* to ensure that the selected siRNAs were species cross-reactive. The design process used the SNCA transcripts NM_007308.1 from human (NCBI Geneld 6622) and XM_00 1095402.1 from rhesus (NCBI Geneld 706985) from the NCBI Refseq collection as follows:

Species	SNCA sequence ref
human	NM_007308.1
Monkey	XM_001095402.1

siRNA Design and Specificity Prediction

The predicted specificity of all possible 19mers was predicted from each sequence. The SNCA siRNAs were used in a comprehensive search against the human and rhesus transcriptomes (defined as the set of NM_ and XM_ records within the NCBI Refseq set) using the FASTA algorithm. The Python script OfftargetFasta.py' was then used to parse the alignments and generate a score based on the position and number of mismatches between the siRNA and any potential Off-target' transcript. The off-target score is weighted to emphasize differences in the 'seed' region of siRNAs, in positions 2-9 from the 5' end of the molecule. The off-target score is calculated as follows: mismatches between the oligo and the transcript are given penalties. A mismatch in the seed region in positions 2-9 of the oligo is given a penalty of 2.8; mismatches in the putative cleavage sites 10 and 11 are given a penalty of 1.2, and all other mismatches a penalty of 1. The off-target score for each oligo-transcript pair is then calculated by summing the mismatch penalties. The lowest off-target score from all the oligo-transcript pairs is then determined and used in subsequent sorting of oligos. Both siRNAs strands were assigned to a category of specificity according to the calculated scores: a score above 3 qualifies

as highly specific, equal to 3 as specific and between 2.2 and 2.8 as moderate specific. In picking which oligos to synthesize, we sorted from high to low by the off-target score of the antisense strand and took the 92 best (lowest off-target score) oligo pairs.

Table 2 provides the sequences of 92 sense and antisense strands, 19 nucloetides each. Table 2 also provides the sequences of each strand including a 2 nculeotide "overhange" of NN. Table 3 provides sequences of the of 92 sense and antisense strands including dTdsdT overhangs.

Table 2: SNCA sense and antisense strand sequences and corresponding SNCA sense and antisense strand sequences with 2 base overhangs

Numbering for target sequence is based on human NM_007308. 1.

Strand	Start of target sequence	Unmodified sequence (5' to 3')	SEQ ID NO:	Sequence with 3' dinucleotide overhangs (5' to 3')	SEQ ID NO:
sense	325	GUAUCAAGACUACGAACCU	1	GUAUCAAGACUACGAACCUNN	**185**
antis	325	AGGUUCGUAGUCUUGAUAC	2	AGGUUCGUAGUCUUGAUACNN	**186**
sense	625	CUAAGUGACUACCACUUAU	3	CUAAGUGACUACCACUUAUNN	**187**
antis	625	AUAAGUGGUAGUCACUUAG	4	AUAAGUGGUAGUCACUUAGNN	**188**
sense	321	AAGGGUAUCAAGACUACGA	5	AAGGGUAUCAAGACUACGANN	**189**
antis	321	UCGUAGUCUUGAUACCCUU	6	UCGUAGUCUUGAUACCCUUNN	**190**
sense	326	UAUCAAGACUACGAACCUG	7	UAUCAAGACUACGAACCUGNN	**191**
antis	326	CAGGUUCGUAGUCUUGAUA	8	CAGGUUCGUAGUCUUGAUANN	**192**
sense	200	GUGACAAAUGUUGGAGGAG	9	GUGACAAAUGUUGGAGGAGNN	**193**
antis	200	CUCCUCCAACAUUUGUCAC	10	CUCCUCCAACAUUUGUCACNN	**194**
sense	327	AUCAAGACUACGAACCUGA	11	AUCAAGACUACGAACCUGANN	**195**
antis	327	UCAGGUUCGUAGUCUUGAU	12	UCAGGUUCGUAGUCUUGAUNN	**196**
sense	426	GUGCCCAGUCAUGACAUUU	13	GUGCCCAGUCAUGACAUUUNN	**197**
antis	426	AAAUGUCAUGACUGGGCAC	14	AAAUGUCAUGACUGGGCACNN	**198**
sense	731	UUAAUGAUACUGUCUAAGA	15	UUAAUGAUACUGUCUAAGANN	**199**
antis	731	UCUUAGACAGUAUCAUUAA	16	UCUUAGACAGUAUCAUUAANN	**200**
sense	429	CCCAGUCAUGACAUUUCUC	17	CCCAGUCAUGACAUUUCUCNN	**201**
antis	429	GAGAAAUGUCAUGACUGGG	18	GAGAAAUGUCAUGACUGGGNN	**202**
sense	543	AAGUGAAUACAUGGUAGCA	19	AAGUGAAUACAUGGUAGCANN	**203**
antis	543	UGCUACCAUGUAUUCACUU	20	UGCUACCAUGUAUUCACUUNN	**204**
sense	802	AGCAUGAAACUAUGCACCU	21	AGCAUGAAACUAUGCACCUNN	**205**
antis	802	AGGUGCAUAGUUUCAUGCU	22	AGGUGCAUAGUUUCAUGCUNN	**206**
sense	803	GCAUGAAACUAUGCACCUA	23	GCAUGAAACUAUGCACCUANN	**207**
antis	803	UAGGUGCAUAGUUUCAUGC	24	UAGGUGCAUAGUUUCAUGCNN	**208**
sense	410	AGUGCUCAGUUCCAAUGUG	25	AGUGCUCAGUUCCAAUGUGNN	**209**
antis	410	CACAUUGGAACUGAGCACU	26	CACAUUGGAACUGAGCACUNN	**210**
sense	518	GCAUUUCGGUGCUUCCCUU	27	GCAUUUCGGUGCUUCCCUUNN	**211**
antis	518	AAGGGAAGCACCGAAAUGC	28	AAGGGAAGCACCGAAAUGCNN	**212**
sense	798	UGUGAGCAUGAAACUAUGC	29	UGUGAGCAUGAAACUAUGCNN	**213**
antis	798	GCAUAGUUUCAUGCUCACA	30	GCAUAGUUUCAUGCUCACANN	**214**
sense	318	AGGAAGGGUAUCAAGACUA	31	AGGAAGGGUAUCAAGACUANN	**215**
antis	318	UAGUCUUGAUACCCUUCCU	32	UAGUCUUGAUACCCUUCCUNN	**216**
sense	537	UCACUGAAGUGAAUACAUG	33	UCACUGAAGUGAAUACAUGNN	**217**
antis	537	CAUGUAUUCACUUCAGUGA	34	CAUGUAUUCACUUCAGUGANN	**218**

Strand	Start of target sequence	Unmodified sequence (5' to 3')	SEQ ID NO:	Sequence with 3' dinucleotide overhangs (5' to 3')	SEQ ID NO:
sense	201	UGACAAAUGUUGGAGGAGC	35	UGACAAAUGUUGGAGGAGCNN	219
antis	201	GCUCCUCCAACAUUUGUCA	36	GCUCCUCCAACAUUUGUCANN	220
sense	544	AGUGAAUACAUGGUAGCAG	37	AGUGAAUACAUGGUAGCAGNN	221
antis	544	CUGCUACCAUGUAUUCACU	38	CUGCUACCAUGUAUUCACUNN	222
sense	548	AAUACAUGGUAGCAGGGUC	39	AAUACAUGGUAGCAGGGUCNN	223
antis	548	GACCCUGCUACCAUGUAUU	40	GACCCUGCUACCAUGUAUUNN	224
sense	482	AGCAGUGAUUGAAGUAUCU	41	AGCAGUGAUUGAAGUAUCUNN	225
antis	482	AGAUACUUCAAUCACUGCU	42	AGAUACUUCAAUCACUGCUNN	226
sense	221	GUGGUGACGGGUGUGACAG	43	GUGGUGACGGGUGUGACAGNN	227
antis	221	CUGUCACACCCGUCACCAC	44	CUGUCACACCCGUCACCACNN	228
sense	328	UCAAGACUACGAACCUGAA	45	UCAAGACUACGAACCUGAANN	229
antis	328	UUCAGGUUCGUAGUCUUGA	46	UUCAGGUUCGUAGUCUUGANN	230
sense	942	CAUCUCACUUUAAUAAUAA	47	CAUCUCACUUUAAUAAUAANN	231
antis	942	UUAUUAUUAAAGUGAGAUG	48	UUAUUAUUAAAGUGAGAUGNN	232
sense	227	ACGGGUGUGACAGCAGUAG	49	ACGGGUGUGACAGCAGUAGNN	233
antis	227	CUACUGCUGUCACACCCGU	50	CUACUGCUGUCACACCCGUNN	234
sense	320	GAAGGGUAUCAAGACUACG	51	GAAGGGUAUCAAGACUACGNN	235
antis	320	CGUAGUCUUGAUACCCUUC	52	CGUAGUCUUGAUACCCUUCNN	236
sense	683	AGUUGUUAGUGAUUUGCUA	53	AGUUGUUAGUGAUUUGCUANN	237
antis	683	UAGCAAAUCACUAACAACU	54	UAGCAAAUCACUAACAACUNN	238
sense	374	UCUUGAGAUCUGCUGACAG	55	UCUUGAGAUCUGCUGACAGNN	239
antis	374	CUGUCAGCAGAUCUCAAGA	56	CUGUCAGCAGAUCUCAAGANN	240
sense	358	AUCUUUGCUCCCAGUUUCU	57	AUCUUUGCUCCCAGUUUCUNN	241
antis	358	AGAAACUGGGAGCAAAGAU	58	AGAAACUGGGAGCAAAGAUNN	242
sense	552	CAUGGUAGCAGGGUCUUUG	59	CAUGGUAGCAGGGUCUUUGNN	243
antis	552	CAAAGACCCUGCUACCAUG	60	CAAAGACCCUGCUACCAUGNN	244
sense	639	CUUAUUUCUAAAUCCUCAC	61	CUUAUUUCUAAAUCCUCACNN	245
antis	639	GUGAGGAUUUAGAAAUAAG	62	GUGAGGAUUUAGAAAUAAGNN	246
sense	425	UGUGCCCAGUCAUGACAUU	63	UGUGCCCAGUCAUGACAUUNN	247
antis	425	AAUGUCAUGACUGGGCACA	64	AAUGUCAUGACUGGGCACANN	248
sense	675	UGUUCAGAAGUUGUUAGUG	65	UGUUCAGAAGUUGUUAGUGNN	249
antis	675	CACUAACAACUUCUGAACA	66	CACUAACAACUUCUGAACANN	250
sense	329	CAAGACUACGAACCUGAAG	67	CAAGACUACGAACCUGAAGNN	251
antis	329	CUUCAGGUUCGUAGUCUUG	68	CUUCAGGUUCGUAGUCUUGNN	252
sense	809	AACUAUGCACCUAUAAAUA	69	AACUAUGCACCUAUAAAUANN	253
antis	809	UAUUUAUAGGUGCAUAGUU	70	UAUUUAUAGGUGCAUAGUUNN	254
sense	794	AAUAUGUGAGCAUGAAACU	71	AAUAUGUGAGCAUGAAACUNN	255
antis	794	AGUUUCAUGCUCACAUAUU	72	AGUUUCAUGCUCACAUAUUNN	256
sense	487	UGAUUGAAGUAUCUGUACC	73	UGAUUGAAGUAUCUGUACCNN	257
antis	487	GGUACAGAUACUUCAAUCA	74	GGUACAGAUACUUCAAUCANN	258
sense	538	CACUGAAGUGAAUACAUGG	75	CACUGAAGUGAAUACAUGGNN	259
antis	538	CCAUGUAUUCACUUCAGUG	76	CCAUGUAUUCACUUCAGUGNN	260
sense	534	CUUUCACUGAAGUGAAUAC	77	CUUUCACUGAAGUGAAUACNN	261
antis	534	GUAUUCACUUCAGUGAAAG	78	GUAUUCACUUCAGUGAAAGNN	262
sense	427	UGCCCAGUCAUGACAUUUC	79	UGCCCAGUCAUGACAUUUCNN	263
antis	427	GAAAUGUCAUGACUGGGCA	80	GAAAUGUCAUGACUGGGCANN	264
sense	567	UUUGUGUGCUGUGGAUUUU	81	UUUGUGUGCUGUGGAUUUUNN	265
antis	567	AAAAUCCACAGCACACAAA	82	AAAAUCCACAGCACACAAANN	266
sense	806	UGAAACUAUGCACCUAUAA	83	UGAAACUAUGCACCUAUAANN	267

Strand	Start of target sequence	Unmodified sequence (5' to 3')	SEQ ID NO:	Sequence with 3' dinucleotide overhangs (5' to 3')	SEQ ID NO:
antis	806	UUAUAGGUGCAUAGUUUCA	84	UUAUAGGUGCAUAGUUUCANN	268
sense	738	UACUGUCUAAGAAUAAUGA	85	UACUGUCUAAGAAUAAUGANN	269
antis	738	UCAUUAUUCUUAGACAGUA	86	UCAUUAUUCUUAGACAGUANN	270
sense	876	UUGUAUAUAAAUGGUGAGA	87	UUGUAUAUAAAUGGUGAGANN	271
antis	876	UCUCACCAUUUAUAUACAA	88	UCUCACCAUUUAUAUACAANN	272
sense	322	AGGGUAUCAAGACUACGAA	89	AGGGUAUCAAGACUACGAANN	273
antis	322	UUCGUAGUCUUGAUACCCU	90	UUCGUAGUCUUGAUACCCUNN	274
sense	324	GGUAUCAAGACUACGAACC	91	GGUAUCAAGACUACGAACCNN	275
antis	324	GGUUCGUAGUCUUGAUACC	92	GGUUCGUAGUCUUGAUACCNN	276
sense	334	CUACGAACCUGAAGCCUAA	93	CUACGAACCUGAAGCCUAANN	277
antis	334	UUAGGCUUCAGGUUCGUAG	94	UUAGGCUUCAGGUUCGUAGNN	278
sense	884	AAAUGGUGAGAAUUAAAAU	95	AAAUGGUGAGAAUUAAAAUNN	279
antis	884	AUUUUAAUUCUCACCAUUU	96	AUUUUAAUUCUCACCAUUUNN	280
sense	553	AUGGUAGCAGGGUCUUUGU	97	AUGGUAGCAGGGUCUUUGUNN	281
antis	553	ACAAAGACCCUGCUACCAU	98	ACAAAGACCCUGCUACCAUNN	282
sense	323	GGGUAUCAAGACUACGAAC	99	GGGUAUCAAGACUACGAACNN	283
antis	323	GUUCGUAGUCUUGAUACCC	100	GUUCGUAGUCUUGAUACCCNN	284
sense	620	AACACCUAAGUGACUACCA	101	AACACCUAAGUGACUACCANN	285
antis	620	UGGUAGUCACUUAGGUGUU	102	UGGUAGUCACUUAGGUGUUNN	286
sense	619	AAACACCUAAGUGACUACC	103	AAACACCUAAGUGACUACCNN	287
antis	619	GGUAGUCACUUAGGUGUUU	104	GGUAGUCACUUAGGUGUUUNN	288
sense	682	AAGUUGUUAGUGAUUUGCU	105	AAGUUGUUAGUGAUUUGCUNN	289
antis	682	AGCAAAUCACUAACAACUU	106	AGCAAAUCACUAACAACUUNN	290
sense	732	UAAUGAUACUGUCUAAGAA	107	UAAUGAUACUGUCUAAGAANN	291
antis	732	UUCUUAGACAGUAUCAUUA	108	UUCUUAGACAGUAUCAUUANN	292
sense	515	UCAGCAUUUCGGUGCUUCC	109	UCAGCAUUUCGGUGCUUCCNN	293
antis	515	GGAAGCACCGAAAUGCUGA	110	GGAAGCACCGAAAUGCUGANN	294
sense	737	AUACUGUCUAAGAAUAAUG	111	AUACUGUCUAAGAAUAAUGNN	295
antis	737	CAUUAUUCUUAGACAGUAU	112	CAUUAUUCUUAGACAGUAUNN	296
sense	804	CAUGAAACUAUGCACCUAU	113	CAUGAAACUAUGCACCUAUNN	297
antis	804	AUAGGUGCAUAGUUUCAUG	114	AUAGGUGCAUAGUUUCAUGNN	298
sense	554	UGGUAGCAGGGUCUUUGUG	115	UGGUAGCAGGGUCUUUGUGNN	299
antis	554	CACAAAGACCCUGCUACCA	116	CACAAAGACCCUGCUACCANN	300
sense	943	AUCUCACUUUAAUAAUAAA	117	AUCUCACUUUAAUAAUAAANN	301
antis	943	UUUAUUAUUAAAGUGAGAU	118	UUUAUUAUUAAAGUGAGAUNN	302
sense	674	UUGUUCAGAAGUUGUUAGU	119	UUGUUCAGAAGUUGUUAGUNN	303
antis	674	ACUAACAACUUCUGAACAA	120	ACUAACAACUUCUGAACAANN	304
sense	428	GCCCAGUCAUGACAUUUCU	121	GCCCAGUCAUGACAUUUCUNN	305
antis	428	AGAAAUGUCAUGACUGGGC	122	AGAAAUGUCAUGACUGGGCNN	306
sense	580	GAUUUUGUGGCUUCAAUCU	123	GAUUUUGUGGCUUCAAUCUNN	307
antis	580	AGAUUGAAGCCACAAAAUC	124	AGAUUGAAGCCACAAAAUCNN	308
sense	813	AUGCACCUAUAAAUACUAA	125	AUGCACCUAUAAAUACUAANN	309
antis	813	UUAGUAUUUAUAGGUGCAU	126	UUAGUAUUUAUAGGUGCAUNN	310
sense	621	ACACCUAAGUGACUACCAC	127	ACACCUAAGUGACUACCACNN	311
antis	621	GUGGUAGUCACUUAGGUGU	128	GUGGUAGUCACUUAGGUGUNN	312
sense	936	UUAUCCCAUCUCACUUUAA	129	UUAUCCCAUCUCACUUUAANN	313
antis	936	UUAAAGUGAGAUGGGAUAA	130	UUAAAGUGAGAUGGGAUAANN	314
sense	349	CUAAGAAAUAUCUUUGCUC	131	CUAAGAAAUAUCUUUGCUCNN	315
antis	349	GAGCAAAGAUAUUUCUUAG	132	GAGCAAAGAUAUUUCUUAGNN	316

Strand	Start of target sequence	Unmodified sequence (5' to 3')	SEQ ID NO:	Sequence with 3' dinucleotide overhangs (5' to 3')	SEQ ID NO:
sense	433	GUCAUGACAUUUCUCAAAG	133	GUCAUGACAUUUCUCAAAGNN	317
antis	433	CUUUGAGAAAUGUCAUGAC	134	CUUUGAGAAAUGUCAUGACNN	318
sense	350	UAAGAAAUAUCUUUGCUCC	135	UAAGAAAUAUCUUUGCUCCNN	319
antis	350	GGAGCAAAGAUAUUUCUUA	136	GGAGCAAAGAUAUUUCUUANN	320
sense	1036	AAGAAGGAGGAAUUUUAGA	137	AAGAAGGAGGAAUUUUAGANN	321
antis	1036	UCUAAAAUUCCUCCUUCUU	138	UCUAAAAUUCCUCCUUCUUNN	322
sense	424	AUGUGCCCAGUCAUGACAU	139	AUGUGCCCAGUCAUGACAUNN	323
antis	424	AUGUCAUGACUGGGCACAU	140	AUGUCAUGACUGGGCACAUNN	324
sense	673	GUUGUUCAGAAGUUGUUAG	141	GUUGUUCAGAAGUUGUUAGNN	325
antis	673	CUAACAACUUCUGAACAAC	142	CUAACAACUUCUGAACAACNN	326
sense	640	UUAUUUCUAAAUCCUCACU	143	UUAUUUCUAAAUCCUCACUNN	327
antis	640	AGUGAGGAUUUAGAAAUAA	144	AGUGAGGAUUUAGAAAUAANN	328
sense	638	ACUUAUUUCUAAAUCCUCA	145	ACUUAUUUCUAAAUCCUCANN	329
antis	638	UGAGGAUUUAGAAAUAAGU	146	UGAGGAUUUAGAAAUAAGUNN	330
sense	720	UUAGGUGUCUUUUAAUGAU	147	UUAGGUGUCUUUUAAUGAUNN	331
antis	720	AUCAUUAAAAGACACCUAA	148	AUCAUUAAAAGACACCUAANN	332
sense	353	GAAAUAUCUUUGCUCCCAG	149	GAAAUAUCUUUGCUCCCAGNN	333
antis	353	CUGGGAGCAAAGAUAUUUC	150	CUGGGAGCAAAGAUAUUUCNN	334
sense	357	UAUCUUUGCUCCCAGUUUC	151	UAUCUUUGCUCCCAGUUUCNN	335
antis	357	GAAACUGGGAGCAAAGAUA	152	GAAACUGGGAGCAAAGAUANN	336
sense	421	CCAAUGUGCCCAGUCAUGA	153	CCAAUGUGCCCAGUCAUGANN	337
antis	421	UCAUGACUGGGCACAUUGG	154	UCAUGACUGGGCACAUUGGNN	338
sense	404	UGUACAAGUGCUCAGUUCC	155	UGUACAAGUGCUCAGUUCCNN	339
antis	404	GGAACUGAGCACUUGUACA	156	GGAACUGAGCACUUGUACANN	340
sense	405	GUACAAGUGCUCAGUUCCA	157	GUACAAGUGCUCAGUUCCANN	341
antis	405	UGGAACUGAGCACUUGUAC	158	UGGAACUGAGCACUUGUACNN	342
sense	721	UAGGUGUCUUUUAAUGAUA	159	UAGGUGUCUUUUAAUGAUANN	343
antis	721	UAUCAUUAAAAGACACCUA	160	UAUCAUUAAAAGACACCUANN	344
sense	345	AAGCCUAAGAAAUAUCUUU	161	AAGCCUAAGAAAUAUCUUUNN	345
antis	345	AAAGAUAUUUCUUAGGCUU	162	AAAGAUAUUUCUUAGGCUUNN	346
sense	412	UGCUCAGUUCCAAUGUGCC	163	UGCUCAGUUCCAAUGUGCCNN	347
antis	412	GGCACAUUGGAACUGAGCA	164	GGCACAUUGGAACUGAGCANN	348
sense	418	GUUCCAAUGUGCCCAGUCA	165	GUUCCAAUGUGCCCAGUCANN	349
antis	418	UGACUGGGCACAUUGGAAC	166	UGACUGGGCACAUUGGAACNN	350
sense	684	GUUGUUAGUGAUUUGCUAU	167	GUUGUUAGUGAUUUGCUAUNN	351
antis	684	AUAGCAAAUCACUAACAAC	168	AUAGCAAAUCACUAACAACNN	352
sense	516	CAGCAUUUCGGUGCUUCCC	169	CAGCAUUUCGGUGCUUCCCNN	353
antis	516	GGGAAGCACCGAAAUGCUG	170	GGGAAGCACCGAAAUGCUGNN	354
sense	941	CCAUCUCACUUUAAUAAUA	171	CCAUCUCACUUUAAUAAUANN	355
antis	941	UAUUAUUAAAGUGAGAUGG	172	UAUUAUUAAAGUGAGAUGGNN	356
sense	566	CUUUGUGUGCUGUGGAUUU	173	CUUUGUGUGCUGUGGAUUUNN	357
antis	566	AAAUCCACAGCACACAAAG	174	AAAUCCACAGCACACAAAGNN	358
sense	226	GACGGGUGUGACAGCAGUA	175	GACGGGUGUGACAGCAGUANN	359
antis	226	UACUGCUGUCACACCCGUC	176	UACUGCUGUCACACCCGUCNN	360
sense	346	AGCCUAAGAAAUAUCUUUG	177	AGCCUAAGAAAUAUCUUUGNN	361
antis	346	CAAAGAUAUUUCUUAGGCU	178	CAAAGAUAUUUCUUAGGCUNN	362
sense	414	CUCAGUUCCAAUGUGCCCA	179	CUCAGUUCCAAUGUGCCCANN	363
antis	414	UGGGCACAUUGGAACUGAG	180	UGGGCACAUUGGAACUGAGNN	364
sense	722	AGGUGUCUUUUAAUGAUAC	181	AGGUGUCUUUUAAUGAUACNN	365

Strand	Start of target sequence	Unmodified sequence (5' to 3')	SEQ ID NO:	Sequence with 3' dinucleotide overhangs (5' to 3')	SEQ ID NO:
antis	722	GUAUCAUUAAAAGACACCU	182	GUAUCAUUAAAAGACACCUNN	366
sense	330	AAGACUACGAACCUGAAGC	183	AAGACUACGAACCUGAAGCNN	367
antis	330	GCUUCAGGUUCGUAGUCUU	184	GCUUCAGGUUCGUAGUCUUNN	368

Table 3: SNCA sense and antisense strand sequences with dTsdT overhangs

Numbering for target sequence is based on human NM_007308. 1.

Strand	Start of target sequence	Sequence with 3'dTsdT overhangs (5' to 3')	SEQ ID NO:
sense	325	GUAUCAAGACUACGAACCUdTsdT	369
antis	325	AGGUUCGUAGUCUUGAUACdTsdT	370
sense	625	CUAAGUGACUACCACUUAUdTsdT	371
antis	625	AUAAGUGGUAGUCACUUAGdTsdT	372
sense	321	AAGGGUAUCAAGACUACGAdTsdT	373
antis	321	UCGUAGUCUUGAUACCCUUdTsdT	374
sense	326	UAUCAAGACUACGAACCUGdTsdT	375
antis	326	CAGGUUCGUAGUCUUGAUAdTsdT	376
sense	200	GUGACAAAUGUUGGAGGAGdTsdT	377
antis	200	CUCCUCCAACAUUUGUCACdTsdT	378
sense	327	AUCAAGACUACGAACCUGAdTsdT	379
antis	327	UCAGGUUCGUAGUCUUGAUdTsdT	380
sense	426	GUGCCCAGUCAUGACAUUUdTsdT	381
antis	426	AAAUGUCAUGACUGGGCACdTsdT	382
sense	731	UUAAUGAUACUGUCUAAGAdTsdT	383
antis	731	UCUUAGACAGUAUCAUUAAdTsdT	384
sense	429	CCCAGUCAUGACAUUUCUCdTsdT	385
antis	429	GAGAAAUGUCAUGACUGGGdTsdT	386
sense	543	AAGUGAAUACAUGGUAGCAdTsdT	387
antis	543	UGCUACCAUGUAUUCACUUdTsdT	388
sense	802	AGCAUGAAACUAUGCACCUdTsdT	389
antis	802	AGGUGCAUAGUUUCAUGCUdTsdT	390
sense	803	GCAUGAAACUAUGCACCUAdTsdT	391
antis	803	UAGGUGCAUAGUUUCAUGCdTsdT	392
sense	410	AGUGCUCAGUUCCAAUGUGdTsdT	393
antis	410	CACAUUGGAACUGAGCACUdTsdT	394
sense	518	GCAUUUCGGUGCUUCCCUUdTsdT	395
antis	518	AAGGGAAGCACCGAAAUGCdTsdT	396
sense	798	UGUGAGCAUGAAACUAUGCdTsdT	397
antis	798	GCAUAGUUUCAUGCUCACAdTsdT	398
sense	318	AGGAAGGGUAUCAAGACUAdTsdT	399
antis	318	UAGUCUUGAUACCCUUCCUdTsdT	400
sense	537	UCACUGAAGUGAAUACAUGdTsdT	401
antis	537	CAUGUAUUCACUUCAGUGAdTsdT	402
sense	201	UGACAAAUGUUGGAGGAGCdTsdT	403
antis	201	GCUCCUCCAACAUUUGUCAdTsdT	404
sense	544	AGUGAAUACAUGGUAGCAGdTsdT	405
antis	544	CUGCUACCAUGUAUUCACUdTsdT	406
sense	548	AAUACAUGGUAGCAGGGUCdTsdT	407

Strand	Start of target sequence	Sequence with 3'dTsdT overhangs (5' to 3')	SEQ ID NO:
antis	548	GACCCUGCUACCAUGUAUUdTsdT	408
sense	482	AGCAGUGAUUGAAGUAUCUdTsdT	409
antis	482	AGAUACUUCAAUCACUGCUdTsdT	410
sense	221	GUGGUGACGGGUGUGACAGdTsdT	411
antis	221	CUGUCACACCCGUCACCACdTsdT	412
sense	328	UCAAGACUACGAACCUGAAdTsdT	413
antis	328	UUCAGGUUCGUAGUCUUGAdTsdT	414
sense	942	CAUCUCACUUUAAUAAUAAdTsdT	415
antis	942	UUAUUAUUAAAGUGAGAUGdTsdT	416
sense	227	ACGGGUGUGACAGCAGUAGdTsdT	417
antis	227	CUACUGCUGUCACACCCGUdTsdT	418
sense	320	GAAGGGUAUCAAGACUACGdTsdT	419
antis	320	CGUAGUCUUGAUACCCUUCdTsdT	420
sense	683	AGUUGUUAGUGAUUUGCUAdTsdT	421
antis	683	UAGCAAAUCACUAACAACUdTsdT	422
sense	374	UCUUGAGAUCUGCUGACAGdTsdT	423
antis	374	CUGUCAGCAGAUCUCAAGAdTsdT	424
sense	358	AUCUUUGCUCCCAGUUUCUdTsdT	425
antis	358	AGAAACUGGGAGCAAAGAUdTsdT	426
sense	552	CAUGGUAGCAGGGUCUUUGdTsdT	427
antis	552	CAAAGACCCUGCUACCAUGdTsdT	428
sense	639	CUUAUUUCUAAAUCCUCACdTsdT	429
antis	639	GUGAGGAUUUAGAAAUAAGdTsdT	430
sense	425	UGUGCCCAGUCAUGACAUUdTsdT	431
antis	425	AAUGUCAUGACUGGGCACAdTsdT	432
sense	675	UGUUCAGAAGUUGUUAGUGdTsdT	433
antis	675	CACUAACAACUUCUGAACAdTsdT	434
sense	329	CAAGACUACGAACCUGAAGdTsdT	435
antis	329	CUUCAGGUUCGUAGUCUUGdTsdT	436
sense	809	AACUAUGCACCUAUAAAUAdTsdT	437
antis	809	UAUUUAUAGGUGCAUAGUUdTsdT	438
sense	794	AAUAUGUGAGCAUGAAACUdTsdT	439
antis	794	AGUUUCAUGCUCACAUAUUdTsdT	440
sense	487	UGAUUGAAGUAUCUGUACCdTsdT	441
antis	487	GGUACAGAUACUUCAAUCAdTsdT	442
sense	538	CACUGAAGUGAAUACAUGGdTsdT	443
antis	538	CCAUGUAUUCACUUCAGUGdTsdT	444
sense	534	CUUUCACUGAAGUGAAUACdTsdT	445
antis	534	GUAUUCACUUCAGUGAAAGdTsdT	446
sense	427	UGCCCAGUCAUGACAUUUCdTsdT	447
antis	427	GAAAUGUCAUGACUGGGCAdTsdT	448
sense	567	UUUGUGUGCUGUGGAUUUUdTsdT	449
antis	567	AAAAUCCACAGCACACAAAdTsdT	450
sense	806	UGAAACUAUGCACCUAUAAdTsdT	451
antis	806	UUAUAGGUGCAUAGUUUCAdTsdT	452
sense	738	UACUGUCUAAGAAUAAUGAdTsdT	453
antis	738	UCAUUAUUCUUAGACAGUAdTsdT	454
sense	876	UUGUAUAUAAAUGGUGAGAdTsdT	455
antis	876	UCUCACCAUUUAUAUACAAdTsdT	456

Strand	Start of target sequence	Sequence with 3'dTsdT overhangs (5' to 3')	SEQ ID NO:
sense	322	AGGGUAUCAAGACUACGAAdTsdT	457
antis	322	UUCGUAGUCUUGAUACCCUdTsdT	458
sense	324	GGUAUCAAGACUACGAACCdTsdT	459
antis	324	GGUUCGUAGUCUUGAUACCdTsdT	460
sense	334	CUACGAACCUGAAGCCUAAdTsdT	461
antis	334	UUAGGCUUCAGGUUCGUAGdTsdT	462
sense	884	AAAUGGUGAGAAUUAAAAUdTsdT	463
antis	884	AUUUUAAUUCUCACCAUUUdTsdT	464
sense	553	AUGGUAGCAGGGUCUUUGUdTsdT	465
antis	553	ACAAAGACCCUGCUACCAUdTsdT	466
sense	323	GGGUAUCAAGACUACGAACdTsdT	467
antis	323	GUUCGUAGUCUUGAUACCCdTsdT	468
sense	620	AACACCUAAGUGACUACCAdTsdT	469
antis	620	UGGUAGUCACUUAGGUGUUdTsdT	470
sense	619	AAACACCUAAGUGACUACCdTsdT	471
antis	619	GGUAGUCACUUAGGUGUUUdTsdT	472
sense	682	AAGUUGUUAGUGAUUUGCUdTsdT	473
antis	682	AGCAAAUCACUAACAACUUdTsdT	474
sense	732	UAAUGAUACUGUCUAAGAAdTsdT	475
antis	732	UUCUUAGACAGUAUCAUUAdTsdT	476
sense	515	UCAGCAUUUCGGUGCUUCCdTsdT	477
antis	515	GGAAGCACCGAAAUGCUGAdTsdT	478
sense	737	AUACUGUCUAAGAAUAAUGdTsdT	479
antis	737	CAUUAUUCUUAGACAGUAUdTsdT	480
sense	804	CAUGAAACUAUGCACCUAUdTsdT	481
antis	804	AUAGGUGCAUAGUUUCAUGdTsdT	482
sense	554	UGGUAGCAGGGUCUUUGUGdTsdT	483
antis	554	CACAAAGACCCUGCUACCAdTsdT	484
sense	943	AUCUCACUUUAAUAAUAAAdTsdT	485
antis	943	UUUAUUAUUAAAGUGAGAUdTsdT	486
sense	674	UUGUUCAGAAGUUGUUAGUdTsdT	487
antis	674	ACUAACAACUUCUGAACAAdTsdT	488
sense	428	GCCCAGUCAUGACAUUUCUdTsdT	489
antis	428	AGAAAUGUCAUGACUGGGCdTsdT	490
sense	580	GAUUUUGUGGCUUCAAUCUdTsdT	491
antis	580	AGAUUGAAGCCACAAAAUCdTsdT	492
sense	813	AUGCACCUAUAAAUACUAAdTsdT	493
antis	813	UUAGUAUUUAUAGGUGCAUdTsdT	494
sense	621	ACACCUAAGUGACUACCACdTsdT	495
antis	621	GUGGUAGUCACUUAGGUGUdTsdT	496
sense	936	UUAUCCCAUCUCACUUUAAdTsdT	497
antis	936	UUAAAGUGAGAUGGGAUAAdTsdT	498
sense	349	CUAAGAAAUAUCUUUGCUCdTsdT	499
antis	349	GAGCAAAGAUAUUUCUUAGdTsdT	500
sense	433	GUCAUGACAUUUCUCAAAGdTsdT	501
antis	433	CUUUGAGAAAUGUCAUGACdTsdT	502
sense	350	UAAGAAAUAUCUUUGCUCCdTsdT	503
antis	350	GGAGCAAAGAUAUUUCUUAdTsdT	504
sense	1036	AAGAAGGAGGAAUUUUAGAdTsdT	505

Strand	Start of target sequence	Sequence with 3'dTsdT overhangs (5' to 3')	SEQ ID NO:
antis	1036	UCUAAAAUUCCUCCUUCUUdTsdT	506
sense	424	AUGUGCCCAGUCAUGACAUdTsdT	507
antis	424	AUGUCAUGACUGGGCACAUdTsdT	508
sense	673	GUUGUUCAGAAGUUGUUAGdTsdT	509
antis	673	CUAACAACUUCUGAACAACdTsdT	510
sense	640	UUAUUUCUAAAUCCUCACUdTsdT	511
antis	640	AGUGAGGAUUUAGAAAUAAdTsdT	512
sense	638	ACUUAUUUCUAAAUCCUCAdTsdT	513
antis	638	UGAGGAUUUAGAAAUAAGUdTsdT	514
sense	720	UUAGGUGUCUUUUAAUGAUdTsdT	515
antis	720	AUCAUUAAAAGACACCUAAdTsdT	516
sense	353	GAAAUAUCUUUGCUCCCAGdTsdT	517
antis	353	CUGGGAGCAAAGAUAUUUCdTsdT	518
sense	357	UAUCUUUGCUCCCAGUUUCdTsdT	519
antis	357	GAAACUGGGAGCAAAGAUAdTsdT	520
sense	421	CCAAUGUGCCCAGUCAUGAdTsdT	521
antis	421	UCAUGACUGGGCACAUUGGdTsdT	522
sense	404	UGUACAAGUGCUCAGUUCCdTsdT	523
antis	404	GGAACUGAGCACUUGUACAdTsdT	524
sense	405	GUACAAGUGCUCAGUUCCAdTsdT	525
antis	405	UGGAACUGAGCACUUGUACdTsdT	526
sense	721	UAGGUGUCUUUUAAUGAUAdTsdT	527
antis	721	UAUCAUUAAAAGACACCUAdTsdT	528
sense	345	AAGCCUAAGAAAUAUCUUUdTsdT	529
antis	345	AAAGAUAUUUCUUAGGCUUdTsdT	530
sense	412	UGCUCAGUUCCAAUGUGCCdTsdT	531
antis	412	GGCACAUUGGAACUGAGCAdTsdT	532
sense	418	GUUCCAAUGUGCCCAGUCAdTsdT	533
antis	418	UGACUGGGCACAUUGGAACdTsdT	534
sense	684	GUUGUUAGUGAUUUGCUAUdTsdT	535
antis	684	AUAGCAAAUCACUAACAACdTsdT	536
sense	516	CAGCAUUUCGGUGCUUCCCdTsdT	537
antis	516	GGGAAGCACCGAAAUGCUGdTsdT	538
sense	941	CCAUCUCACUUUAAUAAUAdTsdT	539
antis	941	UAUUAUUAAAGUGAGAUGGdTsdT	540
sense	566	CUUUGUGUGCUGUGGAUUUdTsdT	541
antis	566	AAAUCCACAGCACACAAAGdTsdT	542
sense	226	GACGGGUGUGACAGCAGUAdTsdT	543
antis	226	UACUGCUGUCACACCCGUCdTsdT	544
sense	346	AGCCUAAGAAAUAUCUUUGdTsdT	545
antis	346	CAAAGAUAUUUCUUAGGCUdTsdT	546
sense	414	CUCAGUUCCAAUGUGCCCAdTsdT	547
antis	414	UGGGCACAUUGGAACUGAGdTsdT	548
sense	722	AGGUGUCUUUUAAUGAUACdTsdT	549
antis	722	GUAUCAUUAAAAGACACCUdTsdT	550
sense	330	AAGACUACGAACCUGAAGCdTsdT	551
antis	330	GCUUCAGGUUCGUAGUCUUdTsdT	552

siRNA sequence selection

A total of 92 sense and 92 antisense human SNCA derived siRNA oligos were synthesized. The nucleotides were modified as described below. The sense and their respective antisense oligos were annealed into duplexes.

Table 4 provides the sequences and modifications of the sense and antisense strands of the 92 synthesized duplexes.

Synthesis of dsRNA

The sense and antisense strands of the dsRNA duplexes were synthesized on a MerMade 192 synthesizer at 1 μηοϊ scale. The strands were modified as follows and as described in Table 4. In the sense strand, all pyrimidines (U, C) were replaced with corresponding 2'-0-Methyl bases (2' O-Methyl C and 2'-0-Methyl U). In the antisense strand, pyrimidines adjacent to (towards the 5' position) a ribo A nucleoside were replaced with their corresponding 2-O-Methyl nucleosides. A two base dTsdT extension at the 3' end of both sense and antisense sequences was introduced. The sequence file was converted to a text file to make it compatible for loading in the MerMade 192 synthesis software.

The synthesis of each strand of the dsRNA used solid supported oligonucleotide synthesis using phosphoramidite chemistry.

Synthesis was performed at 1 μηοϊ scale in 96-well plates. The amidite solutions were prepared at 0.1 M concentration and ethyl thio tetrazole (0.6 M in acetonitrile) was used as an activator. The synthesized sequences were cleaved and deprotected in 96-well plates, using methylamine in the first step and triethylamine 3HF in the second step. The crude sequences thus obtained were precipitated using acetone: ethanol (80:20) mix and the pellet were re-suspended in 0.5 M sodium acetate buffer. Samples from each sequence were analyzed by LC-MS and the resulting mass data confirmed the identity of the sequences. UV detection was used for sample quantitation. A selected set of samples were also analyzed for purity by IEX chromatography.

All sequences were purified on an AKTA explorer purification system using a Source 15Q column. A column temperature of 65°C was maintained during purification. Sample injection and collection was performed in 96-well (1.8 mL -deep well) plates. A single peak corresponding to the full length sequence was collected in the eluent.

The purified sequences were desalted on a Sephadex G25 column using an AKTA purifier. The desalted sequences were analyzed for concentration (by UV measurement at A260) and purity (by ion exchange HPLC). For the preparation of duplexes, equimolar amounts of sense and antisense strand were heated in the required buffer *(e.g.,* 1x PBS) at 95°C for 2-5

minutes and slowly cooled to room temperature. Integrity of the duplex was confirmed by HPLC analysis.

Table 4: Chemically modified SNCA sense and antisense strand sequences with dTsdT overhangs

Numbering for target sequence is based on human NM_007308. 1. Modifications: sense strand - all pyrimidines (U, C) are 2'OMe; antisense strand - pyrimidines adjacent to A (UA, CA) are 2'OMe; 3' end is thio (dTsdT). Residues in lower case indicate chemical modification.

Duplex No.	Strand	Start of target sequence	Sequence with 3'dTsdT overhangs (5' to 3')	SEQ ID NO:
AD-21752	sense	325	GuAucAAGAcuAcGAAccudTsdT	553
	antis	325	AGGUUCGuAGUCUUGAuACdTsdT	554
AD-21753	sense	625	cuAAGuGAcuAccAcuuAudTsdT	555
	antis	625	AuAAGUGGuAGUcACUuAGdTsdT	556
AD-21754	sense	321	AAGGGuAucAAGAcuAcGAdTsdT	557
	antis	321	UCGuAGUCUUGAuACCCUUdTsdT	558
AD-21755	sense	326	uAucAAGAcuAcGAAccuGdTsdT	559
	antis	326	cAGGUUCGuAGUCUUGAuAdTsdT	560
AD-21756	sense	200	GuGAcAAAuGuuGGAGGAGdTsdT	561
	antis	200	CUCCUCcAAcAUUUGUcACdTsdT	562
AD-21757	sense	327	AucAAGAcuAcGAAccuGAdTsdT	563
	antis	327	UcAGGUUCGuAGUCUUGAUdTsdT	564
AD-21758	sense	426	GuGcccAGucAuGAcAuuudTsdT	565
	antis	426	AAAUGUcAUGACUGGGcACdTsdT	566
AD-21759	sense	731	uuAAuGAuAcuGucuAAGAdTsdT	567
	antis	731	UCUuAGAcAGuAUcAUuAAdTsdT	568
AD-21760	sense	429	cccAGucAuGAcAuuucucdTsdT	569
	antis	429	GAGAAAUGUcAUGACUGGGdTsdT	570
AD-21761	sense	543	AAGuGAAuAcAuGGuAGcAdTsdT	571
	antis	543	UGCuACcAUGuAUUcACUUdTsdT	572
AD-21762	sense	802	AGcAuGAAAcuAuGcAccudTsdT	573
	antis	802	AGGUGcAuAGUUUcAUGCUdTsdT	574
AD-21763	sense	803	GcAuGAAAcuAuGcAccuAdTsdT	575
	antis	803	uAGGUGcAuAGUUUcAUGCdTsdT	576
AD-21764	sense	410	AGuGcucAGuuccAAuGuGdTsdT	577
	antis	410	cAcAUUGGAACUGAGcACUdTsdT	578
AD-21765	sense	518	GcAuuucGGuGcuucccuudTsdT	579
	antis	518	AAGGGAAGcACCGAAAUGCdTsdT	580
AD-21766	sense	798	uGuGAGcAuGAAAcuAuGcdTsdT	581
	antis	798	GcAuAGUUUcAUGCUcAcAdTsdT	582
AD-21767	sense	318	AGGAAGGGuAucAAGAcuAdTsdT	583
	antis	318	uAGUCUUGAuACCCUUCCUdTsdT	584
AD-21768	sense	537	ucAcuGAAGuGAAuAcAuGdTsdT	585
	antis	537	cAUGuAUUcACUUcAGUGAdTsdT	586
AD-21769	sense	201	uGAcAAAuGuuGGAGGAGcdTsdT	587
	antis	201	GCUCCUCcAAcAUUUGUcAdTsdT	588
AD-21770	sense	544	AGuGAAuAcAuGGuAGcAGdTsdT	589

Duplex No.	Strand	Start of target sequence	Sequence with 3'dTsdT overhangs (5' to 3')	SEQ ID NO:
	antis	544	CUGCuACcAUGuAUUcACUdTsdT	590
AD-21771	sense	548	AAuAcAuGGuAGcAGGGucdTsdT	591
	antis	548	GACCCUGCuACcAUGuAUUdTsdT	592
AD-21772	sense	482	AGcAGuGAuuGAAGuAucudTsdT	593
	antis	482	AGAuACUUcAAUcACUGCUdTsdT	594
AD-21773	sense	221	GuGGuGAcGGGuGuGAcAGdTsdT	595
	antis	221	CUGUcAcACCCGUcACcACdTsdT	596
AD-21774	sense	328	ucAAGAcuAcGAAccuGAAdTsdT	597
	antis	328	UUcAGGUUCGiiAGUCUUGAdTsdT	598
AD-21775	sense	942	cAucucAcuuuAAiiAAiiAAdTsdT	599
	antis	942	UuAUiiAUiiAAAGUGAGAUGdTsdT	600
AD-21776	sense	227	AcGGGuGiiGAcAGcAGiiAGdTsdT	601
	antis	227	CuACUGCUGUcAcACCCGUdTsdT	602
AD-21777	sense	320	GAAGGGuAucAAGAciiAcGdTsdT	603
	antis	320	CGuAGUCUUGAuACCCUUCdTsdT	604
AD-21778	sense	683	AGuuGuuAGuGAiiiiiiGcuAdTsdT	605
	antis	683	uAGcAAAUcACiiAAcAACUdTsdT	606
AD-21779	sense	374	uciiiiGAGAiiciiGciiGAcAGdTsdT	607
	antis	374	CUGUcAGcAGAUCUcAAGAdTsdT	608
AD-22077	sense	358	AucuuuGcucccAGiiiiiiciidTsdT	609
	antis	358	AGAAACUGGGAGcAAAGAUdTsdT	610
AD-21780	sense	552	cAuGGiiAGcAGGGiiciiiiiiGdTsdT	611
	antis	552	cAAAGACCCUGCuACcAUGdTsdT	612
AD-22078	sense	639	cuuAuuiicuAAAiicciicAcdTsdT	613
	antis	639	GUGAGGAUUuAGAAAuAAGdTsdT	614
AD-21781	sense	425	uGuGcccAGucAuGAcAuudTsdT	615
	antis	425	AAUGUcAUGACUGGGcAcAdTsdT	616
AD-21782	sense	675	uGuucAGAAGuuGuuAGuGdTsdT	617
	antis	675	cACuAAcAACUUCUGAAcAdTsdT	618
AD-21783	sense	329	cAAGAcuAcGAAccuGAAGdTsdT	619
	antis	329	CUUcAGGUUCGuAGUCUUGdTsdT	620
AD-22079	sense	809	AAcuAuGcAccuAuAAAuAdTsdT	621
	antis	809	uAUUuAuAGGUGcAuAGUUdTsdT	622
AD-21784	sense	794	AAuAuGuGAGcAuGAAAcudTsdT	623
	antis	794	AGUUUcAUGCUcAcAuAUUdTsdT	624
AD-21785	sense	487	uGAuuGAAGuAucuGuAccdTsdT	625
	antis	487	GGuAcAGAuACUUcAAUcAdTsdT	626
AD-21786	sense	538	cAcuGAAGuGAAuAcAuGGdTsdT	627
	antis	538	CcAUGuAUUcACUUcAGUGdTsdT	628
AD-21787	sense	534	cuuucAcuGAAGuGAAuAcdTsdT	629
	antis	534	GuAUUcACUUcAGUGAAAGdTsdT	630
AD-21788	sense	427	uGcccAGucAuGAcAuuucdTsdT	631
	antis	427	GAAAUGUcAUGACUGGGcAdTsdT	632
AD-22080	sense	567	uuuGuGuGcuGuGGAuuuudTsdT	633
	antis	567	AAAAUCcAcAGcAcAcAAAdTsdT	634
AD-21789	sense	806	uGAAAcuAuGcAccuAuAAdTsdT	635
	antis	806	UuAuAGGUGcAuAGUUUcAdTsdT	636
AD-21790	sense	738	uAcuGucuAAGAAuAAuGAdTsdT	637
	antis	738	UcAUuAUUCUuAGAcAGuAdTsdT	638

Duplex No.	Strand	Start of target sequence	Sequence with 3'dTsdT overhangs (5' to 3')	SEQ ID NO:
AD-21791	sense	876	uuGuAuAuAAAuGGuGAGAdTsdT	639
	antis	876	UCUcACcAUUuAuAuAcAAdTsdT	640
AD-21792	sense	322	AGGGuAucAAGAcuAcGAAdTsdT	641
	antis	322	UUCGuAGUCUUGAuACCCUdTsdT	642
AD-21793	sense	324	GGuAucAAGAcuAcGAAccdTsdT	643
	antis	324	GGUUCGuAGUCUUGAuACCdTsdT	644
AD-21794	sense	334	cuAcGAAccuGAAGccuAAdTsdT	645
	antis	334	UuAGGCUUcAGGUUCGuAGdTsdT	646
AD-21795	sense	884	AAAuGGuGAGAAuuAAAAudTsdT	647
	antis	884	AUUUuAAUUCUcACcAUUUdTsdT	648
AD-21796	sense	553	AuGGuAGcAGGGucuuuGudTsdT	649
	antis	553	AcAAAGACCCUGCuACcAUdTsdT	650
AD-21797	sense	323	GGGuAucAAGAcuAcGAAcdTsdT	651
	antis	323	GUUCGuAGUCUUGAuACCCdTsdT	652
AD-21798	sense	620	AAcAccuAAGuGAcuAccAdTsdT	653
	antis	620	UGGuAGUcACUuAGGUGUUdTsdT	654
AD-21799	sense	619	AAAcAccuAAGuGAcuAccdTsdT	655
	antis	619	GGuAGUcACUuAGGUGUUUdTsdT	656
AD-21800	sense	682	AAGuuGuuAGuGAuuuGcudTsdT	657
	antis	682	AGcAAAUcACuAAcAACUUdTsdT	658
AD-21801	sense	732	uAAuGAuAcuGucuAAGAAdTsdT	659
	antis	732	UUCUuAGAcAGuAUcAUuAdTsdT	660
AD-21802	sense	515	ucAGcAuuucGGuGcuuccdTsdT	661
	antis	515	GGAAGcACCGAAAUGCUGAdTsdT	662
AD-21803	sense	737	AuAcuGucuAAGAAuAAuGdTsdT	663
	antis	737	cAUuAUUCUuAGAcAGuAUdTsdT	664
AD-21804	sense	804	cAuGAAAcuAuGcAccuAudTsdT	665
	antis	804	AuAGGUGcAuAGUUUcAUGdTsdT	666
AD-21805	sense	554	uGGuAGcAGGGucuuuGuGdTsdT	667
	antis	554	cAcAAAGACCCUGCuACcAdTsdT	668
AD-22081	sense	943	AucucAcuuuAAuAAuAAAdTsdT	669
	antis	943	UUuAUuAUuAAAGUGAGAUdTsdT	670
AD-21806	sense	674	uuGuucAGAAGuuGuuAGudTsdT	671
	antis	674	ACuAAcAACUUCUGAAcAAdTsdT	672
AD-21807	sense	428	GcccAGucAuGAcAuuucudTsdT	673
	antis	428	AGAAAUGUcAUGACUGGGCdTsdT	674
AD-21808	sense	580	GAuuuuGuGGcuucAAucudTsdT	675
	antis	580	AGAUUGAAGCcAcAAAAUCdTsdT	676
AD-21809	sense	813	AuGcAccuAuAAAuAcuAAdTsdT	677
	antis	813	UuAGuAUUuAuAGGUGcAUdTsdT	678
AD-21810	sense	621	AcAccuAAGuGAcuAccAcdTsdT	679
	antis	621	GUGGuAGUcACUuAGGUGUdTsdT	680
AD-22472	sense	936	uuAucccAucucAcuuuAAdTsdT	681
	antis	936	UiiAAAGUGAGAUGGGAiiAAdTsdT	682
AD-2181 1	sense	349	ciiAAGAAAuAucuuuGcucdTsdT	683
	antis	349	GAGcAAAGAuAUUUCUiiAGdTsdT	684
AD-21812	sense	433	GucAuGAcAuuucucAAAGdTsdT	685
	antis	433	CUUUGAGAAAUGUcAUGACdTsdT	686
AD-21813	sense	350	iiAAGAAAuAucuuuGcuccdTsdT	687

Duplex No.	Strand	Start of target sequence	Sequence with 3'dTsdT overhangs (5' to 3')	SEQ ID NO:
	antis	350	GGAGcAAAGAuAUUUCUuAdTsdT	688
AD-21814	sense	1036	AAGAAGGAGGAAuuuuAGAdTsdT	689
	antis	1036	UCuAAAAUUCCUCCUUCUUdTsdT	690
AD-21815	sense	424	AuGuGcccAGucAuGAcAudTsdT	691
	antis	424	AUGUcAUGACUGGGcAcAUdTsdT	692
AD-21816	sense	673	GuuGuucAGAAGuuGuuAGdTsdT	693
	antis	673	CuAAcAACUUCUGAAcAACdTsdT	694
*	sense	640	uuAuuucuAAAuccucAcudTsdT	695
	antis	640	AGUGAGGAUUiiAGAAAiiAAdTsdT	696
AD-22082	sense	638	AcuuAiiiiiicuAAAiicciicAdTsdT	697
	antis	638	UGAGGAUUiiAGAAAiiAAGUdTsdT	698
AD-21817	sense	720	uuAGGuGucuuiiuAAiiGAiidTsdT	699
	antis	720	AUcAUuAAAAGAcACCiiAAdTsdT	700
AD-21818	sense	353	GAAAuAuciiiiiiGciicccAGdTsdT	701
	antis	353	CUGGGAGcAAAGAuAUUUCdTsdT	702
AD-21819	sense	357	uAuciiiiiiGciicccAGiiiiiicdTsdT	703
	antis	357	GAAACUGGGAGcAAAGAuAdTsdT	704
AD-21820	sense	421	ccAAiiGiiGcccAGiicAiiGAdTsdT	705
	antis	421	UcAUGACUGGGcAcAUUGGdTsdT	706
AD-21821	sense	404	iiGuAcAAGiiGciicAGiiiiccdTsdT	707
	antis	404	GGAACUGAGcACUUGuAcAdTsdT	708
AD-21822	sense	405	GuAcAAGiiGciicAGiiiiccAdTsdT	709
	antis	405	UGGAACUGAGcACUUGuACdTsdT	710
AD-21823	sense	721	uAGGuGucuuuiiAAuGAuAdTsdT	711
	antis	721	uAUcAUuAAAAGAcACCiiAdTsdT	712
AD-21824	sense	345	AAGccuAAGAAAuAuciiiiiidTsdT	713
	antis	345	AAAGAiiAUUUCUuAGGCUUdTsdT	714
AD-21825	sense	412	iiGciicAGiiiiccAAiiGiiGccdTsdT	715
	antis	412	GGcAcAUUGGAACUGAGcAdTsdT	716
AD-21826	sense	418	GuuccAAuGuGcccAGucAdTsdT	717
	antis	418	UGACUGGGcAcAUUGGAACdTsdT	718
AD-21827	sense	684	GuuGuuAGuGAuiiiiGcuAiidTsdT	719
	antis	684	AuAGcAAAUcACiiAAcAACdTsdT	720
AD-21828	sense	516	cAGcAuuucGGuGcuucccdTsdT	721
	antis	516	GGGAAGcACCGAAAUGCUGdTsdT	722
AD-21829	sense	941	ccAucucAcuuiiAAiiAAiiAdTsdT	723
	antis	941	iiAUuAUiiAAAGUGAGAUGGdTsdT	724
AD-21830	sense	566	cuuuGuGuGcuGuGGAuuudTsdT	725
	antis	566	AAAUCcAcAGcAcAcAAAGdTsdT	726
AD-2183 1	sense	226	GAcGGGuGuGAcAGcAGuAdTsdT	727
	antis	226	uACUGCUGUcAcACCCGUCdTsdT	728
AD-21832	sense	346	AGccuAAGAAAuAiiciiiiiGdTsdT	729
	antis	346	cAAAGAuAUUUCUiiAGGCUdTsdT	730
AD-21833	sense	414	cucAGuuccAAuGuGcccAdTsdT	731
	antis	414	UGGGcAcAUUGGAACUGAGdTsdT	732
AD-21834	sense	722	AGGuGucuuuuAAuGAiiAcdTsdT	733
	antis	722	GuAUcAUiiAAAAGAcACCUdTsdT	734
AD-21835	sense	330	AAGAcuAcGAAccuGAAGcdTsdT	735
	antis	330	GCUUcAGGUUCGuAGUCUUdTsdT	736

Example 3. In vitro screening of siRNAs for mRNA suppression

The duplexes described in Table 4 were assayed for RNA suppression in tissue culture cells.

Cell culture and transfections;

NCI-H460 (ATCC, Manassas, VA) or BE(2)M17 cells were grown to near confluence at 37°C in an atmosphere of 5% $C0_2$ in RPMI (ATCC) supplemented with 10% FBS, streptomycin, and glutamine (ATCC) before being released from the plate by trypsinization. Reverse transfection was carried out by adding 5 μï of Opti-MEM to 5 μï of siRNA duplexes per well into a 96-well plate along with 10 μï of Opti-MEM plus 0.2 μï of Lipofectamine RNAiMax per well (Invitrogen, Carlsbad CA. cat # 13778-150) and incubated at room temperature for 15 minutes. 80 μï of complete growth media without antibiotic containing 2.0 xlO^4 Hela cells were then added. Cells were incubated for 24 hours prior to RNA purification.

Total RNA isolation using MagMAX-96 Total RNA Isolation Kit (Applied Biosvstem, Forer City CA, part #: AMI 830):

Cells were harvested and lysed in 140 μï of Lysis/Binding Solution then mixed for 1 minute at 850 rpm using and Eppendorf Thermomixer (with constant mixing speed throughout the process). 20 μï of magnetic beads and Lysis/Binding Enhancer mixture were added into cell-lysate and mixed for 5 minutes. Magnetic beads were captured using magnetic stand and the supernatant was removed without disturbing the beads. After removing supernatant, magnetic beads were washed with Wash Solution 1 (isopropanol added) and mixed for 1 minute. Beads were capture again and supernatant removed. Beads were then washed with 150 μï Wash Solution 2 (ethanol added), captured and supernatant was removed. 50 μï of DNase mixture (MagMax turbo DNase Buffer and Turbo DNase) was then added to the beads and they were mixed for 10 to 15 minutes. After mixing, 100 μï of RNA Rebinding Solution was added and mixed for 3 minutes. Supernatant was removed and magnetic beads were washed again with 150 μï Wash Solution 2 and mixed for 1 minute and supernatant was removed completely. The magnetic beads were mixed for 2 minutes to dry before RNA was eluted with 50 μï of water.

cDNA synthesis using ABI High capacity cDNA reverse transcription kit (Applied Biosvstems. Foster City. CA. Cat. No. 4368813):

A master mix of 2 μï 10X Buffer, 0.8 μï 25X dNTPs, 2 μï Random primers, 1 μï Reverse Transcriptase, 1 μï RNase inhibitor and 3.2 μï of H2O per reaction were added into 10 μï total RNA. cDNA was generated using a Bio-Rad C-1000 or S-1000 thermal cycler (Hercules, CA)

through the following steps: 25°C for 10 minutes, 37°C for 120 minutes, 85°C for 5 seconds, and hold at 4°C.

Real time PCR:

2 μï of cDNA were added to a master mix containing 0.5 μï GAPDH TaqMan Probe (Applied Biosystems, Cat. No. 43263 17E), 0.5 μï synuclein TaqMan probe (Applied Biosystems, Cat. No. Hs00240906) and 5 μï Roche Probes Master Mix (Roche, Cat. No. 04887301001) in a total of 10 μï per well in a LightCycler 480 384 well plate (Roche, Cat. No. 0472974001). Real time PCR was done in a LightCycler 480 Real Time PCR machine (Roche) using the AACt assay. Each duplex was tested in two independent transfections and each transfection was assayed in duplicate.

Real-time data were analyzed using the AACt method. Each sample was normalized to GAPDH expression and knockdown was assessed relative to cells transfected with the non-targeting duplex AD- 1955.

Results

Experiments were performed at 0.1 nM and 10 nM final duplex concentrations for single dose screens with 79 synuclein duplexes. described in Table 4. Table 5 shows the results of the single dose *in vitro* siRNA screen. After making the determinations reported in Table 5, it was determined that certain of the oligonucleotides were subject to concentration artifacts now thought to arise from incomplete thawing of plates containing the oligonucleotides. Subsequent analysis of two large datasets (unrelated to SNCA) indicated that any such concentration artifacts would not alter the assay results more than that which can be accounted for by normal experimental variability. Moreover, these concentration artifacts are unlikely to substantially affect our conclusions about lead selection.

Table 5: Fraction of target remaining following treatment with 0.1 nM and 10 nM duplex in the NCI-H460 and BE(2)M 17 cell lines

	NCI-H460		BE(2)M17	
Duplex No.	**0.1 nM**	**10 nM**	**0.1 nM**	**10 nM**
AD-21752	0.09	0.02	0.13	0.11
AD-21753	0.51	0.10	0.47	0.23
AD-21754	0.76	0.43	0.60	0.48
AD-21755	0.62	0.22	0.46	0.26
AD-21756	0.66	0.41	0.72	0.39
AD-21757	0.33	0.26	0.57	0.33
AD-21758	0.10	0.05	0.36	0.19

	NCI-H460		BE(2)M17	
Duplex No.	0.1 nM	10 nM	0.1 nM	10 nM
AD-21759	0.20	0.19	0.42	0.26
AD-21760	0.03	0.02	0.12	0.07
AD-21762	0.14	0.16	0.34	0.19
AD-21763	0.29	0.10	0.31	0.19
AD-21764	0.81	0.63	0.84	0.82
AD-21765	0.06	0.02	0.16	0.11
AD-21766	0.17	0.05	0.35	0.15
AD-21767	0.74	0.59	1.07	1.26
AD-21768	0.52	0.16	0.75	0.29
AD-21769	0.17	0.24	0.34	0.08
AD-21770	0.45	0.38	0.74	0.47
AD-21771	0.18	0.15	0.42	0.20
AD-21772	0.02	0.02	0.09	0.07
AD-21773	0.25	0.13	0.50	0.33
AD-21775	0.64	0.56	0.66	0.60
AD-21776	0.55	0.30	0.68	0.50
AD-21777	0.65	0.65	0.97	1.02
AD-21778	0.22	0.10	0.54	0.20
AD-21780	0.19	0.03	0.29	0.09
AD-21781	0.03	0.02	0.18	0.11
AD-21782	0.23	0.03	0.67	0.09
AD-21783	0.15	0.12	0.46	0.34
AD-21784	0.19	0.18	0.30	0.21
AD-21785	0.07	0.04	0.26	0.11
AD-21786	0.07	0.04	0.15	0.08
AD-21787	0.62	0.16	0.75	0.36
AD-21788	0.02	0.02	0.09	0.08
AD-21789	0.14	0.13	0.27	0.22
AD-21790	0.09	0.08	0.14	0.14
AD-21791	0.45	0.34	0.72	0.55
AD-21792	0.76	0.59	0.74	0.59
AD-21793	0.23	0.08	0.34	0.14
AD-21794	0.29	0.26	0.49	0.41
AD-21795	0.28	0.27	0.39	0.28
AD-21796	0.15	0.08	0.28	0.19
AD-21797	0.16	0.04	0.26	0.11
AD-21798	0.07	0.06	0.21	0.17
AD-21799	0.39	0.51	0.62	0.51
AD-21800	0.35	0.46	0.58	0.47
AD-21801	0.08	0.10	0.14	0.13
AD-21802	0.66	0.23	0.64	0.25
AD-21803	0.63	0.63	0.68	0.56

	NCI-H460		BE(2)M17	
Duplex No.	0.1 nM	10 nM	0.1 nM	10 nM
AD-21804	0.08	0.07	0.15	0.16
AD-21805	0.41	0.08	0.46	0.15
AD-21806	0.06	0.07	0.14	0.13
AD-21807	0.06	0.02	0.21	0.10
AD-21808	0.03	0.02	0.12	0.07
AD-21809	0.11	0.10	0.23	0.24
AD-21810	0.05	0.06	0.14	0.18
AD-21811	0.28	0.09	0.44	0.18
AD-21812	0.04	0.04	0.24	0.16
AD-21813	0.07	0.04	0.18	0.09
AD-21814	0.65	0.64	0.72	0.67
AD-21815	0.40	0.26	0.67	0.46
AD-21816	0.16	0.07	0.25	0.16
AD-21817	0.07	0.08	0.13	0.13
AD-21818	0.55	0.12	0.75	0.40
AD-21820	0.28	0.06	0.44	0.15
AD-21821	0.02	0.02	0.09	0.07
AD-21822	0.01	0.01	0.07	0.09
AD-21823	0.12	0.06	0.22	0.13
AD-21824	0.02	0.01	0.09	0.05
AD-21825	0.25	0.13	0.36	0.17
AD-21826	0.03	0.02	0.13	0.07
AD-21828	0.75	0.40	0.75	0.34
AD-21829	0.26	0.07	0.39	0.10
AD-21830	0.02	0.01	0.08	0.05
AD-21831	0.52	0.25	0.71	0.40
AD-21832	0.33	0.09	0.66	0.21
AD-21833	0.65	0.15	0.80	0.29
AD-21834	0.54	0.46	0.69	0.55
AD-21835	0.42	0.54	0.49	0.35

A subset of 20 duplexes that showed robust silencing in the 10 nM and 0.1 nM screens were assayed over a range of concentrations from 100 nM to 10 fM using 10 fold serial dilutions to determine their IC50 in BE(2)M17 cells. IC50s were defined using a 4 parameter fit model in XLfit. Table 6 shows the average IC50s for this subset of duplexes.

Table 6: Average IC50s for a subset of synuclein duplexes

Duplex ID	Average IC50 in [pM]
AD-21752	33.7
AD-21760	9.0

Duplex ID	Average IC50 in [pM]
AD-21765	19.5
AD-21772	3.4
AD-21781	22.4
AD-21786	41.2
AD-21788	15.2
AD-21790	6.7
AD-21801	1.8
AD-21804	9.3
AD-21806	10.4
AD-21808	28.1
AD-21810	17.3
AD-21813	47.3
AD-21817	15.4
AD-21821	11.8
AD-21822	3.4
AD-21824	19.8
AD-21826	30.8
AD-21830	11.9

Example 4; Silencing of human SNCA by intraparenchymal infusion in transgenic mice

To determine the ability of several of the msot potent SNCA siRNAs (AD21822, AD-21830, AD-21772, AD-2801, and AD-21810) to reduce levels of human SNCA siRNA *in vivo*, infusion studies were conducted in transgenic mice. A transgenic mouse strain (Mayo, unpublished) was generated using a P1 artificial chromosome (PAC) comprising the entire human SNCA locus. The expression pattern of human SNCA in this transgenic mouse strain mirrors that of endogenous mouse SNCA. To assess target silencing, solutions of siRNA (30 mg/ml in PBS) were infused via osmotic minipumps (Alzet Corp.) at a flow rate of 0.25 microliters per hour directly into the CA1 region of the hippocampus (a site of high SNCA expression) in one hemisphere of the brain. Controls included an siRNA targeting Luciferase (AD-1955) and PBS infusion. After 15 days, brains were dissected and 2 mm thick coronal sections encompassing the infusion site were obtained. Tissue punches (1.2 mm diameter) were taken from the hippocampus at the infusion site. Total RNA was isolated and used to generate cDNA. Levels of human SNCA and mouse GAPDH were measured by Taqman® analysis (Applied Biosystems, Inc.). Levels of SNCA were normalized to GAPDH for each tissue punch, and group mean values were normalized to the PBS infused animals. As shown in Table 7,

infusion with AD-21830, AD21822, AD-21772, AD-2801 and AD-21810 reduced human SNCA expression by 76%, 85%, 90%, 76% and 59%, respectively, compared to PBS infusion.

Table 7: Levels of SNCA relative to GAPDH.

Treatment	n	Mean relative hu SNCA	St. Dev
PBS	8	100%	53%
AD-1955	13	77%	55%
AD-21830	4	24%	12%
AD-21822	4	15%	18%
AD-21772	5	10%	3%
AD-21801	4	24%	11%
AD-21810	5	41%	17%

Example 5; Silencing of mouse SNCA by intraparenchymal infusion

To determine the ability of a potent, rodent cross-reactive SNCA siRNA (AD21822) to reduce the level of mouse SNCA siRNA *in vivo*, infusion studies were conducted in C57/B1 mice. To assess target silencing, solutions of siRNA (30 mg/ml in PBS) were infused via osmotic minipumps (Alzet Corp.) at a flow rate of 0.25 microliters per hour directly into the CA1 region of the hippocampus (a site of high SNCA expression) in one hemisphere of the brain. Control animals were infused with PBS. After 14 days, brains were dissected and 1 mm thick coronal sections centered on the infusion site and 1 mm posterior to the infusion site were obtained. Tissue pieces encompassing the hippocampus were dissected from the infused side of each thick section. Total RNA was isolated and used to generate cDNA. Levels of mouse SNCA and mouse GAPDH were measured by Taqman® analysis (Applied Biosystems, Inc.). Levels of SNCA were normalized to GAPDH for each tissue piece, and group mean values were normalized to the PBS samples taken at the infusion site. As shown in Table 8, infusion with AD21822 reduced mouse SNCA expression by 95% at the infusion site, and by 57% at a distance of 1 mm posterior to the infusion site.

Table 8: Levels of SNCA relative to GAPDH.

Sample	n	Mean relative SNCA	St. Dev
PBS, infusion site	5	100%	7%

AD-2 1822, infusion site	6	5%	3%
PBS, 1 mm posterior	5	115%	6%
AD-2 1822, 1 mm posterior	6	43%	28%

Example 6. Silencing of rat SNCA by intraparenchymal infusion

To determine the ability of two potent rodent cross-reactive SNCA siRNAs (AD21822 and AD21810) to reduce the level of rat SNCA mRNA *in vivo*, infusion studies were conducted in Sprague-Dawley rats. To assess target silencing, solutions of siRNA (15 mg/ml in PBS) were infused via osmotic minipumps (Alzet Corp.) at a flow rate of 1 microliter per hour directly into the substantia nigra in one hemisphere of the brain. Control animals were infused with an siRNA targeting Luciferase (AD1955) or PBS. After 7 days of infusion, brains were dissected and 1 mm thick coronal sections centered on the infusion site, 1 mm anterior to the infusion site and 1mm posterior to the infusion site were obtained. Tissue pieces encompassing the substantia nigra were dissected from the infused side of each section. Total RNA was isolated and used to generate cDNA. Levels of rat SNCA and rat GAPDH were measured by Taqman® analysis (Applied Biosystems, Inc.). Levels of SNCA were normalized to GAPDH for each tissue piece, and group mean values were normalized to the PBS samples taken at the infusion site. Levels of rat Tyrosine Hydroxylase (TH) mRNA, a marker of dopaminergic neurons in the substantia nigra, were also measured by Taqman® and normalized to GAPDH as described for SNCA. Infusion with AD2 1822 reduced rat SNCA expression by 50% at the infusion site, by 57% at a distance of 1 mm anterior to the infusion site and by 59% at a distance of 1 mm posterior to the infusion site relative to the PBS group (infusion site). Infusion with AD21 810 reduced rat SNCA expression by 90% at the infusion site, by 84% at a distance of 1 mm anterior to the infusion site and by 92% at a distance of 1 mm posterior to the infusion site relative to the PBS group (infusion site).

To explore duration of target inhibition, the two potent SNCA siRNAs (AD21822 and AD21810) were infused via osmotic minipumps (Alzet Corp.) at a flow rate of 0.5 microliters per hour into the substantia nigra in one hemisphere of the brain of Sprague-Dawley rats for 7 days. The concentration of siRNA in the pumps was 8 mg/ml. Control animals received PBS. Pumps were disconnected at the end of the infusion period. Groups of animals were sacrificed at the end of infusion, as well as 1 week, 2 weeks and 4 weeks after the end of infusion. Brain fragments were collected as described above. Measurement of rat SNCA (normalized to rat GAPDH) in the fragments nearest the infusion site demonstrated suppression of expression by 70% (AD21810) and 78% (AD21822) at the end of infusion; 72% (AD21810) and 88%

(AD2 1822) one week after the end of infusion; 43% (AD2 1810) and 79% (AD2 1822) two weeks after the end of infusion; and 56% (AD21810) and 65% (AD21822) four weeks after the end of infusion (compared to PBS infused animals).

To further study the potency of one of the siRNAs, AD21822 was infused at a rate of 0.25 ul per hour for 7 or 28 days into the substantia nigra of one hemisphere of the brain of Sprague-Dawley rats at siRNA concentrations of 2, 1 and 0.3 mg/ml. Control animals received PBS. At the end of infusion, brain fragments encompassing the infusion site were collected and analyzed as described above. After 7 days of infusion, rat SNCA levels (normalized to rat GAPDH) were reduced by 80%, 54%, and 55% at 2 mg/ml, 1 mg/ml and 0.3 mg/ml, respectively. After 28 days of infusion, rat SNCA levels (normalized to rat GAPDH) were reduced by 82%, 69%, and 54% at 2 mg/ml, 1 mg/ml and 0.3 mg/ml, respectively.

Example 7. *Inhibition of SNCA in humans*

A human subject is treated with a dsRNA targeted to a SNCA gene to inhibit expression of the SNCA gene to treat a condition.

A subject in need of treatment is selected or identified. The subject can have a neurodegenerative disease, *e.g.,* Parkinson's disease, sporadic Parkinson's disease, Alzeimer's disease, multiple system atrophy, Lewy body dementia, or any other disease associated with SNCA expression.

The identification of the subject can occur in a clinical setting, or elsewhere, *e.g.,* in the subject's home through the subject's own use of a self-testing kit.

At time zero, a suitable first dose of an anti-SNCA siRNA is administered to the subject. The dsRNA is formulated as described herein. After a period of time following the first dose, *e.g.,* 7 days, 14 days, and 2 1 days, the subject's condition is evaluated by any suitable means, including, *e.g.,* a reduction in disease symptoms. This measurement can be accompanied by a measurement of SNCA expression in said subject, and/or the products of the successful siRNA-targeting of SNCA mRNA. Other relevant criteria can also be measured. The number and strength of doses are adjusted according to the subject's needs.

After treatment, the symptoms of subject's neurodegenerative disease are reduced or arrested relative to the symptoms existing prior to the treatment, or relative to those measured in a similarly afflicted but untreated subject.

Example 8. *Method of treating a patient diagnosed with a synucleinopathy*

A patient diagnosed with a synucleinopathy can be administered a pharmaceutical composition containing a dsRNA that targets the SNCA gene. The composition can be delivered

directly to the brain by a device that includes an osmotic pump and mini-cannula and is bilaterally implanted into the patient.

Prior to implantation of the device, the patient receives an MRI with stereotactic frame. A computer-guided trajectory is used for delivery of the cannula to the brain. The mini-pump device is implanted into the abdomen, and then the patient is hospitalized for 2-3 days to monitor for hemorrhaging.

Approximately two weeks post-implantation of the pump, the patient can receive an MRI to check the implanted device. If the human is healing well, and no complications have occurred as a result of implanting the device, then the anti-SNCA composition can be infused into the pump, and into the cannula. A test dose of the anti-SNCA agent can be administered prior to the initiation of the therapeutic regimen.

MRIs taken at 3 months, six months, and one year following the initial treatment can be used to monitor the condition of the device, and the reaction of the patient to the device and treatment with the dsRNA. Clinicians should watch for the development of edema and an inflammatory response. Following the one-year anniversary of the initiation of the treatment, MRIs can be performed as needed.

The patient can be monitored for an improvement or stabilization in disease symptoms throughout the course of the therapy. Monitoring can include serial clinical assessments and functional neuroimaging, *e.g.,* by MRI.

Example 9. *Human SNCA mRNA sequence*

Shown below is the sequence of the full length mRNA of human SNCA (transcript variant NACP 112; GenBank Access. No. NM_007308.1 (NCBI Geneld 6622); SEQ ID NO:737).

```
  1 gaattcatta gccatggatg tattcatgaa aggactttca aaggccaagg agggagttgt
 61 ggctgctgct gagaaaacca aacagggtgt ggcagaagca gcaggaaaga caaaagaggg
121 tgttctctat gtaggctcca aaaccaagga gggagtggtg catggtgtgg caacagtggc
181 tgagaagacc aaagagcaag tgacaaatgt tggaggagca gtggtgacgg gtgtgacagc
241 agtagcccag aagacagtgg agggagcagg gagcattgca gcagccactg gctttgtcaa
301 aaaggaccag ttgggcaagg aagggtatca agactacgaa cctgaagcct aagaaatatc
361 tttgctccca gtttcttgag atctgctgac agatgttcca tcctgtacaa gtgctcagtt
421 ccaatgtgcc cagtcatgac atttctcaaa gtttttacag tgtatctcga agtcttccat
481 cagcagtgat tgaagtatct gtacctgccc ccactcagca tttcggtgct tccctttcac
541 tgaagtgaat acatggtagc agggtctttg tgtgctgtgg attttgtggc ttcaatctac
601 gatgttaaaa caaattaaaa acacctaagt gactaccact tatttctaaa tcctcactat
```

661 ttttttgttg ctgttgttca gaagttgtta gtgatttgct atcatatatt ataagatttt
721 taggtgtctt ttaatgatac tgtctaagaa taatgacgta ttgtgaaatt tgttaatata
781 tataatactt aaaaatatgt gagcatgaaa ctatgcacct ataaatacta aatatgaaat
841 tttaccattt tgcgatgtgt tttattcact tgtgtttgta tataaatggt gagaattaaa
901 ataaaacgtt atctcattgc aaaaatattt tatttttatc ccatctcact ttaataataa
961 aaatcatgct tataagcaac atgaattaag aactgacaca aaggacaaaa atataaagtt
1021 attaatagcc atttgaagaa ggaggaattt tagaagaggt agagaaaatg gaacattaac
1081 cctacactcg gaattc

Example 10. *Rhesus monkey SNCA mRNA sequence*

Shown below is the sequence of the predicted full length mRNA of rhesus monkey SNCA (isoformNACP140); GenBank Access. No. XM_001095402.1 (NCBI Geneld 706985); SEQ ID NO:738).

1 atgcgagggc aaagtgctct cggcgcgccc ccactttccc gccttgcgcg gccaggcagg
61 cggctggaat tggtggttca ccctgcgccc cctgccccat ccccatccga gataggcaac
121 gaagagcacg ctgcagggaa agcagcgagc gctgggaggg gagcgtggag aggcgctgac
181 aaatcagcgg tgggggcgga gagccgagga gaaggagaag gaggaggacg aggaggagga
241 ggacggcgac gaccagaagg ggcccgagag agggggcgag cgaccgagcg ccgcgacgcg
301 ggagtgaggt gcgtgcgggc tgcagcgcag accccggccc ggcccctccg agagcgtcct
361 gagcgctccc tcacgccttc ccttcaaacc ttctgccttt ttctccatcc tcgtgagcgg
421 agaactggga gtggccattc gacgacaggc tccaaaacca aggagggagt ggtgcacggt
481 gtggcaacag tggctgagaa gaccaaagag caagtgacaa atgttggagg agcggtggtg
541 acgggtgtga cagcagtagc ccagaagaca gtggagggag cagggagcat tgcagcagcc
601 actggcttca tcaaaaagga ccagttgggc aagaatgaag aaggagcccc acaggaagga
661 attctacaag atatgcctgt ggatcctgac aatgaggctt atgaaatgcc ttctgaggaa
721 gggtatcaag actacgaacc tgaagcctaa gaaatatctt tgctcccagt ttcttgagat
781 ctgctgacag acgttccatc ttgtacaagt gctcagttcc aatgtgccca gtcatgacat
841 ttctcaaagt ttttacagta tattttgaag tcttccatca gcagtgattg aagtatctgt
901 acctgccccc attcagcatt tcggtgcttc cctttcactg aagtgaatac atggtagcag
961 ggtctttgtg tgctgtggat tttgtggctt caatctatga tgttaaaaca atttaaaaac
1021 acctaagtga ctaccactta tttctaaatc ctcactattt ttttgttgct gttgttcaga
1081 agttgttagt gatttgctat cgtatattat aagattttta ggtgtctttt aatgatactg
1141 tctaagaata atgatgtatt gtgaaatttg ttaatatata taatacttaa aaatatgtga
1201 gcatgaaact atgcacctat aaatactaac tatgaaattt taccgttttg tgatgtgttt
1261 tattaacttg tgtttgtata taaatggtga gaattaaaat aaaatgtcgt ctcattgcaa
1321 acaaaatttt atttttatcc catctcactt taataataaa aatcttgctt ataagcaaca
1381 tgcattgaga actgacacaa tggacataaa gttattaata ggcatttgaa gaaggaggaa

```
1441 ttttagaaga ggtagagaaa atgaacatta accctacact gggaattccc tgaagcagca
1501 ctgccagaag tgtgttttgt ggtgccttaa gtggctgtga ttaaaaaaaa aaaaaagtgg
1561 gctccaggga acgaagcagt gtaaaagatg attttgacta catcctcctt agagatccat
1621 gagacacttt agcacatatt agcacattca aggctctgag acaatgtggt taacttagtt
1681 taactcagca gtccccacta aaaaaaaaaa aatcatcaaa aattctctct ctctattcct
1741 ttttctctcg ctccccttttt ttccaggaaa tgcctttaaa caccttgggg aactatcagg
1801 atcaccttaa agaagatcag ttctccagac tgataaaaat ttcatgatct cttttaaatg
1861 ttgccaaata tatgaattct aggatttttc cttgggaaag gtttttctct ttcagggaag
1921 atctattaac tccccatggg tgctgaaaat aaacttgatg gtgaaaaatt ctatataaat
1981 taatttaaaa tttttttggt ttctcttttt aattattctg gggcatagtc atttttaaaa
2041 gtcactagta gaaagtataa tttcaagaca gaatattcta gacatgctag cagtttatat
2101 gtattcatga gtaatgtgat atatattggg cactggtgag gcaggaagga ggaatgagtg
2161 actataagga tggttaccat agaaacttcc ttttttacct
```

Other embodiments are in the claims.

CLAIMS

We claim:

1. A double-stranded ribonucleic acid (dsRNA) for inhibiting expression of alpha-synuclein (SNCA), comprising a sense strand and an antisense strand comprising a region of complementarity to an mRNA encoding SNCA, wherein each strand is at least 15 nucleotides in length, and wherein the sense strand and antisense strand comprise at least 15 contiguous nucleotides of sequences selected from Tables 2, 3, 4, or 6.

2. The dsRNA of claim 1, wherein the sense strand comprises SEQ ID NO:l, 17, 27, 41, 63, 75, 79, 85, 107, 113, 119, 123, 127, 135, 147, 155, 157, 161, 165, or 173 and/or the antisense strand comprises SEQ ID NO:2, 18, 28, 42, 64, 76, 80, 86, 108, 114, 120, 124, 128, 136, 148, 156, 158, 162, 166, or 174.

3. The dsRNA of claim 1, wherein the antisense strand is complementary to at least 15 contiguous nucleotides of SEQ ID NO: 157.

4. The dsRNA of claim 1, wherein the antisense strand is complementary to at least the first 11 nucleotides of SEQ ID NO: 158.

5. The dsRNA of claim 1, wherein the sense strand sequence comprises SEQ ID NO: 157 and the antisense strand sequence comprises SEQ ID NO: 158.

6. The dsRNA of claim 1, wherein the sense strand comprises SEQ ID NO:553, 569, 579, 593, 615, 627, 631, 637, 659, 665, 671, 675, 679, 687, 699, 707, 709, 713, 717, or 725; and/or the antisense strand comprises SEQ ID NO:554, 570, 580, 594, 616, 628, 632, 638, 660, 666, 672, 676, 680, 688, 700, 708, 710, 714, 718, or 726.

7. The dsRNA of claim 1, wherein the sense strand sequence comprises SEQ ID NO:707 and the antisense strand sequence comprises SEQ ID NO: 708.

8. The dsRNA of claim 1, wherein the dsRNA is selected from the group consisting of: AD-21752, AD-21760, AD-21765, AD-21772, AD-21781, AD-21786, AD-21788, AD-21790, AD-21801, AD-21804, AD-21806, AD-21808, AD-21810, AD-21813, AD-21817, AD-21821, AD-21822, AD-21824, AD-21826, and AD-21830.

9. The dsRNA of claim 1, wherein the dsRNA is AD-21822.

10. The dsRNA of claim 1, wherein the SNCA is human SNCA encoded by NM_007308.1.

11. The dsRNA of claim 1, wherein the SNCA is monkey SNCA encoded by XM_001095402.1.

12. The dsRNA of claim 1, wherein the region of complementarity is between 19 and 21 nucleotides in length.

13. The dsRNA of claim 1, wherein the region of complementarity is 19 nucleotides in length.

14. The dsRNA of claim 1, wherein at least one strand comprises a 3' overhang of at least 1 nucleotide.

15. The dsRNA of claim 1, wherein the dsRNA comprises a nucleotide overhang having 1 to **4** nucleotides.

16. The dsRNA of claim 1, wherein each strand comprises a 3' overhang consisting of 2 nucleotides.

17. The dsRNA of claim 1, wherein each strand comprises a 3' overhang consisting of dTsdT.

18. The dsRNA of claim 1, wherein said dsRNA comprises at least one modified nucleotide.

19. The dsRNA of claim 18, wherein said modified nucleotide is selected from the group of: a 2'-0-methyl modified nucleotide, a nucleotide comprising a 5'-phosphorothioate group, and a terminal nucleotide linked to a cholesteryl derivative or dodecanoic acid bisdecylamide group.

20. The dsRNA of claim 18, 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.

21. The dsRNA of claim 18, comprising at least one 2'-0-methyl modified nucleotide and at least one 2'-deoxythymidine-3'-phosphate nucleotide comprising a 5'-phosphorothioate group.

22. The dsRNA of claim 18, wherein the sense strand comprises all 2'-0-methyl modified pyrimidines and the antisense strand comprises 2'-0-methyl modified pyrimidines when the pyrimidine is adjacent to A; and wherein each strand comprises dTdT at the 3' end.

23. The dsRNA of claim 18, wherein the sense strand comprises all 2'-0-methyl modified pyrimidines and the antisense strand comprises 2'-0-methyl modified pyrimidines when the pyrimidine is adjacent to A; and wherein each strand comprises dTsdT at the 3' end.

24. The dsRNA of claim 18, wherein the sense strand comprises all 2'-0-methyl modified pyrimidines and the antisense strand comprises 2'-0-methyl modified pyrimidines when a) the

pyrimidine is adjacent to A, or b) the pyrimidine is a uracil adjacent to a U or a G; and wherein each strand comprises dTsdT at the 3' end.

25. The dsRNA of claim 1, further comprising a ligand.

26. The dsRNA of claim 26, wherein the ligand is conjugated to the 3'-end of the sense strand of the dsRNA.

27. A composition for inhibiting expression of a SNCA gene comprising the dsRNA of claim 1 and a pharmaceutical formulation.

28. The composition of claim 27, wherein the pharmaceutical formulation is a lipid formulation.

29. The composition of claim 27, wherein the pharmaceutical formulation is a LNP formulation, a LNPO 1 formulation, a XTC-SNALP formulation, a SNALP formulation, or a LNP 11 formulation.

30. An isolated cell containing the dsRNA of claim 1.

31. A vector comprising a nucleotide sequence that encodes at least one strand of the dsRNA of claim 1.

32. A cell comprising the vector of claim 31.

33. The dsRNA of claim 1, wherein said dsRNA, upon contact with a cell expressing said SNCA, inhibits expression of said SNCA gene by at least 40% compared to a cell not so contacted.

34. The dsRNA of claim 1, wherein the dsRNA has an IC50 of 10 pM or less.

35. The dsRNA of claim 1, wherein administration of 0.1 nM of the dsRNA to a NCI-H460 cell or a BE(2)M17 cell results in greater than 50% inhibition of SNCA mRNA expression as measured by a real time PCR assay or administration of 10 nM of the dsRNA to a NCI-H460 cell or a BE(2)M17 cell results in greater than 66% inhibition of SNCA mRNA expression as measured by a real time PCR assay.

36. A method of inhibiting SNCA expression in a cell, the method comprising:

(a) introducing into the cell the dsRNA of claim 1; and

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

37. The method of claim 36, wherein expression is inhibited by at least 20%, 40%, 60%, or at least 80%.

38. A method of treating a disorder mediated by SNCA expression comprising administering to a human in need of such treatment a therapeutically effective amount of the dsRNA of claim 1.

39. A method of treating, preventing or managing a neurodegenerative disorder comprising administering to a patient in need of such treatment, prevention or management a therapeutically or prophylactically effective amount of a dsRNA of claim 1.

40. The method of claim 39, wherein the neurodegenerative disorder is a synucleinopathy.

41. The method of claim 39, wherein the neurodegenerative disorder is Parkinson's disease.

42. The method of claim 39, wherein the neurodegenerative disorder is Alzheimer's disease, multiple system atrophy, or Lewy body dementia.

43. A method of treating a human comprising: identifying a human diagnosed as having a neurodegenerative disorder or at risk for developing the neurodegenerative disorder and administering the dsRNA of claim 1.

44. The method of claim 43, further comprising administering an additional composition.

45. The method of claim 43, further comprising administering a second dsRNA.

46. A method of inhibiting SNCA expression in the brain, the method comprising administering an effective amount of the dsRNA of claim 1 into the brain of a subject to obtain suppression of expression of SNCA mRNA, thereby inhibiting SNCA expression in the brain.

47. The method of claim 46, wherein the administration is by infusion.

48. The method of claim 46, wherein the dsRNA comprises a sense strand of SEQ ID NO: 709 or 679 and/or an antisense strand of SEQ ID NO: 710 or 680.

49. The method of claim 46, wherein the dsRNA is AD-2 1822.

50. The method of claim 46, wherein the dsRNA is AD-2 1810.

51. The method of claim 46, wherein the administration of the dsRNA results in greater than 40%, 50%, 60%, 70%, 80%, or 90% inhibition of SNCA mRNA expression.

52. The method of claim 47, wherein the administration of the dsRNA results in greater than 65% inhibition of SNCA mRNA expression at the end of infusion, greater than 70% inhibition of SNCA mRNA expression one week after infusion, greater than 40% inhibition of SNCA mRNA

expression two weeks after infusion, or greater than 50% inhibition of SNCA mRNA expression four weeks after infusion.

53. The method of claim 47, wherein the administration of the dsRNA results in greater than 50%, 60%, 70%, or 80% inhibition of SNCA mRNA expression after 7 or 28 days of infusion.