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(54) Title: SMALL INTERFERING RNA (SIRNA) MOLECULES FOR MODULATING SUPEROXIDE DISMUTASE (SOD)

(57) Abstract: The invention pertains to using double stranded ribonucleic acid molecules such as small interfering RNA (siRNA) molecules to target an SOD gene to interfere with gene expression and SOD protein production. Method are disclosed for inhibiting expression of a target protein in a subject with a neurological disorder by introducing a small interference ribonucleic acid (siRNA) molecule into the subject with the neurological disorder, such as amyotrophic lateral sclerosis.



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SMALL INTERFERING RNA (SIRNA) MOLECULES FOR MODULATING SUPEROXIDE DISMUTASE (SOD)

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PRIORITY

This application claims priority from U.S. Provisional Application No. 60/636,752 filed December 16, 2004, the contents of which is incorporated herein by reference in its entirety.

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BACKGROUND OF THE INVENTION

Amyotrophic lateral sclerosis (ALS) is the most commonly diagnosed progressive motor neuron disease. The disease is characterized by degeneration of motor neurons in the cortex, brainstem and spinal cord (Principles of Internal Medicine, 1991 McGraw-Hill, Inc., New York; Tandan *et al.* (1985) *Ann. Neurol.*, 18:271-280, 419-431). The cause of the disease is unknown and ALS may only be diagnosed when the patient begins to experience asymmetric limb weakness and fatigue, localized fasciculation in the upper limbs and/or spasticity in the legs which typifies onset. There is increasing evidence that there is a genetic component to at least some incidences of ALS.

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In almost all instances, sporadic ALS and autosomal dominant familial ALS (FALS) are clinically similar (Mulder *et al.* (1986) *Neurology*, 36:511-517). It has been shown that in some but not all FALS pedigrees the disease is linked to a genetic defect on chromosome 21q (Siddique *et al.*, (1991) *New Engl. J. Med.*, 324:1381-1384).

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In particular, mutations in the SOD-1 gene which is localized on chromosome 21q, appear to be associated with the familial form of ALS. The deleterious effects of various mutations on SOD-1 are most likely mediated through a gain of toxic function rather than a loss of SOD-1 activity (Al-Chalabi and Leigh, (2000) *Curr. Opin. Neurol.*, 13, 397-405; Alisky *et al.* (2000) *Hum. Gene Ther.*, 11, 2315-2329). While the toxicity is unclear, there exists evidence to suggest that elimination of the protein itself will ameliorate the toxicity.

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In the last few years, advances in nucleic acid chemistry and gene transfer have inspired new approaches to engineer specific interference with gene expression and protein production. For antisense strategies, stoichiometric amounts of single-stranded

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nucleic acid complementary to the messenger RNA (mRNA) for the gene of interest are introduced into the cell. Some difficulties with antisense-based approaches relate to delivery, stability, and dose requirements. In general, cells do not have an uptake
5 mechanism for single-stranded nucleic acids, hence uptake of unmodified single-stranded material is extremely inefficient. While waiting for uptake into cells, the single-stranded material is also subject to degradation.

A need exists to develop therapies that can alter the course of neurodegenerative diseases or prolong the survival time of patients with such diseases. In particular, a need
10 exists to reduce the SOD-1 protein produced in the brain and spinal cord of ALS patients. Preventing the formation of wild type or mutant SOD-1 protein may stop disease progression and allow for amelioration of ALS symptoms.

SUMMARY OF THE INVENTION

The invention pertains to nucleic acid chemistry and gene transfer to engineer
15 specific interference with gene expression and protein production. In particular, the invention relates to using double stranded ribonucleic acid molecules such as small interfering RNA (siRNA) molecules to target an SOD gene to interfere with gene expression and SOD protein production. The invention relies on generating a small
20 number of siRNA molecules that are able to interfere with SOD gene expression and SOD protein production irrelevant of any particular mutation in the SOD gene.

Although antisense strategies have been used to silence genes, the difficulties associated with antisense technology relating to delivery, stability, dose requirements and degradation, limit the use of this technology. An alternative approach is to use small
25 interfering RNA (siRNA) molecules. With siRNA interference, a small double-stranded RNA can be used to cleave and destroy its cognate RNA, thus inhibiting the expression of the gene and the protein it encodes. The siRNA works by first assembling into an RNA-induced silencing complex (RISC), and then activating the complex by unwinding its RNA strands. The unwound RNA strands subsequently guide the complex to the
30 complementary RNA molecules, where the complex cleaves and destroys the cognate RNA, which results in the RNA interference.

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Accordingly, in one aspect the invention pertains to a method of inhibiting expression of a target protein in a subject with a neurological disorder by introducing a small interference ribonucleic acid (siRNA) molecule into the subject with the neurological disorder. The siRNA comprises a first strand and a second strand hybridized together, and at least one strand of the siRNA is complementary to the nucleotide sequence of a target gene encoding the target protein. The siRNA interacts with an RNA induced silencing complex (RISC) to activate and direct the RISC to the target gene. The destruction of the gene product is promoted, e.g., by cleavage of the mRNA sequence. This in turn prevents transcription and translation of the gene into its corresponding protein, thereby inhibiting expression of the target protein.

The method of the invention can be used to ameliorate any neurological disorder such as Amyotrophic Lateral Sclerosis (ALS), multiple sclerosis, Down's syndrome, Huntington's disease, Parkinson's disease, Spinocerebellar ataxia, Spinomuscular atrophy, Creutzfeldt-Jakob disease, Gerstmann-Straussler-Scheinker disease, and Alzheimer's disease.

The small interfering RNA can be about 15 to about 25 bases in length, preferably about 19 to about 23 bases in length. The small interfering RNA can either be an unmodified small interfering RNA or a modified RNA molecule, for example, modified to be a locked base molecule.

The siRNA are designed to target a gene that encodes a target protein. The target gene can be any gene in the disease causing pathway. For example, the target gene can be the SOD gene and the target protein can be the SOD protein. Preferably, the target gene is the SOD-1 gene, SOD-2 gene, and SOD-3 gene, and the target protein is the SOD-1 protein, SOD-2 protein and the SOD-3 protein, respectively. The SOD-1 gene can be a wild type gene or a mutant gene with at least one mutation. Likewise, the SOD-1 protein can be a wild type protein or a mutant protein with at least one mutation.

The methods of the present invention can be used to substantially inhibit expression of a target gene. As used herein, the term substantially inhibit is intended to mean inhibition of the target gene by at least 10%, more preferably about 20%, more preferably about 30%, 40%, 50%, 60%, 70%, 80%, 90% and 100%. Likewise, the expression of the target protein can be inhibited by at least 10%, more preferably about 20%, more preferably about 30%, 40%, 50%, 60%, 70%, 80%, 90% and 100%.

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In another aspect, the invention pertains to a method of inhibiting expression of a SOD-1 protein in a subject with a neurological disorder by introducing a small interference ribonucleic acid (siRNA) molecule into the subject with the neurological disorder. The siRNA comprises a first strand and a second strand hybridized together, where at least one strand of the siRNA is complementary to the nucleotide sequence of an SOD-1 gene encoding the SOD-1 protein. The siRNA interacts with an RNA induced silencing complex (RISC) to activate and direct the RISC to the SOD-1 gene. Destruction of the SOD-1 gene product is promoted, thereby substantially inhibiting expression of the SOD-1 protein.

In yet another aspect, the invention pertains to a method of ameliorating amyotrophic lateral sclerosis (ALS) in subject by introducing a small interference ribonucleic acid (siRNA) molecule into the subject with the ALS, where the siRNA comprises a first strand and a second strand hybridized together. In some embodiment, ALS is familial ALS, which has been linked to SOD1 mutations. At least one strand of the siRNA is complementary to a nucleotide sequence of wild type SOD-1 gene. The siRNA interacts with an RNA induced silencing complex (RISC) to activate and direct the RISC to the wild type SOD-1 gene. Destruction of the wild type SOD-1 gene product is promoted to inhibit expression of the wild type SOD-1 protein, thereby ameliorating ALS in the subject. In another embodiments, ALS is sporadic ALS. The target genes in sporadic ALS can be identified by performing gene expression profiling on both the mouse and human sporadic patients to identify differentially expressed genes that are common to both. If genes can be found that are altered in both the mouse and human, those genes can be targeted using the methods of this invention.

In some embodiments, at least one strand of the small interfering RNA is complementary to an exon region of a SOD gene. For example, at least one strand of the small interfering RNA is complementary to the region of Exon 3 of the wild type SOD-1 gene. The small interfering RNA is about 15 – 25 bases in length, preferably about 19 bases in length. The small interfering RNA can be an unmodified small interfering RNA or a modified RNA molecule. The SOD-1 gene can be inhibited by at least 10%, more preferably about 20%, more preferably about 30%, 40%, 50%, 60%, 70%, 80%, 90% and 100%. Likewise, the expression of the SOD-1 protein can be inhibited by at least 10%, more preferably about 20%, more preferably about 30%, 40%, 50%, 60%, 70%,

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80%, 90% and 100%. The SOD-1 gene can be the wild type SOD-1 gene or a mutant SOD-1 gene with at least one mutation. The SOD-1 protein can be the wild type SOD-1 protein or a mutant SOD-1 protein with at least one mutation.

5 In another aspect, the invention discloses methods of assessing the ability of an unmodified siRNA sequence to enter the cell. This selection method facilitates the selection of siRNA sequences that exhibit greater potency by virtue of improved access to the cytosolic site of action. The method of identifying a siRNA molecule useful for treating neurological disorders comprises incubating mammalian cells capable of
10 expressing a target gene in the presence of dsRNA test compound in the absence and presence of a transfection reagent; incubating mammalian cells in the presence of a control nucleic acid compound, in the absence and presence of a transfection reagent; assaying the incubated mammalian cells for target gene expression; comparing the expression levels of the target gene. The siRNA molecule is useful for treating
15 neurological disorders when the expression level in the presence of the dsRNA and in the absence of the transfection reagent is substantially modified when compared to the control levels (i.e., the siRNA molecule in the presence of the transfection agent, the control nucleic acid in the presences and absence of the transfection reagent). The assaying step can further include assaying for protein activity. The target gene can be a
20 SOD gene, i.e., SOD-1. The method allows for selection of siRNA sequences with improved cell permeability and ability to reach and contact their intracellular target based on their ability to modify target gene expression in cultured cells without the use of transfection reagents. Identified siRNA sequences can then be evaluated further in vivo for their ability to modify the function and/or expression level of a target gene.

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BRIEF DESCRIPTION OF THE FIGURES

Figure 1 is a bar chart depicting the effect of incubation with various siRNA on SOD-1 protein expression in HeLa cells;

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Figure 2 is a bar chart depicting decreased SOD levels in spinal cord of mice following intrathecal delivery of siRNA.

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DETAILED DESCRIPTION OF THE INVENTION

The practice of the present invention employs, unless otherwise indicated, conventional methods of microbiology, molecular biology and recombinant DNA techniques within the skill of the art. Such techniques are explained fully in the literature. (See, e.g., Sambrook, *et al.* Molecular Cloning: A Laboratory Manual (Current Edition); DNA Cloning: A Practical Approach, Vol. I & II (D. Glover, ed.); Oligonucleotide Synthesis (N. Gait, ed., Current Edition); Nucleic Acid Hybridization (B. Hames & S. Higgins, eds., Current Edition); Transcription and Translation (B. Hames & S. Higgins, eds., Current Edition); CRC Handbook of Parvoviruses, vol. I & II (P. Tijessen, ed.); Fundamental Virology, 2nd Edition, Vol. I & II (B. N. Fields and D. M. Knipe, eds.))

So that the invention is more clearly understood, the following terms are defined:

The phrase "double-stranded ribonucleic acid molecule" or "dsRNA" as used herein refers to any RNA molecule, fragment or segment containing two strands forming an RNA duplex, notwithstanding the presence of single stranded overhangs of unpaired nucleotides. Further, as used herein, a double-stranded ribonucleic acid molecule includes single stranded RNA molecules forming functional stem-loop structures, such as small temporal RNAs, short hairpin RNAs and microRNAs, thereby forming the structural equivalent of an RNA duplex with single strand overhangs. The RNA molecule of the present invention may be isolated, purified, native or recombinant, and may be modified by the addition, deletion, substitution and/or alteration of one or more nucleotides, including non-naturally occurring nucleotides, also including those added at 5' and/or 3' ends to increase nuclease resistance.

The double-stranded ribonucleic acid molecule may be any one of a number of non-coding RNAs (i.e., RNA which is not mRNA, tRNA or rRNA), including, preferably, a small interfering RNA, but may also comprise a small temporal RNA, small nuclear RNA, small nucleolar RNA, short hairpin RNA or a microRNA having either a double-stranded structure or a stem loop configuration comprising an RNA duplex with or without single strand overhangs. The double-stranded RNA molecule may be very large, comprising thousands of nucleotides, or preferably in the case of siRNA protocols involving mammalian cells, may be small, in the range of about 15 to about 25 nucleotides, preferably in the range of about 15 to about 19 nucleotides.

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The phrase "small interfering RNA" or "siRNA" as used herein, refers to a double stranded RNA duplex of any length, with or without single strand overhangs, wherein at least one strand, putatively the antisense strand, is homologous to the target mRNA to be degraded. The difference between antisense and double stranded small interfering molecules is that an antisense molecule is a single stranded oligonucleotide which is complementary to a section of the target RNA and must hybridize or bind to it in a 1:1 ratio in order to cause its degradation. In contrast, siRNA provides a substrate for the RNA-induced silencing complex (RISC), and unlike antisense, is inactive until incorporated into this macromolecular complex. This RISC complex is then guided by the unwound siRNA to its target gene. Once the target gene is located, it is destroyed by cleaving the target gene into small pieces, and thereby preventing its expression.

In a preferred embodiment, the siRNA of the present invention comprises a double-stranded RNA duplex of at least about 15, or preferably at least about 19, nucleotides with no overhanging nucleotides. In another embodiment, the siRNA of the present invention has nucleotide overhangs. For example, the siRNA may have two nucleotide overhangs, thus the siRNA will comprise a 21 nucleotide sense strand and a 21 nucleotide antisense strand paired so as to have a 19 nucleotide duplex region. The number of nucleotides in the overhang can be in the range of about 1 to about 6 homologous nucleotide overhangs at each of the 5' and 3' ends, preferably, about 2-4, more preferably, about 3 homologous nucleotide overhangs at each of the 5' and 3' ends. The nucleotides overhang can be modified, for example to increase nuclease resistance. For example, the 3' overhang can comprise 2' deoxynucleotides, e.g., TT, for improved nuclease resistance.

The term "homology" or "identity" as used herein refers to the percentage of likeness between nucleic acid molecules. To determine the homology or percent identity of two amino acid sequences or of two nucleic acid sequences, the sequences are aligned for optimal comparison purposes (e.g., gaps can be introduced in one or both of a first and a second amino acid or nucleic acid sequence for optimal alignment and non-homologous sequences can be disregarded for comparison purposes). In a preferred embodiment, the length of a reference sequence aligned for comparison purposes is at least 30%, preferably at least 40%, more preferably at least 50%, even more preferably at least 60%, and even more preferably at least 70%, 80%, or 90% of the length of the

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reference sequence. The amino acid residues or nucleotides at corresponding amino acid positions or nucleotide positions are then compared. When a position in the first sequence is occupied by the same amino acid residue or nucleotide as the corresponding position in the second sequence, then the molecules are identical at that position (as used
5 herein amino acid or nucleic acid “identity” is equivalent to amino acid or nucleic acid “homology”). The percent identity between the two sequences is a function of the number of identical positions shared by the sequences, taking into account the number of gaps, and the length of each gap, which need to be introduced for optimal alignment of
10 the two sequences.

The comparison of sequences and determination of percent identity between two sequences can be accomplished using a mathematical algorithm. For example, the percent identity between two amino acid sequences can be determined using the Needleman and Wunsch ((1970) *J. Mol. Biol.* (48):444-453) algorithm which has been
15 incorporated into the GAP program in the GCG software package,, using either a Blossom 62 matrix or a PAM250 matrix, and a gap weight of 16, 14, 12, 10, 8, 6, or 4 and a length weight of 1, 2, 3, 4, 5, or 6. In another example, the percent identity between two nucleotide sequences is determined using the GAP program in the GCG software package,, using a NWSgapdna.CMP matrix and a gap weight of 40, 50, 60, 70,
20 or 80 and a length weight of 1, 2, 3, 4, 5, or 6. In yet another example, the percent identity between two amino acid or nucleotide sequences is determined using the algorithm of E. Meyers and W. Miller (CABIOS, 4:11-17 (1989)) which has been incorporated into the ALIGN program (version 2.0), using a PAM120 weight residue table, a gap length penalty of 12 and a gap penalty.

25 The phrase “homologous” particularly refers to a nucleotide sequence that has at least 80% sequence identity, preferably at least 90% sequence identity, more preferably at least 95% sequence identity, and even more preferably at least 98% and 99% sequence identity, to a portion of mRNA transcribed from the target gene, e.g., the SOD-1 gene. The most preferred embodiment of the invention comprises a siRNA having
30 100% sequence identity with the target mRNA, the e.g., SOD-1 protein. Specifically, the small interfering RNA must be of sufficient homology to guide the RNA-induced silencing complex (RISC) to the target mRNA for degradation. Limited mutations in siRNA relative to the target mRNA are also within the scope of the invention.

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The term "complement" refers to a nucleotide sequence which is complementary to an indicated sequence and which is able to hybridize to the indicated sequences.

5 In a preferred embodiment of the invention, at least a portion of one strand of the double-stranded ribonucleic acid molecule (i.e., the antisense strand) homologous to a portion of mRNA transcribed from the SOD-1 gene, preferably the human SOD-1 gene, and most preferably to exon 3 of the human SOD-1 gene. The double-stranded ribonucleic acid can be a small interfering RNA molecule selected from the siRNAs shown in Tables 1, 2, and 3.

10 Also included within the present invention are sequence variants of the polynucleic acids as selected from any of the nucleotide sequences as given in any of the given SEQ ID numbers or listed in Tables 1-3 with sequence variants containing either deletion and/or insertions of one or more nucleotides, especially insertions or deletions of 1 or more codons, mainly at the extremities of oligonucleotides (either 3' or 5'), or
15 substitutions of some non-essential nucleotides by others (including modified nucleotides an/or inosine). Other preferred variant polynucleic acids of the present invention include sequences which are redundant as a result of the degeneracy of the genetic code compared any of the above-given polynucleic acids of the present invention. These variant polynucleic acid sequences will thus encode the same amino
20 acid sequence as the polynucleic acids they are derived from.

Particularly preferred variant polynucleic acids of the present invention include also sequences which hybridize under stringent conditions with any of the polynucleic acid sequences of the present invention. Particularly, sequences which show a high degree of homology (similarity) to any of the polynucleic acids of the invention as
25 described above. Particularly sequences which are at least 80%, 85%, 90%, 95% or more homologous to said polynucleic acid sequences of the invention. Preferably said sequences will have less than 20%, 15%, 10%, or 5% variation of the original nucleotides of said polynucleic acid sequence.

Polynucleic acid sequences according to the present invention which are
30 homologous to the sequences as represented by a SEQ ID NO can be characterized and isolated according to any of the techniques known in the art, such as amplification by means of sequence-specific primers, hybridization with sequence-specific probes under

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more or less stringent conditions, serological screening methods or via the LiPA typing system.

5 The term “inhibit” or “inhibiting” as used herein refers to a measurable reduction of expression of a target gene or a target protein. The term also refers to a measurable reduction in the activity of a target protein. Preferably a reduction in expression is at least about 10%. More preferably the reduction of expression is about 20%, 30%, 40%, 50%, 60%, 80%, 90% and even more preferably, about 100%.

10 The phrase “a disorder associated with SOD activity” or “a disease associated with SOD activity” as used herein refers to any disease state associated with the expression of SOD protein (e.g., SOD-1, SOD-2, SOD-3, and the like). In particular, this phrase refers to the gain of toxic function associated with SOD protein production. The SOD protein can be a wild type SOD protein or a mutant SOD protein and can be derived from a wild type SOD gene or an SOD gene with at least one mutation.

15 The phrase “a disorder associated with SOD-1 activity” or “a disease associated with SOD-1 activity” as used herein refers to any disease state associated with the expression of SOD-1 protein, for example, ALS. In particular, this phrase refers to the gain of toxic function associated with SOD-1 protein production. The SOD-1 protein can be a wild type SOD-1 protein or a mutant SOD-1 protein and can be derived from a wild type SOD-1 gene or an SOD-1 gene with at least one mutation.

20 The term “subject” as used herein refers to any living organism in which an immune response is elicited. The term subject includes, but is not limited to, humans, nonhuman primates such as chimpanzees and other apes and monkey species; farm animals such as cattle, sheep, pigs, goats and horses; domestic mammals such as dogs and cats; laboratory animals including rodents such as mice, rats and guinea pigs, and the like. The term does not denote a particular age or sex. Thus, adult and newborn subjects, as well as fetuses, whether male or female, are intended to be covered.

25 The terms “neurological disorder” and “neurodegenerative disorder,” “neuromuscular disorder,” as used interchangeably herein refer to an impairment or absence of a normal neurological function or presence of an abnormal neurological function in a subject. For example, neurological disorders can be the result of disease, injury, and/or aging. As used herein, neurological disorder also includes neurodegeneration which causes morphological and/or functional abnormality of a

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neural cell or a population of neural cells. Non-limiting examples of morphological and functional abnormalities include physical deterioration and/or death of neural cells, abnormal growth patterns of neural cells, abnormalities in the physical connection
5 between neural cells, under- or over production of a substance or substances, *e.g.*, a neurotransmitter, by neural cells, failure of neural cells to produce a substance or substances which it normally produces, production of substances, *e.g.*, neurotransmitters, and/or transmission of electrical impulses in abnormal patterns or at abnormal times. Neurodegeneration can occur in any area of the brain of a subject and is seen with many
10 disorders including, for example, Amyotrophic Lateral Sclerosis (ALS), multiple sclerosis, Down's syndrome, Huntington's disease, Parkinson's disease, Spinocerebellar ataxia, Spinomuscular atrophy, Creutzfeldt-Jakob disease, Gerstmann-Straussler-Scheinker disease, and Alzheimer's disease.

"Amyotrophic lateral sclerosis" or "ALS" are terms understood in the art and as
15 used herein to denote a progressive neurodegenerative disease that affects upper motor neurons (motor neurons in the brain) and/or lower motor neurons (motor neurons in the spinal cord) and results in motor neuron death. As used herein, the term "ALS" includes all of the classifications of ALS known in the art, including, but not limited to classical ALS (typically affecting both lower and upper motor neurons), Primary Lateral Sclerosis
20 (PLS, typically affecting only the upper motor neurons), Progressive Bulbar Palsy (PBP or Bulbar Onset, a version of ALS that typically begins with difficulties swallowing, chewing and speaking), Progressive Muscular Atrophy (PMA, typically affecting only the lower motor neurons) and familial ALS (a genetic version of ALS).

The term "therapeutically effective amount" refers to an amount effective, at
25 dosages and for periods of time necessary, to achieve the desired therapeutic result. A therapeutically effective amount of the pharmacological agent may vary according to factors such as the disease state, age, sex, and weight of the individual, and the ability of the pharmacological agent to elicit a desired response in the individual. A therapeutically effective amount is also one in which any toxic or detrimental effects of
30 the pharmacological agent are outweighed by the therapeutically beneficial effects.

The term "prophylactically effective amount" refers to an amount effective, at dosages and for periods of time necessary, to achieve the desired prophylactic result. Typically, since a prophylactic dose is used in subjects prior to or at an earlier stage of

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disease, the prophylactically effective amount will be less than the therapeutically effective amount.

5 I. RNA Interference

 In one aspect, the invention pertains to using a double stranded RNA molecule to interfere with gene expression and protein production. Antisense technology is the most commonly cited approach for achieving post-transcriptional gene silencing. However, RNA interference with double stranded RNA (dsRNA) molecules has numerous
10 advantages over antisense technology. For example, cellular uptake of unmodified antisense nucleic acid is very inefficient, therefore a large amount of antisense nucleic acid needs to be synthesized and applied in order to achieve and maintain a sufficient concentration in the target cells, which is usually at or above the level of the endogenous target mRNA. Therefore, a successful antisense strategy requires the introduction of
15 large amounts of single-stranded antisense nucleic acid (DNA or RNA) into cells. In contrast, the cellular uptake of double-stranded RNA is more efficient, thereby permitting RNA interference to occur with much smaller amounts of dsRNA.

 When double-stranded RNA (dsRNA) is introduced into a cell, it has the ability to silence the expression of a homologous gene within the cell, i.e., “interfere” with gene
20 expression. In 1998, Fire *et al.* demonstrated the efficacy of RNA interference by injecting the gut of *C. elegans* with a dsRNA that had been prepared *in vitro* (Fire, *et al.* (1998) *Nature*, 391, 806-811). The injection of dsRNA into *C. elegans* resulted in loss of expression of the homologous target gene, not only throughout the worm, but also in its progeny.

25 The difference between antisense and double stranded small interfering molecules is that antisense molecule is a single stranded oligonucleotide which is complementary to a section of the target RNA and must hybridize or bind to it in a 1:1 ratio in order to cause it's degradation. In contrast, siRNA provides a substrate for the RNA-induced silencing complex (RISC), and unlike antisense, is inactive until
30 incorporated into this macromolecular complex.

 More specifically, in eukaryotes, the current model of the RNA interference mechanism involves both an initiation and an effector step. In the initiation step, a processing enzyme cleaves the introduced dsRNA into small interfering RNAs of 21-23

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nucleotides. In the effector step, each siRNA is incorporated into an RNA induced silencing complex ("RISC"), comprising a helicase, an exonucleolytic nuclease, and an endonucleolytic nuclease. The siRNA, now incorporated into the RISC, serves as a guide molecule, directing the RISC to the homologous mRNA transcript for degradation (Hammond, *et al.*, "Post-transcriptional gene silencing by double-stranded RNA," *Nature Rev. Gen.*, 2, 110-119). The RISC complex is led to the intended mRNA by the incorporated siRNA molecule and catalyzes the cleavage of multiple copies of the mRNA, whereas the antisense sequence is destroyed after mediating the cleavage a single mRNA molecule. Double stranded small interfering molecules have the advantage of being more stable than single stranded RNA, and being more effective at inhibition at lower concentrations than single stranded RNA. In addition, siRNA does not require the use of viral vectors.

Other double stranded RNA molecules are also included within the scope of the invention. A growing number of RNAs do not function as messenger RNAs, transfer RNAs or ribosomal RNAs. These so-called "non-coding" RNAs describe a wide variety of RNAs of incredibly diverse function, ranging from the purely structural to the purely regulatory (Riddihough, (2002) *Science*, 296, 1259). The non-coding RNA that has generated the most interest, however, is the "small interfering RNA" or "siRNA" associated with the phenomenon of RNA interference ("RNAi"). Other representative non-coding RNAs include small nuclear RNAs, involved in the splicing of pre-mRNAs in eukaryotes (Will *et al.*, (2001) *Curr. Opin. Cell Biol.*, 13, 290), small nucleolar RNAs, which direct 2'-O-ribose methylation and pseudouridylation of rRNA and tRNA (Kiss, (2001) *EMBO J.*, 20, 3617) and "micro-RNAs" ("miRNAs"), very small RNAs of approximately 22 nucleotides in length which appear to be involved in various aspects of mRNA regulation and degradation. Two miRNAs characterized in some detail are the "small temporal RNAs" ("stRNAs") lin4 and let7, which control developmental timing in the nematode worm *C. elegans* and repress the translation of their target genes by binding to the 3' untranslated regions of their mRNAs (Riddihough, (2002) *Supra*); Ruvkun, (2001) *Science*, 294, 797; Grosshans, *et al.*, (2002) *J. Cell. Biol.* 156, 17). Also known are the short hairpin RNAs ("shRNAs"), patterned from endogenously encoded triggers of the RNA interference pathway (Paddison, *et al.*, (2002) *Genes and Dev.*, 16:948-958).

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In the present invention, siRNA are introduced into the cell rather than large dsRNA molecules, thus circumventing the initiation step of the mechanism. Although composed of two structural elements that resemble oligonucleotides used in antisense gene inhibition, the siRNA molecule has clear structural distinctions from the former. A
5 siRNA molecule is composed of two complementary strands of RNA that must be hybridized with one another. There must be base-pair overhangs at each end of the molecule. Although the two oligonucleotides used for siRNA are the same length as those used for antisense, they will not be incorporated into the RISC complex unless
10 they form this RNA duplex.

Where the siRNA of the present invention is delivered to a cell for the purposes of inhibiting expression of a target gene within the cell, at least one strand of the small interfering RNA is homologous to a portion of mRNA transcribed from the target gene, e.g., wild type SOD-1. In a preferred embodiment, the siRNA strand is at least 85%
15 homologous to a portion of mRNA transcribed from the target gene. Preferably, the siRNA strand is 90% homologous, more preferably is 95% homologous, and even more preferably, is 98% and 99% homologous to a portion of mRNA transcribed from the target gene, e.g., wild type SOD-1. In the most preferred embodiment, at least one strand of the siRNA is 100% homologous to a portion of mRNA transcribed from the
20 target gene, e.g., wild type SOD-1.

In one embodiment, at least one siRNA molecule can be delivered to the cell, for example an siRNA molecule associated with a region of the SOD-1 gene, e.g., the exon 3 region of the SOD-1 gene. In another embodiment, a plurality of siRNA molecules can be delivered to the cell, for example, a plurality of siRNA molecules associated with
25 one region of the SOD-1 gene, e.g., exon 3 region. In another embodiment, the plurality of siRNA molecules can be associated with different regions of the SOD-1 gene, for example, exon-1, and exon-3; or exon-2, exon-3, and exon-4; or exon-1, exon-2, exon-4, and exon-4, and so forth. Thus, it will be appreciated that the scope of the invention covers any combination of siRNA molecules that can target and interfere with one or
30 more desired regions of the SOD-1 gene.

The target gene may be an endogenous gene in relation to the cell, as in the case of a regulatory gene or a gene coding for a native protein, or it may be heterologous in relation to the cell, as in the case of a viral or bacterial gene, transposon, or transgene.

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In either case, uninhibited expression of the target gene may result in a disease or a condition. To inhibit expression of the target gene, the cell is contacted with the siRNA in an amount sufficient to inhibit expression of the target gene, e.g., wild type SOD-1.

5 The cell receiving the siRNA of the present invention may be isolated, within a tissue, or within an organism. It may be an animal cell, a plant cell, a fungal cell, a protozoan, or a bacterium. An animal cell may be derived from vertebrates or invertebrates, but in a preferred embodiment of the invention, the cell is derived from a mammal, such as a rodent or a primate, and even more preferably, is derived from a
10 human. The cell may be of any type, including neural cells, neuronal cells, epithelial cells, endothelial cells, muscle cells or nerve cells. Representative cell types include, but are not limited to, microglia, myoblasts, fibroblasts, astrocytes, neurons, oligodendrocytes, macrophages, myotubes, lymphocytes, NIH3T3 cells, PC12 cells, and neuroblastoma cells. Such delivery may be accomplished either *in vitro* or *in vivo* by
15 standard techniques.

 The siRNA can be obtained by chemical synthesis or by DNA-vector based RNA interference technology. Custom siRNAs can be generated on order from Dharmacon Research, Inc., Lafayette, Colo. Other sources for custom siRNA preparation include Xeragon Oligonucleotides, Huntsville, Ala. and Ambion of Austin, Tex. Alternatively,
20 siRNAs can be chemically synthesized using ribonucleoside phosphoramidites and a DNA/RNA synthesizer. In the present invention, the siRNA molecules were chemically synthesized using the Invitrogen commercially available technique with ribonucleoside phosphoramidites and a DNA/RNA synthesizer.

 Using DNA vector based siRNA technology, a small DNA insert (about 70 bp)
25 encoding a short hairpin RNA targeting the gene of interest is cloned into a commercially available vector. The insert-containing vector can be transfected into the cell, and it expresses the short hairpin RNA. The hairpin RNA is rapidly processed by the cellular machinery into 19-22 nt double stranded RNA (siRNA). The following is a list of commercially available GenScript siRNA expression vectors: U6 like promoter:
30 pRNA-U6.1/Neo, pRNA-U6.1/Hygro, pRNA-U6.1/Zeo, pRNAT-U6.1/Neo (with GFP marker), pRNAT-U6.1/Hygro (with GFP marker). H1 like promoter: pRNA-H1.1/Neo, pRNA-H1.1/Hygro, pRNA-H1.1/Zeo, pRNAT-H1.1/Neo (with GFP marker), pRNAT-H1.1/Hygro (with GFP marker).

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To improve hybridization, locked bases, which differ from native RNA bases in that they contain a 2'-O, 4'-C methylene bridge, can be used. By chemically modifying the siRNA, enhanced hybridization and improved biostability, can be achieved. The
5 siRNA can be chemically modified at either or both the 5' and 3' end bases to increase stability, hybridization, and cellular uptake. The molecules can be modified using the locked base technology described by Proligo in U.S. 6,794,499 and U. S. 6,670,461, incorporated herein by reference.

The siRNA can be chemically modified, for example, by N-type modification to
10 produce a linked nucleic acid (LNA). LNA is a synthetic nucleic acid analogue, incorporating "internally bridged" nucleoside analogues. Synthesis of LNA, and properties thereof, have been described by a number of authors: Nielsen *et al.*, (1997) *J. Chem. Soc. Perkin Trans. 1*, 3423; Koshkin *et al.*, (1998) *Tetrahedron Letters* 39, 4381; Singh & Wengel (1998) *Chem. Commun.* 1247; and Singh *et al.*, (1998) *Chem. Commun.*
15 455. LNA exhibits greater thermal stability when paired with DNA, than do conventional DNA/DNA heteroduplexes.

A sugar engineered into an N-type (RNA-like) pucker usually conveys an increase in helical thermostability when hybridized with complementary RNA (Freier *et al.* (1997) *Nucleic Acids Res.* 25, 4429-4443). Prominent examples of such N-type
20 nucleic acid analogues are 2'-O-alkylated RNA (Manoharan (1999) *Biochim. Biophys. Acta* 1489, 117-130), 2'-F-RNA (Kawasaki *et al.* (1993) *J. Med. Chem.* 36, 831-841), phosphoramidates (Gryaznov (1999) *Biochim. Biophys. Acta* 1489, 131-140), HNA (Hendrix *et al.* (1997) *Chem. Eur. J.* 3, 1513-1520), and LNA (Koshkin, *et al.* (1998) *Tetrahedron* 54, 3607-3630; Obika, *et al.* (1998) *Tetrahedron Lett.* 39, 5401-5404; Wengel, (1999) *Acc. Chem. Res.* 32, 301-310; and Petersen (2003) *Trends Biotechnol.*
25 21, 74-81).

In LNA, the furanose conformation is chemically locked in an N-type (C3'-endo) conformation by the introduction of a 2'-O,4'-C methylene linkage. LNAs have shown high thermal affinities when hybridized with either DNA ($T_m = 1-8^\circ\text{C}$ per modification)
30 (Koshkin *et al.* (1998) *Tetrahedron* 54, 3607-3630; Obika, *et al.* (1998) *Tetrahedron Lett.* 39, 5401-5404; Wengel. (1999) *Acc. Chem. Res.* 32, 301-310; Petersen, *et al.* (2003) *Trends Biotechnol.* 21, 74-81; Kværn, *et al.* (2000) *J. Org. Chem.* 65, 5167-5176; and Braasch, *et al.* (2001) *Chem. Biol.* 8, 1-7), RNA ($T_m = 2-10^\circ\text{C}$ per modification)

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(Braasch, (2001) *Chem. Biol.* 8, 1-7; Bondensgaard, et al. (2000) *Chem. Eur. J.* 6, 2687-2695; and Kurreck et al. (2002) *Nucleic Acids Res.* 30, 1911-1918) or LNA ($T_m > 5^\circ\text{C}$ per modification) (Koshkin, (1998) *J. Am. Chem. Soc.* 120, 13252-13253).

5

II. SOD and SOD Mutations

The invention pertains to eliminating the SOD-1 protein, particularly wild type SOD-1 protein in cells by causing the degradation of the mRNA encoding SOD-1 protein using dsRNA, interference, specifically with siRNA molecules. The siRNA
10 generated will target the human wild type SOD-1 mRNA in regions that do not contain mutations. This strategy allows the silencing of the bulk of familial mutations without designing individual molecules for each mutation. While the target of the siRNA will be the wild type SOD-1 protein, sequences that target mutations in SOD-1 are also within the scope of the invention.

15 The SOD-1 gene is localized to chromosome 21q22.1. SOD-1 sequences are disclosed in PCT publication WO 94/19493 are oligonucleotide sequences encoding SOD-1 and generally claimed is the use of an antisense DNA homolog of a gene encoding SOD-1 in either mutant and wild-type forms in the preparation of a medicament for treating a patient with a disease (Brown et al., 1994).

20 The nucleic acid sequence of human SOD-1 gene can be found at Genbank accession no. NM_000454. The nucleotide sequence of human SOD-1 is also presented in SEQ ID NO: 1. the underlined regions are the exon regions. The corresponding SOD-1 protein sequence is presented in SEQ ID NO: 2. The siRNA molecules were designed around exon 3 of the SOD-1 gene. The entire sequence of exon 3 is disclosed
25 in SEQ ID NO: 3. siRNA molecules that can be used to inhibit the SOD-1 gene are disclosed in Table 1, and preferred siRNA molecules that inhibit expression of the SOD-1 gene are described in the Examples section.

The siRNA molecules are all sequences are listed in the 5' – 3' direction, with the sense sequence of the pair listed first. All sequences were rigorously tested for
30 similarity with known human mRNAs in GeneBank using the Blast algorithm for short, nearly exact matches. Examples of some preferred sequences are shown in the Examples section. These and other siRNA sequences can readily be made using the methods and sequences disclosed in the invention.

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RNA interference with siRNA produces a measurable reduction of expression of a target gene or a target protein. Preferably a reduction in expression is at least about 10%. More preferably the reduction of expression is about 20%, 30%, 40%, 50%, 60%, 80%, 90% and even more preferably, about 100%.

III. Delivery of Double Stranded RNA

Previous methods of delivering double stranded RNA primarily involve transfection (for general transfection protocols, see Elbashir, *et al.*, (2001) *Nature*, 411, 494-498; Elbashir, *et al.*, (2001b) *Genes & Dev.*, 15, 188-200). The efficiency of transfection depends on cell type, passage number and the confluency of the cells. The time and the manner of formation of dsRNA are also critical. One example of transfection of siRNA molecules includes using U6 and CMV promoters in any suitable transfection vector.

Yet another method of delivering double stranded molecules to a cell involves using cell-penetration enhancing peptides conjugated to the double stranded molecules. The membrane shuttling proteins such as the *Drosophila* homeobox protein Antennapedia, the HIV-1 transcriptional factor TAT and VP22 from HSV-1 can be conjugated to the siRNA molecule to increase its cellular uptake and thus efficacy.

Other techniques for dsRNA uptake include electroporation, injection, liposome-facilitated transport, and microinjection. Although direct microinjection of dsRNA into cells is generally considered to be the most effective means known for inducing RNA interference, the characteristics of this technique severely limit its practical utility. In particular, direct microinjection can only be performed *in vitro*, which limits its application to gene therapy. Furthermore, only one cell at a time can be microinjected, which limits the technique's efficiency. As a means of introducing dsRNA into cells, electroporation is also relatively impractical because it is not possible *in vivo*. Finally, while dsRNA can be introduced into cells using liposome-facilitated transportation or passive uptake. The siRNA sequences can be assessed for their ability to inhibit gene expression in cultured cells in the absence of transfection reagent. In a preferred embodiment, the siRNA is delivered intraspinally without a gene therapy vector. Delivery of siRNA molecules can also be accomplished by passive cellular uptake *in vivo* (see United States Patent Application 20040248174).

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It is also possible to introduce dsRNA indirectly into cells, by transforming the cells with expression vectors containing DNA coding for dsRNA (*See, e.g.,* U.S. Pat. No. 6,278,039, U.S. published application 2002/0006664, WO 99/32619, WO 01/29058, 5 WO 01/68836, and WO 01/96584). Cells transformed with the dsRNA-encoding expression vector will then produce dsRNA *in vivo*.

Another delivery method involves delivering naked siRNA molecules directly into the central nervous system of the subject. This can be accomplished by using a ventricular Omayia reservoir spinal catheter (e.g., portacath). Alternatively, direct 10 delivery of the siRNA molecules can be accomplished by using continuous spinal infusion using pump technologies (e.g., for Medtronic pump). For continuous spinal infusion, the lumbar catheterization protocol can be conducted by initially preparing a catheter using for example, polyethylene tubing (PE10) with outer diameter of about 0.6 mm, and a total tubing length of about 4.5 cm. A thin tungsten wire (e.g., with a 15 diameter of about 0.12 mm) can be inserted into the PE10 tube as a guide wire. One end of the tubing can be stretched so the outer diameter shrinks. A triple knot can be made with silk suture at each end of the tubing in order to provide anchor points for the tubing after catheter implantation. An ALZET pump can be filled and primed with at least one siRNA molecule formulated in a delivery vehicle such as saline, dextrose, 20 artificial cerebrospinal fluid, and the like. The siRNA can be delivered at a rate of about 6µl/day. It will be appreciated that the volume of the siRNA formulation, and the rate at which it is delivered will depend on the size and weight of the subject. An adapter tube can be made using 0.69mmID tubing cut to approximately 5mm.

To implant the catheter, the subject, e.g., mice can be anesthetized with 25 ketamine/domitor combination IP injection. The mice can be injected with Buprenex as a pain medication. A 2 cm longitudinal skin incision can be made above vertebrae L5 and L6. While holding the mouse's pelvic girdle firmly, a hole can be made in the muscle at the L5 and L6 junction using a 23 gauge needle. The needle can be gently pressed and spun through the muscle tissue. The catheter with metal wire inside can be 30 pushed into the side of the L5-6 process initially at a 70 degree angle from the vertebral column. The angle can be flattened once resistance is reached until the catheter and wire is about 20-30 degrees from the vertebral column. The catheter with the wire can be pushed through the intervertebral space and dura until the sign of dura penetration (tail

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flick and/or hind limb quiver) occurs. At this point the guide wire is withdrawn in order to protect the spinal cord from damage. The catheter is then fed into the vertebral space until the silk suture knot rests adjacent to the hole in the muscle. A knot is tied through the fascia that rests superficially to the lumbar muscle so that the knot anchors the original silk catheter knot into its place. This keeps the catheter in place. The ALZET pump is attached to the catheter tubing using an adhesive and adaptor tube. The pump is implanted in the skin pocket. The second silk knot is anchored to the fascia at the neck with a suture knot. The incision is closed and the mice are dosed with Antisedan in order to counteract the Domitor.

Where delivery is made *in vivo* to a living organism, administration may be by any procedure known in the art, including but not limited to, oral, parenteral, intraspinal, intracisternal, subdural, rectal, intradermal, transdermal, intramuscular, or topical administration. To facilitate delivery, the dsRNA may be formulated in various compositions with a pharmaceutically acceptable carrier, excipient or diluent. "Pharmaceutically acceptable" means the carrier, excipient or diluent of choice does not adversely affect the biological activity of the dsRNA, or the recipient of the composition.

Suitable pharmaceutical carriers, excipients and/or diluents include, but are not limited to, lactose, sucrose, starch powder, talc powder, cellulose esters of alkoanoic acids, magnesium stearate, magnesium oxide, crystalline cellulose, methyl cellulose, carboxymethyl cellulose, gelatin, glycerin, sodium alginate, gum arabic, acacia gum, sodium and calcium salts of phosphoric and sulfuric acids, polyvinylpyrrolidone and/or polyvinyl alcohol, saline, and water.

For oral administration, the composition may be presented as capsules or tablets, powders, granules or a suspension. The composition may be further presented in convenient unit dosage form, and may be prepared using a controlled-release formulation, buffering agents and/or enteric coatings.

For parenteral administration (i.e., subcutaneous, intravenous, or intramuscular administration), the dsRNA may be dissolved or suspended in a sterile aqueous or non-aqueous isotonic solution, containing one or more of the carriers, excipients or diluents noted above. Such formulations may be prepared by dissolving a composition containing the dsRNA in sterile water containing physiologically compatible substances such as

sodium chloride, glycine, and the like, and having a buffered pH compatible with physiological conditions to produce an aqueous solution. Alternatively, a composition containing the dsRNA may be dissolved in non-aqueous isotonic solutions of
5 polyethylene glycol, propylene glycol, ethanol, corn oil, cottonseed oil, peanut oil, etc.

The dsRNA may be administered by formulation with any suitable carrier that is solid at room temperature but dissolves at body temperature. Such carriers include cocoa butter, synthetic mono-, di-, or tri-glycerides, fatty acids, polyethylene glycols, glycerinated gelatin, hydrogenated vegetable oils, and the like.

10 Intradermal administration of the dsRNA, i.e., administration via injectable preparation, may be accomplished by suspending or dissolving the dsRNA in a non-toxic parenterally acceptable diluent or solvent, e.g., as a solution in 1,3-butanediol, water, Ringer's solution, and isotonic sodium chloride solution. Occasionally, sterile fixed oils or fatty acids are employed as a solvent or suspending medium.

15 For transdermal or topical administration, the dsRNA may be combined with compounds that act to increase the permeability of the skin and allow passage of the dsRNA into the bloodstream. Such enhancers include propylene glycol, polyethylene glycol, isopropanol, ethanol, oleic acid, N-methylpyrrolidone, and the like. Delivery of such compositions may be via transdermal patch or iontophoresis device.

20 Specific formulations of compounds for therapeutic treatment are discussed in Hoover, J. E., Remington's Pharmaceutical Sciences (Easton, Pa.: Mack Publishing Co., 1975) and Liberman, H. A., and Lachman, L., Eds., Pharmaceutical Dosage Forms (New York, N.Y.: Marcel Decker Publishers, 1980).

25 The quantity of dsRNA administered to tissue or to a subject should be an amount that is effective to inhibit expression of the target gene within the tissue or subject, and are readily determined by the practitioner skilled in the art. Specific dosage will depend further upon the dsRNA, e.g., siRNA used, the target gene to be inhibited and the cell type having target gene expression. Quantities will be adjusted for the body weight of the subject and the particular disease or condition being targeted.

30 A stable cell line with a specific gene knocked-out can be established, and its phenotype can be studied. A knock-out mouse line can be established using transgenic dsRNA, e.g., siRNA method (Kunach *et al.* (2003) *Nature Biotechnology* 21:559-561). dsRNA can be inserted into a vector with an inducible promoter to study its effect. The

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dsRNA can be delivered by using for example, a viral vector (Shen *et al.* (2003) *FEBS Lett* 539(1-3):111-114; and Barton *et al.* (2002) *Proc Natl Acad Sci US A* 99(23):14943-14945) and used for gene therapy purpose.

One skilled in the art will appreciate further features and advantages of the invention based on the above-described embodiments. Accordingly, the invention is not to be limited by what has been particularly shown and described, except as indicated by the appended claims. All publications and references cited herein are expressly incorporated herein by reference in their entirety.

EXAMPLES

Example 1: Designing siRNA

Targets for siRNA were designed for wild type SOD-1 mRNA. A general strategy for designing siRNA targets comprises beginning at the start codon for exon 3 of SOD-1 and then scanning the length of exon 3. The potential target site can then be compared to the appropriate genome database, so that any target sequences that have significant homology to non-target genes can be discarded. Multiple target sequences along the length of the gene should be located, so that target sequences are derived from the 3', 5' and medial portions of the mRNA of exon 3. Negative control siRNAs can be generated using the same nucleotide composition as the subject siRNA, but scrambled and checked so as to lack sequence homology to any genes of the cells being transfected (Elbashir *et al.* (2001) *Nature*, 411, 494-498; Ambion siRNA Design Protocol, at www.ambion.com).

In the present invention, generated target sequences were 19 bases long, beginning with start codon of exon 3 (SEQ ID NO: 3). Exon 3 was selected as a target gene for siRNA molecules because it is the stretch of SOD1 mRNA on exon 3 that harbors the fewest (practically zero) disease-associated mutations. This is important because there are numerous different disease-associated SOD-1 mutations on the SOD-1 gene. However, the present invention eliminates the need for separate siRNA molecules for each mutation by creating one siRNA molecule that inhibits SOD-1 gene expression and protein production. The method of the invention is therefore suitable for all FALS patients with various different mutations, regardless of their particular mutation.

The siRNAs were chemically synthesized using ribonucleoside phosphoramidites and a DNA/RNA synthesizer.

5 **Table 1. siRNA molecules that can be used to inhibit the SOD-1 gene.**

SEQ ID NO: 4	5'-UUAUCCUCUAUCCAGAAA-3' (sense)
SEQ ID NO: 5	5'-UUUCUCCAUAGAGGAUUA-3' (antisense)
SEQ ID NO: 6	5'-GUGCAGGUCCUCACUUUA-3' (sense)
SEQ ID NO: 7	5'-UUAAGUGAGGACCUGCAC-3' (antisense)
SEQ ID NO: 8	5'-AGUGCAGGUCCUCACUUUA-3' (sense)
SEQ ID NO: 9	5'-UAAAGUGAGGACCUGCACU-3' (antisense)
SEQ ID NO: 10	5'-UCCUCACUUUAUCCUCUA-3' (sense)
SEQ ID NO: 11	5'-UAGAGGAUUAAGUGAGGA-3' (antisense)
SEQ ID NO: 12	5'-AAUACAGCAGGCUGUACCA-3' (sense)
SEQ ID NO: 13	5'-UGGUACAGCCUGCUGUAUU-3' (antisense)
SEQ ID NO: 14	5'-GCAGGUCCUCACUUUAUC-3' (sense)
SEQ ID NO: 15	5'-GAUUAAGUGAGGACCUGC-3' (antisense)
SEQ ID NO: 16	5'-CCUCACUUUAUCCUCUAU-3' (sense)
SEQ ID NO: 17	5'-AUAGAGGAUUAAGUGAGG-3' (antisense)
SEQ ID NO: 18	5'-UCACUUUAUCCUCUAUCC-3' (sense)
SEQ ID NO: 19	5'-GGAUAGAGGAUUAAGUGA -3' (antisense)
SEQ ID NO: 20	5'-CACUUUAUCCUCUAUCCA-3' (sense)
SEQ ID NO: 21	5'-UGGAUAGAGGAUUAAGUG-3'(antisense)
SEQ ID NO: 22	5'-CUUUAUCCUCUAUCCAGA-3 (sense)
SEQ ID NO: 23	5'-UCUGGAUAGACCAUUAAG-3' (antisense)
SEQ ID NO: 24	5'-UUUAAUCCUCUAUCCAGAA-3' (sense)
SEQ ID NO: 25	5'-UUCUGGAUAGAGGAUUA-3' (antisense)
SEQ ID NO: 26	5'-AAUCCUCUAUCCAGAAAAC-3'(sense)
SEQ ID NO: 27	5'-GUUUUCUGGAUAGAGGAUU-3' (antisense)
SEQ ID NO: 28	5'-AUCCUCUAUCCAGAAAACA-3'(sense)
SEQ ID NO: 29	5'-UGUUUUCAGGAUAGAGGAU-3' (antisense)
SEQ ID NO: 30	5'-CCAGUGCAGGUCCUCACUU-3' (sense)
SEQ ID NO: 31	5'-AAGUGAGGACCUGCACUGG-3' (antisense)
SEQ ID NO: 32	5'-GCUUAAAGGAUUGACAAA-3' (sense)
SEQ ID NO: 33	5'-UUUGUCAAUCCUUUAAGC-3' (antisense)

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Example 2: Testing siRNA In vitro

To quantify the effect the inhibition of expression *in vitro*, attenuation of gene function was assessed by the measurement of mRNA using typical real time
5 fluorescence detection technologies, and by the measurement of immunoreactivity using an enzyme linked immunosorbent assay (ELISA; Bender Medsystems MST222).

Briefly, HeLa cells (ATCC) were plated into 96 well microtiter plates at a density of 4000 cells/well and allowed 12 hours to attach. Following an initial 12 hour
10 incubation, annealed duplex RNA was added to each well at concentrations from 20 nM through 10 uM, in the presence and absence of lipid transfection reagents. Cultures were assayed following 24 – 72 h of RNA treatment. Control sequences with the same base composition but different orders of nucleotides were tested in parallel fashion.

The data showed that siRNA targeted to SOD-1 could decrease SOD-1 expression. Cultured hippocampal neurons were treated with various concentrations of
15 siRNA targeted to SOD-1, and assayed for SOD activity.

Since transfection reagents are generally used in cell-based studies using siRNA to allow the siRNA to enter the cell, but are not usually compatible with most *in vivo* applications, a protocol was developed to quantify the cell penetration of unmodified and modified siRNA sequences. Sequences, from 18 to 30 base-pairs long were added
20 in concentrations from 10 micromolar to 10 picomolar to cell cultures. Cells can be HeLa, human embryonic kidney HEK-293 cells, or any neuroblastoma, glial, microglial, lymphocyte, or other mammalian cell line or primary cell, maintained in serum-free medium. Following addition of the siRNA sequences to the medium, cells are assayed at time points ranging from 24 to 168 hours for target protein levels (via ELISA or
25 Western, or dot immunoblot), target mRNA levels, or target enzyme activity. Minimal effective concentrations of the siRNA and their IC₅₀ values for inhibition of SOD1 expression are then used to rank efficacy and cell penetration.

Over 1000 siRNA duplexes have been designed using rational and computer assisted design tools (see Table 3). The siRNA sequences listed in Table 3 are part of
30 the present invention. Several very potent sequences were identified as shown by Figure 1. Figure 1 depicts a bar graphs showing the decrease in SOD-1 protein following incubation of HeLa cells with various siRNA listed in Table 2 (n = 5, p<0.005).

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Candidate siRNA molecules that show greater than a 10% reduction in SOD-1 protein *in vitro* are tested *in vivo*.

5 **Table 2. Sequences of siRNAs tested in Example 2.**

SEQ ID NO. 34	395Forward	GUGGAAAUGAAGAAAGUACAAAG
SEQ ID NO. 35	395Reverse	CUUUGUACUUUCUUCUUUCCAC
SEQ ID NO. 36	292Forward	GCCGAUGUGUCUAUUGAAGAUUC
SEQ ID NO. 37	292Reverse	GAAUCUUCAAUAGACACAUCGGC
SEQ ID NO. 38	262Forward	GGCAAUGUGACUGCUGACAAAGA
SEQ ID NO. 39	262Reverse	UCUUUGUCAGCAGUCACAUUGCC
SEQ ID NO. 40	97Forward	AAGGUGUGGGGAAGCAUUAAGG
SEQ ID NO. 41	97Reverse	CCUUUAAUGCUUCCCCACACCUU
SEQ ID NO. 42	129Forward	AGGCCUGCAUGGAUUCCAUGUUC
SEQ ID NO. 43	129Reverse	GAACAUGGAAUCCAUGCAGGCCU
SEQ ID NO. 44	289Forward	GUGGCCGAUGUGUCUAUUGAAGA
SEQ ID NO. 45	289Reverse	UCUUCAAUAGACACAUCGGCCAC
SEQ ID NO. 46	102Forward	GUGGGGAAGCAUUAAGGACUGA
SEQ ID NO. 47	102Reverse	UCAGUCCUUUAAUGCUUCCCCAC

Example 3: Testing siRNA *In vivo*: siRNA Knockdown of Mouse SOD1 mRNA

To quantify the effect the inhibition of expression *in vitro*, the siRNA molecules
 10 was introduced into the SOD-93A murine model (GTC Biotherapeutics, Inc.,
 Framingham, MA) for ALS, and the life expectancy measured. The inhibition of RNA
 expression was monitored by isolated blood samples from a mouse pre- and post
 introduction of the siRNA molecule using standard RT-PCR techniques. The expression
 of the SOD-1 protein was determined using ELISA, Western blot techniques, or
 15 TaqMan quantitative PCR.

In vivo experiments were conducted with siRNA molecules that show a
 significant reduction (i.e., greater than 10%, preferably greater than 20%, most
 preferably greater than 50%) of SOD1 levels. siRNA molecules that were showed a
 50% reduction *in vitro* at a concentration of about 50nM siRNA were tested *in vivo*.
 20 This concentration is low enough that therapeutically relevant drug levels should be
 achievable in the spinal cord. Animal testing demonstrates about 25% knockdown in the

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spinal cord via intrathecal delivery of 50 nM siRNA sequence 289 (SEQ ID NO. 44 and 45) (See Figure 2). These experiments were repeated in triplicate and demonstrate statistically significant results ($p < 0.05$). Similar in vivo experiments can be performed using alternative routes of deliver (i.e., oral, parenteral, intraspinal, intracisternal, subdural, rectal, intradermal, transdermal, intramuscular, or topical administration). This experiment demonstrates that the methods of the invention can be used effectively *in vivo*.

Table 3. siRNA sequences of SOD-1 designed using rational and computer assisted design tools.

Sense Strand (5' -3')	Lower Strand (3' -5')
UCAAGCCUGUGAAUAAAAA	AGUUCGGACACUUUUUUU
UCAUGAGUUUGGAGAUAAU	AGUACUCAAAACCUCUAUUA
UUAUCCUCUAUCCAGAAA	AAUUAGGAGAUAGGUCUUU
CAAUGUGACUGCUGACAAA	GUUACACUGACGACUGUUU
UAAUUGGGAUCGCCCAAUA	AUUAACCCUAGCGGGUUUAU
GUAGAAAUGUAUCCUGAUA	CAUCUUUACAUAGGACUAU
GUAGUGAGAAACUGAUUUUA	CAUCACUCUUUGACUAAAU
GUUUUUUGCCAGACUUAAA	CAUAAAACGGUCUGAAUUU
AGAAUUGUAUCCUGAUAAA	UCUUUACAUAGGACUAUUU
GUAUCCUGAUAAACAUUAA	CAUAGGACUAUUUGUAAUU
UAAACACUGUAAUCUUAAA	AUUUGUGACAUUAGAAUUU
GAAGAUUUGUAUAGUUUUUA	CUUCUAAACAUUCAAUAU
AGAUUUGUAUAGUUUUUAU	UCUAAACAUUCAAUAUAU
CGGAGGUCUGGCCUAUAAA	GCCUCCAGACCGGAUUAUU
UGCAGGGCAUCAUCAUUUU	ACGUCCCGUAGUAGUUAUU
UCAUCAUUUCGAGCAGAA	AGUAGUUAAAGCUCGUCUU
GGUGUGGGGAAGCAUUAAA	CCACACCCCUUCGUAAUUU
UGAGUUUGGAGAUAAUACA	ACUCAAAACCUCUAUUUUGU
GUGCAGGUCCUCACUUUAA	CACGUCCAGGAGUGAAAUU
UGCAGGUCCUCACUUUAAU	ACGUCCAGGAGUGAAAUAU
UAAUCCUCUAUCCAGAAAA	AUUAGGAGAUAGGUCUUUU
UGGCCGAUGUGUCUAUUGA	ACCGGCUACACAGUAACU
CGAUGUGUCUAUUGAAGAU	GCUACACAGUAACUUCUA

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ACACUGGUGGUCCAUGAAA	UGUGACCACCAGGUACUUU
CACUGGUGGUCCAUGAAAA	GUGACCACCAGGUACUUUU
ACUGGUGGUCCAUGAAAAA	UGACCACCAGGUACUUUUU
GGGCAAAGGUGGAAAUGAA	CCCGUUUCCACCUUUACUU
CAAAGGUGGAAAUGAAGAA	GUUUCCACCUUUACUUCUU
GGAAAUGAAGAAAGUACAA	CCUUUACUUCUUUCAUGUU
UGAAGAAAGUACAAAGACA	ACUUCUUUCAUGUUUCUGU
UUUGGCUUGUGGUGUAAUU	AAACCGAACACCACAUUAA
AAUUGGGAUCGCCCAAUAA	UUAACCCUAGCGGGUUAUU
AUUGGGAUCGCCCAAUAAA	UAACCCUAGCGGGUUAUUU
CAAUAAACAUUCCCUUGGA	GUUAUUUGUAAGGGAACCU
GUAGUCUGAGGCCCCUUA	CAUCAGACUCCGGGGAAUU
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UGCUAGCUGUAGAAUGUA	ACGAUCGACAUCUUUACAU
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AGGGCAUCAUCAUUUCGA	UCCCGUAGUAGUUAAAGCU
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UGUAUGGCACUUAUUUAUGA	ACAUACCGUGAAUAAUACU

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UUCGAGCAGAAGGAAAGUA	AAGCUCGUCUCCUUUCAU
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CCGAUGUGUCUAUUGAAGA	GGCUACACAGAUAAUUCU
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CUCUCAGGAGACCAUUGCA	GAGAGUCCUCUGGUAACGU
GGAGACCAUUGCAUCAUUG	CCUCUGGUAACGUAGUAAC
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CUAUUUAAAAGAAUCCAAU	GAUAAUUUUCUUAGGUUUA
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GCAGAU	CGUCUACUGAACCCGUUUC
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ACUUGGGCAAAGGUGGAAA	UGAACCCGUUUCACCUUU
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GUUUGGCUUGUGGUGUAAU	CAAACCGAACACCACAUUA
GCUUGUGGUGUAAUUGGGA	CGAACACCACAUUAACCCU
CUUGUGGUGUAAUUGGGAU	GAACACCACAUUAACCCUA

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UGUAAUUGGGAUCGCCCAA	ACAUUAACCCUAGCGGGUU
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UUGGGAUCGCCCAAUAAAC	AACCCUAGCGGGUUUUUG
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GGCCCCUUAACUCAUCUGU	CCGGGGAAUUGAGUAGACA
GCCCCUUAACUCAUCUGUU	CGGGGAAUUGAGUAGACAA
CCUUAACUCAUCUGUUAUC	GGAAUUGAGUAGACAAUAG
UAACUCAUCUGUUAUCCUG	AUUGAGUAGACAAUAGGAC
ACUCAUCUGUUAUCCUGCU	UGAGUAGACAAUAGGACGA
CUGUUAUCCUGCUAGCUGU	GACAAUAGGACGAUCGACA
CUGCUAGCUGUAGAAUGU	GACGAUCGACAUCUUUACA
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GGCACUUAUUAUGAGGCUA	CCGUGAAUAAUACUCCGAU
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AUAAACUCAGUUAAAUG	UAUUUUGAGUCAUUUUUAC

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UGGUUUUGCGUCGUAGUCUC	ACCAAACGCAGCAUCAGAG
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CUCACUUUAAUCCUCUAUC	GAGUGAAAUUAGGAGAUAG
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CUGCUGACAAAGAUGGUGU	GACGACUGUUUCUACCACA
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ACUCUCAGGAGACCAUUGC	UGAGAGUCCUCUGGUAACG
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AGACCAUUGCAUCAUUGGC	UCUGGUAACGUAGUAACCG
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UGGCUUGUGGUGUAAUUGG	ACCGAACACCACAUAUACC
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GUGUAAUUGGGAUCGCCCA	CACAUUAACCCUAGCGGGU
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CGCCCAAUAAACAUUCCCU	GCGGGUUAUUUGUAAGGGA
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CAUCUGUUAUCCUGCUAGC	GUAGACAAUAGGACGAUCG
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UUGUGUGACUUUUUCAGAG	AACACACUGAAAAAGUCUC
GACUUUUUCAGAGUUGCUU	CUGAAAAAGUCUCAACGAA
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UGCUUUAAGUACCUGUAG	ACGAAUUUCAUGGACAUC
CUUUAAGUACCUGUAGUG	GAAUUUCAUGGACAUCAC
UUAAGUACCUGUAGUGAG	AAUUUCAUGGACAUCACUC
UACCUGUAGUGAGAAACUG	AUGGACAUCACUCUUUGAC
GAGAAACUGAUUUUAUGAUC	CUCUUUGACUAAAUACUAG
CUCAGUUAUUUUGUCUGUU	GAGUCAUUUUACAGACAA
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CAGACUAAAUCACAGAUG	GUCUGAAUUUAGUGUCUAC
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GGCGCGGAGGUCUGGCCUA	CCGCGCCUCCAGACCGGAU
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ACAGCAGGCUGUACCAGUG	UGUCGUCCGACAUGGUCAC

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CUGUACCAGUGCAGGUCCU	GACAUGGUCACGUCCAGGA
GUACCAGUGCAGGUCCUCA	CAUGGUCACGUCCAGGAGU
ACCAGUGCAGGUCCUCACU	UGGUCACGUCCAGGAGUGA
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UGGUGUGGGCCGAUGUGUCU	ACCACACCGGCUACACAGA
GGUGUGGGCCGAUGUGUCUA	CCACACCGGCUACACAGAU
GCAUCAUUGGGCCGCACACU	CGUAGUAACCGGCGUGUGA
UCAUUGGGCCGCACACUGGU	AGUAACCGGCGUGUGACCA
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UGAUAAACAUUAAACACUG	ACUAAUUUGUAAUUUGUGAC
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CAUCAUUUCGAGCAGAAG	GUAGUAAAGCUCGUCUUC
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GAUCUCACUCUCAGGAGAC	CUAGAGUGAGAGUCCUCUG
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CUCAGGAGACCAUUGCAUC	GAGUCCUCUGGUAACGUAG
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GUCCAUGAAAAAGCAGAU	CAGGUACUUUUUCGUCUAC
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GUUAUCCUGCUAGCUGUAG	CAAUAGGACGAUCGACAUC
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AGUACCUGUAGUGAGAAAC	UCAUGGACAUACUCUUUG
CUGAUUUUUGAUACAUUGG	GACUAAAUACUAGUGAACC
AUUUAUGAUACAUUGGAAG	UAAAUACUAGUGAACCUUC
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UGUCUGUUUCAUUGACCUG	ACAGACAAAGUUACUGGAC
GUCUGUUUCAUUGACCUGU	CAGACAAAGUUACUGGACA
GACCUGUAUUUUGCCAGAC	CUGGACAUAAAACGGUCUG
GACUUAUUUUCACAGAUUGG	CUGAAUUUAGUGUCUACCC
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UGGGUAUUUUAACUUGUCAG	ACCCAUAUUUUGAACAGUC
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GUAGUCGCGGAGACGGGGU	CAUCAGCGCCUCUGCCCCA
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AGACGGGGUGCUGGUUUGC	UCUGCCCCACGACCAAACG
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UGCAGCGUCUGGGGUUUC	ACGUCGCAGACCCCAAAGG
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SEQUENCES

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SEQ ID NO: 2

ATKAVCVLKGDPVQGIINFEQKESNGPVKVWGSIKGLTEGLHGFHVHEFGDN
TAGCTSAGPHFNPLSRKHGGPKDEERHVGDLGNVTADKDGVADVSIEDSVISLS
5 GDHCCIIGRTL VVHEKADDLGKGGNEESTKTGNAGSRLACGVIGIAQ

SEQ ID NO: 3 Exon3 of hSOD1

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tacttctgg gcttaaagga attgacaaat gggcacttaa aacgatttg tttgtagca ttgattgaa tatagaacta
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CLAIMS:

1. A method of inhibiting expression of a target protein in a subject with a
5 neurological disorder, comprising:
introducing at least one double stranded small interfering ribonucleic acid
(siRNA) molecule into the subject with the neurological disorder, wherein the
siRNA comprises a first strand and a second strand hybridized together, wherein
at least one strand of the siRNA is complementary to the nucleotide sequence of
10 a target gene encoding the target protein;
allowing the siRNA to interact with an RNA induced silencing complex
(RISC) to activate and direct the RISC to the target gene; and
promoting destruction of target mRNA of the target gene, thereby
inhibiting expression of the target protein.
15
2. The method of claim 1, wherein the neurological disorder is selected from the
group consisting of amyotrophic lateral sclerosis (ALS).
3. The method of claim 1, wherein the small interfering RNA is about 15 to about
20 25 bases in length.
4. The method of claim 1, wherein the small interfering RNA is about 19 to about
23 bases in length.
- 25 5. The method of claim 1, wherein the small interfering RNA is selected from the
group consisting of an unmodified small interfering RNA and a modified RNA
molecule.
6. The method of claim 1, wherein the target protein is a SOD protein.
30
7. The method of claim 6, wherein the SOD protein is a wild type SOD-1 protein.

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8. The method of claim 6, wherein the SOD protein is a SOD-1 protein with at least one mutation.
- 5 9. The method of claim 1, wherein the target gene is an SOD gene.
10. The method of claim 9, wherein the SOD gene is a wild type SOD-1 gene.
- 10 11. The method of claim 9, wherein the SOD gene is a SOD-1 gene with at least one mutation.
12. The method of claim 1, wherein the expression of the target protein is inhibited by at least 10%.
- 15 13. The method of claim 1, wherein the step of introducing a double stranded small interfering ribonucleic acid (siRNA) further comprises producing a cDNA corresponding to the target gene from an mRNA, and producing the double stranded siRNA from the cDNA such that the siRNA sequence is identical to at least a portion of the target gene cDNA.
- 20 14. The method of claim 13, wherein the cDNA is SEQ ID No. 1.
15. The method of claim 13, wherein the cDNA is SEQ ID No. 3.
- 25 16. The method of claim 1, wherein the siRNA is selected from Table 3.
17. The method of claim 1, wherein the method further comprises selecting at least one sequence from SEQ ID Nos. 4-47.
- 30 18. The method of claim 1, wherein the siRNA comprises SEQ ID No. 44.
19. The method of claim 1, wherein the siRNA comprises SEQ ID No. 45.

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20. A method of ameliorating amyotrophic lateral sclerosis (ALS) in subject, comprising:
- 5 introducing a small interference ribonucleic acid (siRNA) molecule into the subject with the ALS, wherein the siRNA comprises a first strand and a second strand hybridized together, wherein at least one strand of the siRNA is complementary to a nucleotide sequence of wild type SOD-1 gene;
- allowing the siRNA to interact with an RNA induced silencing complex (RISC) to activate and direct the RISC to the wild type SOD-1 gene; and
- 10 promoting destruction of wild type SOD-1 mRNA to inhibit expression of the wild type SOD-1 protein, thereby modulating ALS in the subject.
21. The method of claim 20, wherein at least one strand of the small interfering RNA is complementary to a region of Exon 3 of the wild type SOD-1 gene.
- 15 22. The method of claim 20, wherein the small interfering RNA is about 15 to about 25 bases in length.
23. The method of claim 20, wherein the small interfering RNA is about 19 to about 20 23 bases in length.
24. The method of claim 20, wherein the small interfering RNA is selected from the group consisting of an unmodified small interfering RNA and a modified RNA molecule.
- 25 25. The method of claim 20, wherein the expression of the target gene is inhibited by at least about 10%.
26. An isolated polynucleic acid consisting of a sequence selected from the group 30 consisting of the sequences listed in Table 3, and the complements thereto.
27. An isolated polynucleic acid consisting of a sequence selected from the group consisting of SEQ ID No. 44 and 45, and the complements thereto.

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28. The isolated polynucleic acid of claim 27, wherein the polynucleic acid is a dsRNA molecule.
- 5 29. A method of identifying a siRNA molecule useful for treating neurological disorders, comprising:
- incubating mammalian cells capable of expressing a target gene in the presence of a dsRNA test compound in the absence and presence of a transfection reagent;
- 10 assaying the incubated mammalian cells for target gene expression;
- comparing the expression levels of the target gene;
- wherein the siRNA molecule is useful for treating neurological disorders when the expression level in the presence of the dsRNA and in the absence of the transfection reagent is substantially modified when compared to the control level.
- 15 30. The method of claim 29, wherein the assaying step further includes assaying for protein activity.
31. The method of claim 29, wherein the target gene is SOD-1.
- 20 32. The method of claim 29, wherein the method further comprises incubating mammalian cells in the presence of a control nucleic acid compound, in the absence and presence of a transfection reagent.

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FIGURE 1

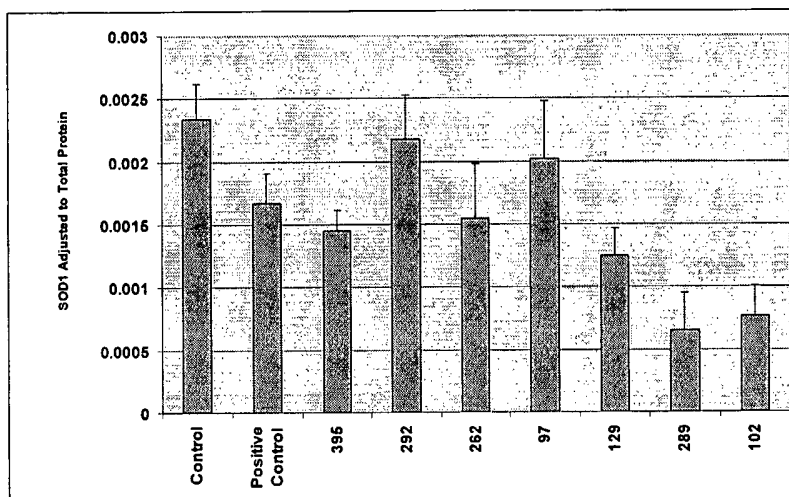


FIGURE 2

