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(54) Title: OLIGONUCLEOTIDE TARGETING SOD1

(57) Abstract: Provided is a siRNA or an oligonucleotide agent for preventing or treating SOD1-associated neurodegenerative diseases or symptoms (such as amyotrophic lateral sclerosis, ALS). The oligonucleotide agent comprises a double-stranded targeting oligonucleotide (siRNA) and a non-targeting single-stranded oligonucleotide (ACO), in which the siRNA targets a mRNA region of the target gene SOD1.

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OLIGONUCLEOTIDE TARGETING *SOD1*

TECHNICAL FIELD

[1] The present application relates to the technical field of nucleic acids, specifically as it relates to an oligonucleotide agent for reducing *SOD1* transcript level and pharmaceutical use thereof.

BACKGROUND OF THE INVENTION

[2] Amyotrophic lateral sclerosis (ALS) is an adult-onset, lethal, paralytic disorder caused by the degeneration of motor neurons. ALS is characterized by progressive, adult-onset degeneration of cranial, brainstem and spinal motor neurons, leading to death by respiratory failure within 3-5 years of diagnosis. ALS presents as a familial or a sporadic form, depending on whether or not there is a family history of the disease, with sporadic ALS (sALS) accounts for 90% of the ALS patients. The most common mutant genes account for ~75% of ALS in the United States: Chromosome 9 Open Reading Frame 72 gene (*C9orf72*; 40%), superoxide dismutase 1 (*SOD1*; 20%), transactive response DNA-binding protein 43 (*TDP43*; 4%) and fused in sarcoma/translocated in liposarcoma (*FUS/TLS*; 4%). Mutations in the *C9orf72* and *SOD1* genes also account for ~5–8% and ~2–3% of apparently sALS, respectively. To date, there is currently no effective therapy available for ALS and new therapies are needed to treat this disease.

[3] Among the known genes underlining ALS, *SOD1* gene remains a major cause of fALS and has been considered to be an important ALS drug target. The first description of the ALS disease dates back to at least 1824 by Charles Bell, however, *SOD1* as the first risk gene of ALS was discovered in 1993. When the first human *SOD1* transgenic mouse model ($hSOD1^{G93A}$) was established in 1994, indicating that the research on ALS entered a new era. *SOD1* mutants cause disease most probably via a gain-of-function and reducing its levels is believed beneficial. Therefore, silencing *SOD1* transcript level is an important strategy for the treatment of ALS.

SUMMARY OF THE INVENTION

[4] To address the problem, the present application provides an oligonucleotide agent with potent inhibitory effect on the expression of superoxide dismutase 1 (*SOD1*). The oligonucleotide agent comprises a small interfering RNA (siRNA), for treating diseases or conditions caused by the mutation of *SOD1* gene such as ALS by targeting *SOD1* mRNA and subsequently downregulating SOD1 protein level in a cell or an individual via RNA interference (RNAi).

[5] In particular, the inventors discovered that an oligonucleotide agent with strong potency for knocking down *SOD1* transcript and high CNS delivery efficiency, comprising: (a) a siRNA; and (b) a non-targeting single-stranded accessary oligonucleotide (ACO), wherein the ACO is 6-22 nucleotides in length, wherein the siRNA and the ACO are covalently linked, with or without one or more linking components, to form the oligonucleotide agent.

[6] In some embodiments, the siRNA comprises a sense strand and an antisense strand forming a duplex structure, wherein the antisense strand comprises a nucleotide sequence comprising at least

10 contiguous nucleotides, with 0, 1, 2 or 3 mismatches, having at least 85% nucleotide sequence complementarity or homology to a portion of the nucleotide sequence of SEQ ID NO: 1 (Table 1).

[7] In some embodiments, the ACO is composed of one or more of RNA, DNA, BNA, LNA, glycerol nucleic acid (GNA) and peptide nucleic acid (PNA). In some embodiments, the ACO is 6-18 nucleotides in length. In some embodiments, the sense strand is at least 10 nucleotides in length. In some embodiments, the sense strand has a nucleotide length ranging from 10-60 nucleotides. In some embodiments, the sense strand has a nucleotide length ranging from 16-25 nucleotides. In some embodiments, the antisense strand has a nucleotide length ranging from 15-35 nucleotides. In some embodiments, the antisense strand has a nucleotide length ranging from 19-25 nucleotides.

[8] In certain embodiments, one strand of the oligonucleotide sequence disclosed in the present application has at least 85%, at least 90%, or at least 95% homology or complementarity to a nucleotide sequence selected from SEQ ID NOs: 2-269. In certain embodiments, the sense strand of the oligonucleotide sequence disclosed in the present application has at least 85%, at least 90%, or at least 95% homology to a nucleotide sequence selected from SEQ ID NOs: 2-269. In certain embodiments, the antisense strand of the oligonucleotide sequence disclosed in the present application has at least 85%, at least 90%, or at least 95% complementarity to a nucleotide sequence selected from SEQ ID NOs: 2-269.

[9] In one aspect of the present application, an oligonucleotide agent capable of inhibiting/down-regulating *SOD1* transcript in a cell is provided. In some embodiments, the oligonucleotide agent comprises a siRNA, wherein the sense strand of the siRNA has a nucleotide sequence that is at least 85%, at least 90%, or at least 95% homology to the nucleotide sequence selected from SEQ ID NOs: 270-537.

[10] In some embodiments, an oligonucleotide agent comprising a siRNA is provided, wherein the antisense strand of the siRNA has a nucleotide sequence that is at least 85%, at least 90%, or at least 95% homology to the nucleotide sequence selected from SEQ ID NOs: 538-805.

[11] In some embodiments, an oligonucleotide agent comprising a siRNA is provided, wherein the siRNA comprising a sense strand and an antisense strand, wherein the sense strand has a nucleotide sequence that is at least 85%, at least 90%, or at least 95% homology to the nucleotide sequence selected from SEQ ID NOs: 270-537, and the antisense strand of the siRNA has a nucleotide sequence that is at least 85%, at least 90%, or at least 95% homology to the nucleotide sequence selected from SEQ ID NOs: 538-805.

[12] In some embodiments, an oligonucleotide agent comprising a siRNA is provided, wherein the siRNA comprises a sense strand and an antisense strand, wherein the sense strand has a nucleotide sequence that is at least 85%, at least 90%, or at least 95% homology to the nucleotide sequence selected from SEQ ID NOs: 808-827, 867, and the antisense strand of the siRNA has a nucleotide sequence that is at least 85%, at least 90%, or at least 95% homology to the nucleotide sequence selected from SEQ ID NOs: 828-849, 868.

[13] In another aspect of the present application, an oligonucleotide agent comprising a siRNA and a non-targeting ACO is provided, wherein the ACO comprises a nucleotide sequence that is at least 90% identical to the nucleotide sequence selected from SEQ ID NO: 865.

[14] In some embodiments, the ACO is conjugated to a linking component. In some embodiments, the 5' end or the 3' end or one or more of internal nucleotides of the ACO is conjugated to a linking component. In some embodiments, the siRNA and the ACO are covalently linked by a linking component. In some embodiments, the sense strand or the antisense strand of the siRNA are covalently linked to the ACO by a linking component. In some embodiments, the linking component is or comprises one or more selected from the group consisting of ethylene glycol chain, an alkyl chain, an alkenyl chain, an alkynyl chain, a peptide, RNA, DNA, carbohydrates, thiol linkage, a phosphodiester, a phosphorothioate, a phosphoramidate, an amide, a carbamate, a tetrazole linkage, and a benzimidazole linkage. In some embodiments, the linking component is or comprises one or more selected from the group consisting of:

a) Spacer phosphoramidite 18 (Phosphoramidous acid, N,N-bis(1-methylethyl)-, 19,19-bis(4-methoxyphenyl)-19-phenyl-3,6,9,12,15,18-hexaoxanonadec-1-yl 2-cyanoethyl ester);

b) Spacer-9 (3-[2-[2-[2-[bis(4-methoxyphenyl)-phenylmethoxy]ethoxy]ethoxy]ethoxy-[di(propan-2-yl)amino]phosphanyl]oxypropanenitrile);

c) Spacer phosphoramidite C3 (6-(4,4'-Dimethoxytrityl)hexyl-1-[(2-cyanoethyl)-(N,N-diisopropyl)]- phosphoramidite); and

d) Spacer-C6 Phosphoramidite (6-(4,4'-Dimethoxytrityl)hexyl-1-[(2-cyanoethyl)-(N,N-diisopropyl)]- phosphoramidite)

e) Divalent linker (DIO) 16-((bis(4-methoxyphenyl)(phenyl)methoxy)methyl)-1,1-bis(4-methoxyphenyl)-18-oxo-1-phenyl-2,5,8,11,14,17-hexaoxahenicosan-CPG.

[15] In some embodiments, the ACO is covalently linked to a 3' end, or a 5' end, or both 3' and 5' ends, one or more of internal nucleotides of the sense strand of the siRNA. In some embodiments, the ACO is covalently linked to a 3' end, or a 5' end, or both 3' and 5' ends, or one or more of internal nucleotides of the antisense strand of the siRNA. In some embodiments, more than one ACO is covalently linked to siRNA. In some embodiments, 2-10 ACOs are covalently linked to the siRNA.

[16] In some embodiments, at least one nucleotide of the siRNA is a chemically modified nucleotide. In some embodiments, at least one nucleotide of the ACO is a chemically modified nucleotide. In some embodiments, at least about 50%, or at least about 60%, or at least about 70%, or at least about 80%, or at least about 90%, or at least about 95%, or about 100% nucleotides of the ACO are chemically modified nucleotides. In some embodiments, at least about 50%, or at least about 60%, or at least about 70%, or at least about 80%, or at least about 90%, or at least about 95%, or about 100% nucleotides of the sense strand of the siRNA are chemically modified nucleotides. In some embodiments, at least about 50%, or at least about 60%, or at least about 70%, or at least about 80%, or at least about 90%, or at least about 95%, or about 100% nucleotides of the antisense strand of the siRNA are chemically modified nucleotides. In some embodiments, the chemical modification

of the at least one chemically modified nucleotide is a 2' sugar modification selected from one or more of: 2'-fluoro-2'-deoxynucleoside (2'-F) modification, 2'-O-methyl (2'-O-Me), modification, and 2'-O-(2-methoxyethyl) (2'-O-MOE) modification. In some embodiments, the chemical modification of the at least one chemically modified nucleotide is a phosphorothioate (PS) backbone modification. In some embodiments, the ACO comprises at least one phosphorothioate (PS) backbone modification. In some embodiments, the ACO comprises 6~17 phosphorothioate (PS) backbone modifications. In some embodiments, the chemical modification of the at least one chemically modified nucleotide is an addition of a 5'-phosophate moiety at the 5' end of the nucleotide sequence. In some embodiments, the chemical modification of the at least one chemically modified nucleotide is an addition of an (E)-vinylphosphonate moiety at the 5' end of the nucleotide sequence. In some embodiments, the chemical modification of the at least one chemically modified nucleotide is an addition of a 5-methyl cytosine moiety at the 5' end of the nucleotide sequence.

[17] In some embodiments, the ACO is conjugated to one or more conjugation groups. In some embodiments, the siRNA is conjugated to one or more conjugation groups. In some embodiments, the sense strand or the antisense strand of the siRNA is conjugated to one or more conjugation groups. In some embodiments, the one or more conjugation groups are selected from: a lipid, a fatty acid, a fluorophore, a ligand, a saccharide, a peptide, and an antibody. In some embodiments, the one or more conjugation groups are selected from: a cell-penetrating peptide, polyethylene glycol, an alkaloid, a tryptamine, a benzimidazole, a quinolone, an amino acid, a cholesterol, glucose and N-acetylgalactosamine.

[18] Aspects of the present application relate to an oligonucleotide agent comprising a siRNA and a non-targeting ACO, wherein the sense strand has a nucleotide sequence that is at least 85%, at least 90%, or at least 95% homology to the nucleotide sequence selected from SEQ ID NOs: 808-827, 850-851, and 856-857, and the antisense strand of the siRNA has a nucleotide sequence that is at least 85%, at least 90%, or at least 95% homology to the nucleotide sequence selected from SEQ ID NOs: 828-849 and 861-863.

[19] Aspects of the present application include a vector comprising the oligonucleotide agent disclosed herein.

[20] Another aspect of the present application provides a cell comprising the oligonucleotide agent disclosed herein. In one embodiment, the cell is a mammalian cell, optionally a human cell. In some embodiments, the cell is a host cell. The aforementioned cell may be *in vitro*, such as a cell line or a cell strain, or may exist or be taken from in a mammalian body, such as a human body.

[21] Yet another aspect of the present application provides a pharmaceutical composition comprising the oligonucleotide agent disclosed herein. In some embodiments, the composition comprises at least one pharmaceutically acceptable carrier selected from an aqueous carrier, liposome or LNP, polymer, micelle, colloid, metal nanoparticle, non-metallic nanoparticle, bioconjugates, and polypeptide. Also provided by the present application includes a kit comprising the oligonucleotide agent or the pharmaceutical composition disclosed herein.

[22] The present application provides a method of decreasing the transcript of a *SOD1* gene or SOD1 protein, comprising administering to a subject a pharmaceutical composition disclosed herein.

[23] The present application also provides a method for treating or delaying the onset or progression of Amyotrophic lateral sclerosis (ALS) in a subject, the method comprising: administering to the subject a pharmaceutical composition disclosed herein. In some embodiments, the subject has sporadic ALS (sALS). In some embodiments, the subject has familial ALS (fALS). In some embodiments, the pharmaceutical composition decreases the transcript of the *SOD1* gene or SOD1 protein.

[24] In some embodiments, the ACO of the oligonucleotide agent improves the stability, bioavailability, biodistribution, and/or cellular uptake of the siRNA as compared to an oligonucleotide agent without the ACO.

[25] In some embodiments, the ACO of the oligonucleotide agent increases the biodistribution of siRNA within one or more target tissues as compared to an oligonucleotide agent without the ACO.

[26] In some embodiments, the ACO of the oligonucleotide agent increases the biodistribution of siRNA within two or more target tissues as compared to an oligonucleotide agent without the ACO.

[27] In some embodiments, the one or more target tissues are selected from: prefrontal cortex, cerebellum, cerebrum, spinal cord, muscle, lung, eye, liver, and kidney.

[28] The present application partially relates to a siRNA comprising an oligonucleotide sequence having a length ranging from 16 to 35 consecutive nucleotides, wherein the continuous oligonucleotide sequence comprises a nucleotide sequence having at least 75%, at least 80%, at least 85%, at least 90%, at least 95% or 100% homology or complementarity to an equal length portion of SEQ ID NO:1, wherein the siRNA inhibits the mRNA transcript of *SOD1* gene by at least 80% as compared to the baseline of *SOD1* mRNA level.

[29] The inventors also discovered that optimal target sequences/sense strand of an siRNA within the *SOD1* gene include sequences having: (1) a GC content between 35% and 65%; (2) less than 5 consecutive identical nucleotides; (3) 3 or less dinucleotide repeats; and (4) 3 or less trinucleotide repeats. As a beneficial consequence, a target sequence (*e.g.*, an isolated nucleic acid sequence comprising the target sequence), upon interacting with the siRNA, can inhibit the *SOD1* mRNA transcript level by at least 80% in a cell as compared to a baseline level of *SOD1* mRNA. Based at least in part on these discoveries, the present disclosure features siRNA, compositions, and pharmaceutical compositions for inhibiting the *SOD1* mRNA transcript level by at least 80% in a cell as compared to baseline levels of *SOD1* mRNA. Also provided herein are methods for preventing or treating a disease or symptom induced by *SOD1* gene mutation or abnormal level of SOD1 protein in a cell in an individual comprising administering any of the siRNA, compositions, and/or pharmaceutical compositions described herein.

[30] Additional aspects and advantages of the present application will become readily apparent to those skilled in this art from the following detailed description, wherein only illustrative embodiments of the present application are shown and described. As will be realized, the present

application is capable of other and different embodiments, and its several details are capable of modifications in various obvious respects, all without departing from the disclosure. Accordingly, the drawings and description are to be regarded as illustrative in nature, and not as restrictive.

INCORPORATION BY REFERENC

[31] All publications, patents, and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each individual publication, patent, or patent application was specifically and individually indicated to be incorporated by reference.

BRIEF DESCRIPTION OF THE DRAWING

[32] The novel features of the invention are set forth with particularity in the appended claims. A better understanding of the features and advantages of the present invention will be obtained by reference to the following detailed description that sets forth illustrative embodiments, in which the principles of the invention are employed, and the accompanying drawings (also "figure" and "FIG." herein), of which:

[33] **FIGs.1A-1E** show the siRNA screen for *SOD1* knockdown *in vitro*. **FIG.1A** shows that HEK293A cells were transfected with each siRNA duplex (268 in total) in duplicate at 10 or 0.1 nM concentrations for 24 hours. *SOD1* expression levels were quantified via RT-qPCR using gene specific primer sets. *TBP* was amplified as an internal reference used to normalize expression data. Shown are the expression values of *SOD1* mRNA of each experimental replicate relative to Mock treatments (dotted line). Mock samples were transfected in absence oligonucleotide. **FIG.1B** shows the knockdown activity and cell viability of the top performing siRNAs which were quantified in HEK293A cells at 6 escalating concentrations (*i.e.*, 0.0064, 0.032, 0.16, 0.8, 4, and 20 nM) via RT-qPCR and PI staining, respectively. Shown are the results for both *SOD1* knockdown and cytotoxicity of the top 5 performing siRNAs (*i.e.*, siSOD1-063, 047, 104, 005, and 258) relative to Mock treatments (dotted line). **FIG.1C** shows that dose response curves were generated in SK-N-AS cells for each of the top 5 siRNAs at 8 treatment concentrations (*i.e.*, 0.00006, 0.0002, 0.001, 0.004, 0.016, 0.063, 0.25, and 1 nM) via RT-qPCR. Data represents mean ± SD from 2 experimental replicates. **FIG.1D** shows that the SK-N-AS cells were transfected at 0.1 nM with different chemically modified variants of each siRNA or non-specific siRNA control (siCon) for 24 hours. Knockdown activity was assessed via RT-qPCR relative to Mock treatment. **FIG.1E** shows that dose response curves were generated in SK-N-AS cells for each of the M3 modified variants (*i.e.*, siSOD1-063M3, 047M3, 104M3, 005M3, and 258M3) via RT-qPCR. Data represents mean ± SD from 3 experimental replicates.

[34] **FIGs.2A-2B** show the secondary screening of siRNA potency and untoward cytotoxicity *in vitro*. Knockdown activity and cell viability in HEK293A cells of the remaining top 25 performing siRNAs identified in the initial screen is shown following transfection at 6 indicated concentrations (*i.e.*, 0.0064, 0.032, 0.16, 0.8, 4, and 20 nM). *SOD1* knockdown and untoward cytotoxicity were quantified by RT-qPCR in **FIG.2A** and PI staining in **FIG.2B** relative to Mock treatments, respectively.

[35] **FIG.3** shows the dose-dependent knockdown of the top 5 siRNA candidates in T98G cells. Dose response curves were generated in T98G cells following transfection for 24 hours with each of the top 5 siRNAs at 8 treatment concentrations (*i.e.*, 0.00006, 0.0002, 0.001, 0.004, 0.016, 0.063, 0.25, and 1 nM). *SOD1* expression levels were quantified via RT-qPCR using gene specific primer sets. *TBP* was amplified as an internal reference used to normalize expression data. Shown are mean values ± SD from 2 experimental replicates relative to controls treated in absence of oligonucleotide.

[36] **FIGs.4A-4B** show the untoward cytotoxicity of the top 5 siRNA candidates *in vitro*. SK-N-AS and T98G cells were transfected with each of the top 5 siRNAs at 4 treatment concentrations (*i.e.*, 1.56, 6.25, 25, and 100nM) in noted excess of their IC_{50} values for *SOD1* knockdown. Treatment with siCon at 100 nM served as a negative control (Neg Con). Mock samples were transfected in absence oligonucleotide. Untoward cytotoxicity was evaluated 72 hours after treatment by quantifying both caspase 3/7 activity in **FIG.4A** and metabolism of WST-8 reagent as a marker for cell viability in **FIG.4B**. Data is shown as mean values ± SD from 2 experimental replicates relative to Mock treatments (dotted lines).

[37] **FIG.5** shows the impact of different chemical modifications patterns on siRNA knockdown activity in T98G cells. T98G cells were transfected at 0.1 nM with 4 different chemically modified variants of each siRNA candidate (*i.e.*, M1, M2, M3, or M4) or a non-specific siRNA control (siCon) for 24 hours. Knockdown activity was assessed via RT-qPCR relative to Mock treatment. Data represents mean ± SD from 2 experimental replicates.

[38] **FIG.6** shows the dose-dependent knockdown of M3-modified siRNAs in T98G cells. Dose response curves were generated in T98G cells following transfection for 24 hours with each M3-modified siRNA candidate (*i.e.*, siSOD1-063M3, 047M3, 104M3, 005M3, 258M3, and 270M3) at gradient treatment concentrations. *SOD1* expression levels were quantified via RT-qPCR using gene specific primer sets. *TBP* was amplified as an internal reference used to normalize expression data. Shown are mean values ± SD from 3 experimental replicates relative to samples treated in absence of oligonucleotide.

[39] **FIGs.7A-7D** show the siRNA-ACO conjugate knockdown activity *in vitro*. **FIG.7A** depicts a visual representation of the siRNA-ACO conjugate structure in which a 14-nt ACO (referred to AC1) is conjugated to the 3'-terminus of the sense strand via short L9 linker (*i.e.*, Spacer-9 linker). **FIG.7B** shows that HEK293A and T98G cells were transfected at 0.25 or 2.5 nM concentrations with exemplary siRNA-ACO (*i.e.*, siSOD1-005M3-AC1) or AC1 only for 24 hours. Mock samples were transfected in absence oligonucleotide. Treatment with siCon served as a negative control for knockdown activity. *SOD1* expression levels were quantified via RT-qPCR using gene specific primer sets. *TBP* was amplified as an internal reference used to normalize expression data. Shown are the expression values of *SOD1* mRNA of each experimental replicate relative to Mock treatments (dotted line). **FIG.7C** shows that dose response curves were generated in T98G cells comparing siRNA knockdown activity of siSOD1-005M3 with AC1 (siSOD1-005M3-AC1) or without AC1 conjugation (siSOD1-005M3) at 10 treatment concentrations (*i.e.*, 0.0003, 0.0009, 0.0027, 0.0082, 0.024, 0.074, 0.22, 0.67, 2, and 6 nM) via RT-qPCR. **FIG.7D** shows that dose response curves were generated comparing knockdown activity of siRNA-ACOs with (siSOD1-005M3-$AC1^{VP}$) or without

(siSOD1-005M3-AC1) 5'VP modification as compared to a published ASO sequence cf. T. Miller et al., New England Journal of Medicine 383, 109-119 (2020)) in both sequence and chemistry (*i.e.*, ASO^{SOD1}, SEQ ID NO: 864).

[40] **FIGs.8A-8B** show the knockdown activity of siRNA-ACO drug candidates *in vitro*. Dose response curves were generated in SK-N-AS (**FIG.8A**) and T98G (**FIG.8B**) cells for each 5'VP-modified siRNA-ACO (*i.e.*, siSOD1-063M3-$AC1^{VP}$, 047M3-$AC1^{VP}$, 104M3-$AC1^{VP}$, 005M3-$AC1^{VP}$, 258M3-$AC1^{VP}$, and 270M3-$AC1^{VP}$) at gradient treatment concentrations. *SOD1* expression levels were quantified via RT-qPCR using gene specific primer sets. *TBP* was amplified as an internal reference to normalize data. Shown are the expression values of *SOD1* mRNA relative to mock transfections. Data represents mean ± SD from 2 experimental replicates.

[41] **FIGs.9A-9B** show the untoward cytotoxicity of siRNA-ACO drug candidates *in vitro*. SK-N-AS and T98G cells were transfected with the lead siRNA-ACO conjugates (*i.e.*, siSOD1-063M3-$AC1^{VP}$, 047M3-$AC1^{VP}$, 104M3-$AC1^{VP}$, 005M3-$AC1^{VP}$, 258M3-$AC1^{VP}$) at gradient treatment concentrations in excess of their IC_{50} values (*i.e.*, 1.56, 6.25, 25, and 100 nM). Treatment with siCon at 100 nM served as a Neg Con. Mock samples were transfected in absence oligonucleotide. Untoward cytotoxicity was evaluated 72 hours after treatment by quantifying both caspase 3/7 activity in **FIG.9A** and metabolism of WST-8 reagent as a marker for cell viability in **FIG.9B**. Data is shown as mean values ± SD from 2 experimental replicates relative to Mock treatments (dotted lines).

[42] **FIG.10** shows siRNA-ACO activity in CNS tissue of $hSOD1^{G93A}$ mice. Adult $hSOD1^{G93A}$ mice were treated via intracerebroventricular (ICV) injection with each siRNA-ACO drug candidate (*i.e.*, siSOD1-047M3-$AC1^{VP}$ and 005M3-$AC1^{VP}$) at a fixed molecular dose (20 nmole). Treatment with aCSF alone was used as a vehicle control to establish baseline expression, while a non-specific siRNA-ACO (*i.e.*, siCON2-$AC1^{VP}$) served as a negative control. Knockdown activity was quantified in tissues from the brain (*i.e.*, frontal cortex, cerebellum, and cerebrum), spinal cord (*i.e.*, cervical, thoracic, and lumbar), and periphery (*i.e.*, liver) via RT-qPCR on day 14 following treatment via ICV injection at a fixed molecular dose (20 nmole) with siRNA-ACO candidates (*i.e.*, siSOD1-047M3-$AC1^{VP}$ and 005M3-$AC1^{VP}$) or their non-conjugated derivatives (*i.e.*, siSOD1-047$M3^{VP}$ and 005$M3^{VP}$). Mouse *Tbp* (*mTbp* or *Tbp*) was amplified as an internal reference to normalize expression data. Shown are the mean expression values ± SD (n=2-6 mice/group) of human *SOD1* (*hSOD1* or *SOD1*) relative to aCSF treatment. The dotted gray line represents 80% knockdown relative to baseline (dashed line).

[43] **FIGs.11A-11B** show the dose-dependent relationship between knockdown activity and tissue accumulation of siRNA-ACO in different CNS tissues. Adult $hSOD1^{G93A}$ mice were treated with siSOD1-047M3-$AC1^{VP}$ (**FIG.11A**) or siSOD1-005M3-$AC1^{VP}$ (**FIG.11B**) at the indicated doses (*i.e.*, 50, 100, 200, or 400 µg) via ICV injection. Knockdown activity of *hSOD1* was quantified via RT-qPCR in select CNS tissues (*i.e.*, cerebellum, cerebrum, and spinal cord) on day 14 following treatment. Animals treated with aCSF alone represent expression levels at baseline and drug quantities detectable in absence of siRNA-ACO treatment (0 µg). **FIGs.11A-11B** show knockdown activity as percent (%) inhibition of *SOD1* relative to baseline (0 µg). Drug concentrations are

shown as siRNA-ACO quantities relative to tissue sample mass (μg/g). Data represents mean ± SD (n=3-4 mice/group).

[44] **FIGs.12A-12C** show the siRNA-ACO delays disease onset and extends animal survival by a single dose ICV injection. Adult *hSOD1*^{G93A} mice were treated with siSOD1-047M3-AC1^{VP} or siSOD1-005M3-AC1^{VP} at the indicated doses (*i.e.*, 50, 100, 200, or 400 μg) via ICV injection on postnatal day (PND) 85 or PND 60, respectively. Growth rates (*i.e.*, percent change in body weight) relative to first day of treatment (dotted line) were plotted to monitor disease progression in **FIG.12A**. Disease onset is plotted as percentage of animals at their peak body weight in each treatment group in **FIG.12B**. Animal survival is plotted as percentage of surviving animals in each treatment group in **FIG.12C**. Animal numbers (n) are indicated in each graph.

[45] **FIGs.13A-13D** show the pathogenic SNPs in target sites of lead siRNA-ACO candidates. **FIGs.13A-13B** show the location of pathogenic SNPs within the target sites of siSOD1-005M3-AC1^{VP} (**FIG.13A**) and siSOD1-047M3-AC1^{VP} (**FIG.13B**). Shown is guide strand sequence including "seed" region (highlighted in grey) complementary to target sites in *hSOD1* transcript containing the indicated pathogenic SNPs. Nucleotide mutation is shown in italic bold in which 'R' signifies a purine substitution. **FIG.13C** shows the luciferase reporter constructs (*i.e.*, pLuc^{SOD1}, pLuc$^{P.E22G}$, pLuc$^{P.F21C}$) containing either consensus sequence or one of the pathogenic mutations (*i.e.*, P.E22G and P.F21C) were co-transfected with siSOD1-047M3-AC1^{VP} or a non-specific scramble control (siCON2-AC1^{VP}) at 18 nM concentrations in HEK293A cells. Dose response curves for luciferase activity were generated following co-transfection with siSOD1-047M3-AC1^{VP} at indicated concentrations (*i.e.*, 0.03, 0.07, 0.22, 0.67, 2.0, 6.0, and 18 nM) in **FIG.13D**. Data represents mean ± SEM from 2 experimental replicates relative to samples treated in absence of siRNA. Statistical significance (*) was determined using Tukey's multiple comparison test to compare the mean values at each data point within the three dose response curves.

[46] **FIGs.14A-14C** show two-doses administration of the siRNA-ACO delay disease onset and prolong survival. Male and female adult *hSOD1*^{G93A} mice were treated twice with siSOD1-047M3-AC1^{VP} at the indicated doses (*i.e.*, 75, 150, or 300 μg) via intrathecal (IT) injection on PND 68 and PND 100. Non-specific control (*i.e.*, siCON3-AC1^{VP}) and ASO^{SOD1} were dosed at 150 μg/injection. Treatment with aCSF served as a vehicle control. Body weight in grams (g) was plotted comparative to background animals (wild type, WT) to monitor disease progression in **FIG.14A**. Disease onset is plotted as percentage of animals at their peak body weight in each treatment group in **FIG.14B**. Animal survival is plotted as percentage of surviving animals in each treatment group in **FIG.14C**. Animal numbers (n) are indicated in each graph.

[47] **FIGs.15A-15D** show the siRNA-ACO treatment improves motor function in *hSOD1*^{G93A} mice. Male and female *hSOD1*^{G93A} mice treated twice with siSOD1-047M3-AC1^{VP} at with the indicated doses (*i.e.*, 75, 150, or 300 μg) via IT on PND 68 and PND 100. Male and female *hSOD1*^{G93A} mice were subject to open field tests during the daytime. Total distance each animal moved was autonomously recorded in centimeters (cm) over the course of 15 minutes. Data is plotted as the mean ± SD distance traveled for each treatment group in **FIG.15A**. Grip strength was assessed in male and female animals organized by treatment group. Grip strength tests were

performed in triplicate and the average value was recorded for each animal. Data is plotted as mean ± SD for grip strength for each treatment group in grams (g) in **FIG.15B**. Animal fatigue and coordination was assessed by rotarod test for 5 minutes. Experiments were performed in triplicate in which the longest latency time to fall was recorded in seconds (s) for each animal and showed in **FIG.15C**. Motor function was scored using the ALS TDI neuroscore (NS) scale for all animals prior to open field, rotarod, and/or grip strength tests. Experiments were performed in triplicate in which the longest latency time to fall was recorded in seconds (s) for each animal in **FIG.15D**. Mean NS ± SD is shown at each treatment group at the indicated time points.

[48] **FIG.16** shows the temporal comparison of rotarod performance for each individual animal following siRNA-ACO treatment as indicated in FIG.14. Latency time to fall in seconds (s) as measured by the rotarod test for each individual animal in their corresponding treatment groups at an early timepoint (*i.e.*, PND 90) in comparison to performance at final timepoint (*i.e.*, the last timepoint before dying, see Table 8). All tests were performed in triplicate in which the longest latency time was recorded for each animal.

[49] **FIG.17** shows the knockdown activity of siRNA on the expression of *SOD1* mRNA in HeLa cells. The indicated siRNAs (*i.e.*, RD-15757, RD-18972, RD-12500, RD-18973, RD-18948 and RD-18949) were directly added into culture medium containing HeLa cell at 1500 nM for 3 days. Cells were transfected in the absence of an oligonucleotide as Mock treatments (not shown). RD-11566 (dsCon2M3v) served as a non-targeting duplex control. *SOD1* mRNA levels were quantified by two-step RT-qPCR using a gene specific primer set in individual PCR reactions. *TBP* was amplified as an internal reference. The values (y-axis) represent the *SOD1* mRNA level relative to Mock treatment after normalized to *TBP* (mean ± SEM of three replicated transfection wells).

[50] **FIGs.18A-18B** show the knockdown activity of siRNA on the expression of *SOD1* mRNA in SK-N-AS cells. The indicated siRNAs (*i.e.*, RD-12926, RD-15757, RD-12500, RD-18947, RD-18948, RD-18949, RD-18946, RD-18972 and RD-18973) were transfected into SK-N-AS cells at indicated concentrations (*i.e.*, 0.0002, 0.001, 0.0039, 0.0156, 0.0625, 0.25, 1 and 4 nM) for 24 hours. Cells were transfected in the absence of an oligonucleotide as Mock treatments (not shown). RD-11566 (dsCon2M3v) served as a non-targeting duplex control (not shown). **FIGs.18A-18B** showed the *SOD1* mRNA level as quantified by two-step RT-qPCR using a gene specific primer set in individual PCR reactions. *TBP* was amplified as an internal reference. The values (y-axis) represent the *SOD1* mRNA level relative to Mock treatment after normalized to *TBP* (mean ± SEM of three replicated transfection wells).

DETAILED DESCRIPTION

[51] While various embodiments of the invention have been shown and described herein, it will be obvious to those skilled in the art that such embodiments are provided by way of example only. Numerous variations, changes, and substitutions may occur to those skilled in the art without departing from the invention. It should be understood that various alternatives to the embodiments of the invention described herein may be employed.

[52] Aspects of the present application include methods of treating Amyotrophic lateral sclerosis (ALS) by administering an effective amount of an oligonucleotide agent comprising a *SOD1*-targeting siRNA. The oligonucleotide agent interferes with *SOD1* mRNA transcript through the RNA silencing mechanism (RNAi). The present inventors have developed *SOD1* siRNAs with potent inhibitory effect, improved delivery, biodistribution, bioavailability, and other pharmacological properties, for use in the treatment of ALS.

[53] The present application is based on investigations related to oligonucleotide agent, compositions and methods that, a targeting oligonucleotide (siRNA etc.), in combination with an oligonucleotide-delivery vehicle (ODV), can downregulate/decrease a gene expression, to improve therapeutic effects for genetic conditions. The term "oligonucleotide-delivery vehicle (ODV)" refers to a structure by conjugating an "accessory oligonucleotide (ACO)" to a molecule, *e.g.*, a duplex oligonucleotide, to facilitate the introduction of the molecule into or uptake by a cell, a tissue, or an organ of an individual.

[54] The present inventors found that some siRNA duplex sequences showed better inhibitory potency on *SOD1* transcript than the sequences having the same or similar target sequence in the prior art. The present inventors also found that chemical modification to the siRNA can improve siRNA activity in vitro. Surprisingly, by conjugated to a single stranded accessory oligonucleotide (ACO, or ODV), the oligonucleotide agent (*i.e.*, ODV-siRNA or siRNA-ACO) achieved CNS delivery, desired biodistribution and bioavailability in CNS tissues when administered in either brain or spinal cord tissue via intracerebroventricular (ICV) and/or intrathecal (IT) injections.

[55] DEFINITIONS

[56] In the present application, the related terms are defined as follows:

[57] Every numerical range given throughout this specification will include every narrower numerical range that falls within such broader numerical range, as if such narrower numerical ranges were all expressly written herein.

[58] The transitional terms/phrases (and any grammatical variations thereof) "comprising", "comprises", "comprise", include the phrases "consisting essentially of", "consists essentially of", "consisting of", and "consists of" and can be interchanged throughout the application. The open term "comprise" also includes a closed term "consisting of" as one option. As used herein, the terms "include," "have" and "comprise" are used synonymously, which terms and variants thereof are intended to be construed as non-limiting.

[59] The terms "Amyotrophic lateral sclerosis" or "ALS" include, but are not limited to, familial ALS (fALS), sporadic ALS (sALS), Lou Gehrig's disease, diseases associated with mutant genes Chromosome 9 Open Reading Frame 72 gene (*C9orf72*; 40%), superoxide dismutase 1 (*SOD1*; 20%), transactive response DNA-binding protein 43 (*TDP43*; 4%) and fused in sarcoma/translocated in liposarcoma (*FUS/TLS*; 4%).

[60] The term "target gene" as used herein can refer to nucleic acid sequences in the form of DNA, RNA or DNA/RNA hybrid, transgenes, viral or bacterial sequences, chromosomes or

extrachromosomal genes that are naturally present in organisms, and/or can be transiently or stably transfected or incorporated into cells and/or chromatins thereof. The target gene can be a protein-coding gene or a non-protein-coding gene (such as a microRNA gene and a long non-coding RNA gene), or a transcript of the protein-coding gene, such as, a message RNA (mRNA) or a complementary DNA (cDNA) of the protein-coding gene. "Target sequence", "target site" or "target" used interchangeably refers to a consecutive oligonucleotide sequence in the sequence of a target gene, such as, the mRNA or cDNA of a target gene, which is homologous or complementary with a sense strand or an antisense strand of a siRNA with or without one or more mismatched based pairs.

[61] As used herein, the terms "*SOD1*" and " *SOD1* gene" can be used interchangeably, and refer to a gene encoding SOD1 protein, preferably a mammalian gene, and more preferably a human gene. As used herein, the term "*SOD1* mRNA" refers to a message RNA (mRNA) generated from the expression of *SOD1* gene, or the transcription of *SOD1* gene. As used herein, the term " *SOD1* cDNA" refers to a complementary DNA (cDNA) generated from the reverse-transcription of a *SOD1* mRNA. As used herein, the term "SOD1 protein" refers to a protein generated from the expression of *SOD1* gene, or translation of the *SOD1* mRNA.

[62] As used herein, the term "baseline expression of *SOD1* gene" or "baseline level of *SOD1* mRNA" used interchangeably refers to the expression of *SOD1* gene of a parallel reference (such as a cell or an individual) without or before the treatment of the siRNA.

[63] The term "oligonucleotide agent" or "oligonucleotide" can be used interchangeably, and refers to polymers of nucleotides, and includes, but is not limited to, single-stranded or double-stranded nucleic acid molecules of DNA, RNA, or DNA/RNA hybrid, oligonucleotide strands containing regularly and irregularly alternating deoxyribosyl portions and ribosyl portions, as well as modified and naturally or unnaturally existing frameworks for such oligonucleotides. The oligonucleotide for inhibiting mRNA transcript level of target gene described herein is a small inhibiting nucleic acid molecule (siRNA), an antisense oligonucleotide molecule (ASO), or an oligonucleotide delivery vehicle (ODV) conjugated siRNA molecule (siRNA-ACO).

[64] The terms "oligonucleotide strand", "strand" and "oligonucleotide sequence" as used herein can be used interchangeably, referring to a generic term for short nucleotide sequences having less than 35 bases (including nucleotides in deoxyribonucleic acid (DNA) or ribonucleic acid (RNA)). In a non-limiting example, the length of a strand can be any length ranging from 16 to 25 nucleotides.

[65] As used herein, the terms "subject" and "individual" are used interchangeably herein to mean any living organism that may be treated with agents of the present application. The term "patient" means a human subject or individual, including disclosure infants, children, and adults.

[66] A "therapeutically effective amount" of a composition is an amount sufficient to achieve a desired therapeutic effect, and therefore does not require cure or complete remission. In embodiments of the present application, therapeutic efficacy is an improvement in any of the disease indicators, and a therapeutically effective amount is sufficient to cause an improvement in a clinically significant condition/symptom in the treated individual. The phrases "therapeutically effective amount" and "effective amount" are used herein to mean an amount sufficient to reduce by

at least about 15 percent, preferably by at least 50 percent, more preferably by at least 90 percent, or to decrease at least about 50 percent, at least about 100 percent, at least about 200 percent, more preferable at least about 500 percent and most preferably prevent, a clinically significant deficit in the activity, function and response of the individual being treated.

[67] The effective amount may vary depending on such factors as the size and weight of the subject, the type of illness, or the particular agents of the application. For example, the choice of the agent of the application could affect what constitutes an "effective amount." One of the ordinary skill in the art would be able to study the factors contained herein and make the determination regarding the effective amount of the agents of the application without undue experimentation.

[68] The regime of administration may affect what constitutes an effective amount. The agent of the application can be administered to the subject either prior to or after the disease diagnosis or condition. Further, several divided dosages, as well as staggered dosages, can be administered daily, weekly, monthly, quarterly or sequentially, or the dose can be continuously infused, or can be a bolus injection. Further, the dosages of the agent(s) of the application could be proportionally increased or decreased as indicated by the exigencies of the therapeutic or prophylactic situation.

[69] The terms "treat," "treated," "treating", or "treatment" as used herein have the meanings commonly understood in the medical arts, and therefore do not require cure or complete remission, and include any beneficial or desired clinical results. Non-limiting examples of such beneficial or desired clinical results are prolonging survival as compared to expected survival without treatment, reduced symptoms including one or more of the followings: weakness and atrophy of proximal skeletal muscles, inability to sit or walk independently, difficulties in swallowing, breathing, etc.

[70] As used herein, "preventing" or "delaying" a disease refers to inhibiting the full development of a disease.

[71] The term "biological sample" refers to any tissue, cell, fluid, or other material derived from an organism (*e.g.*, human subject). In some embodiments, the biological sample is serum or blood.

[72] The term "identity" or "homology" as used herein means that one oligonucleotide strand (sense or antisense strand) of a siRNA has sequence similarity with a coding strand or template strand in a region of a target gene. As used herein, the "identity" or "homology" may be at least about 75%, about 79%, about 80%, about 85%, about 90%, about 95% or 99%. In some embodiments, the siRNA has 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 residues that are different from a reference sequence. To determine the percent identity 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 nucleic acid sequence for optimal alignment and non-homologous sequences can be disregarded for comparison purposes). The nucleotides at corresponding nucleotide positions are then compared. When a position in the first sequence is occupied by the same nucleotide as the corresponding position in the second sequence, then the molecules are identical at that position. The percent identity between the two sequences is a function of the number of identical positions shared by the sequences, considering the number of gaps, and the length of each gap, which need to be introduced for optimal alignment of the two sequences.

[73] The comparison of sequences and determination of percent identity between two sequences can be accomplished using a mathematical algorithm, such as using the Needleman and Wunsch ((1970) J. Mol. Biol. 48:444-453) algorithm which has been incorporated into the GAP program in the GCG software package (available at www.gcg.com). The percent identity between two nucleotide sequences can be determined using the algorithm of E. Meyers and W. Miller ((1989) CABIOS, 4:11-17) 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 of 4. It is understood that the molecules described herein may have additional conservative or non-essential nucleic acid substitutions, which do not have a substantial effect on their functions.

[74] In embodiments of the present application, one target gene is *SOD1*. By "target sequence" is meant a sequence fragment to which the sense oligonucleotide strand or antisense oligonucleotide of the siRNA is homologous or complementary. For example, in some embodiments, a *SOD1* siRNA is homologous or complementary to a target select sequence within human *SOD1* transcript.

[75] As used herein, the term "non-targeting" means that the referenced accessory oligonucleotide (ACO) which conjugates with the targeting oligonucleotide (*e.g.*, siRNA, saRNA, and etc.) does not specifically complement to the target sequence which the targeting oligonucleotide functions, and/or that the referenced oligonucleotide (*i.e.*, ACO) does not share the same target sequence which the targeting oligonucleotide (*e.g.*, siRNA, saRNA, and etc.) specifically attends to function to. The targeting oligonucleotide disclosed herein is a nucleic acid sequence that specifically complements to the target sequence or the region thereof. In some embodiments, the term "non-targeting oligonucleotide" may comprise any referenced oligonucleotide except the "targeting sequence". In some cases, the "specifically complementary" may mean that the complementarity between the targeting oligonucleotide and the target sequence or the region thereof is at least about 95%. The non-targeting oligonucleotide (*i.e.*, ACO) is not to elicit biological activity via any known mechanism, nor intended to elicit activities indicative of ASO (*i.e.*, "mixmer" or "gapmer") function onto a complementary nucleic acid sequence (*i.e.*, mRNA) in a certain subject, an organ of the subject, a tissue of the subject, or a cell of the subject, when the oligonucleotide is administered. The non-targeting oligonucleotide (*i.e.*, ACO) is to facilitate the introduction of the targeting oligonucleotide (*e.g.*, siRNA, saRNA, and etc.) it conjugates into a certain subject, an organ of the subject, a tissue of the subject, a cell of the subject, or a cell nucleus of the subject, when the oligonucleotide conjugate is administered.

[76] As used herein, the term "gapmer" refers to a short DNA antisense oligonucleotide (ASO) structure with modified RNA segments on both sides of the central DNA structure. In some embodiments, at least one of the modified RNA segments comprises one or more of modified nucleotides selected from locked nucleic acids (LNA), and 2'-OMe or 2'-F modified nucleotides to increase affinity to the target, increase nuclease resistance, reduce immunogenicity, and/or decrease toxicity. In some embodiments, a gapmer comprises at least one nucleotide modified with a phosphorothioate (PS) group. In some embodiments, the gamper is designed to hybridize to a target piece of RNA and silence the gene transcript through the induction of RNase H cleavage. As an example, the ASO drug "Tofersen" is a gapmer that knockdowns *SOD1* mRNA for treatment of ALS.

A possible example of a dual-action oligonucleotide (DAO) with a gapmer ASO disclosed in the present application could be "siSOD1-Tofersen conjuagte".

[77] As used herein, the term "mixmer" refers to an antisense oligonucleotide (ASO) characterized as a mixture of DNA and chemically-modified nucleic acid analogs in structure. Optionally, a mixmer is composed of fully modified nucleotides or nucleic acid analogs. In some embodiments, a mixmer is designed to bind and mask complementary RNA sequence to sterically block proteins, factors, or other RNAs from interacting with targeted RNA. In some embodiments, a mixmers is designed to alter pre-mRNA splicing by displacing the spliceosome. In some embodiments, a mixmer is designed to bind and sequester microRNAs (miRNAs) in which it is adopted yet another name called an "antagomir" or an "anti-miR".

[78] As used herein, the terms “sense strand” and “sense oligonucleotide strand” are interchangeable. The sense oligonucleotide strand of siRNA molecule can include, for example, a first nucleic acid strand of siRNA comprising a fragment of a sequence in the human genome or the sequence of a target gene.

[79] As used herein, the terms “antisense strand” and “antisense oligonucleotide strand” are interchangeable. The antisense oligonucleotide strand of a siRNA molecule can include, for example, a second nucleic acid strand in a duplex of siRNA that is complementary to the sense oligonucleotide strand. An antisense strand of a siRNA may be complementary to a consecutive fragment of a target gene sequence and is capable of binding to the consecutive fragment with 0, 1, 2, 3, 4 or 5 mismatches without affecting the function of the siRNA.

[80] As used herein, the term “coding strand” refers to the DNA strand in the target gene that cannot be transcribed, the nucleotide sequence of which is identical to the sequence of the RNA produced by transcription (in RNA the T in DNA is replaced by U). The coding strand of the double-stranded DNA sequence of the target gene promoter described in the present application refers to the promoter sequence on the same DNA strand as the DNA coding strand of the target gene.

[81] As used herein, the term “template strand” refers to another strand of double-stranded DNA of a target gene that is complementary to the coding strand and that can be transcribed as a template into RNA that is complementary to the transcribed RNA base (A-U, G-C). During transcription, RNA polymerase binds to the template strand and moves along the 3 '→ 5' direction of the template strand, catalyzing RNA synthesis in the 5'→ 3' direction. The template strand of the double-stranded DNA sequence of the target gene promoter described in the present application refers to the promoter sequence on the same DNA strand as the DNA template strand of the target gene.

[82] As used herein, the term “overhang” refers to an oligonucleotide strand end (5' or 3 ') with non-base paired nucleotide(s) resulting from another strand extending beyond one of the strands within the siRNA. Single-stranded regions extending beyond the 3 'and/or 5' ends of the duplexes are referred to as overhangs. In some embodiments, the overhang is from 0 to 6 nucleotides in length. It is understood that an overhang of 0 nucleotides means that there is no overhang.

[83] The term "natural overhang" as used herein refers to an overhang which consists of one or more nucleotides identical to or complementary to the corresponding position on the target sequence. A natural overhang on a sense strand consists of one or more nucleotides identical to the corresponding position on the mRNA target. A natural overhang on an antisense strand consists of one or more nucleotides complementary to the corresponding position on the mRNA target.

[84] As used herein, the terms "gene silencing", "knockdown of gene expression", "gene downregulation", "decreasing gene expression" and "downregulating gene expression" can be used interchangeably, and means a decrease or downregulation in transcription, translation, expression or activity of a certain nucleic acid sequence as determined by measuring the transcription level, mRNA level, protein level, enzymatic activity, methylation state, chromatin state or configuration, translation level or the activity or state in a cell or biological system of a gene. These activities or states can be determined directly or indirectly. In addition, "gene downregulation" or "downregulating gene expression" refers to a decrease in activity associated with a nucleic acid sequence, regardless of the mechanism of such downregulation. For example, gene downregulation occurs at the transcriptional or post-transcriptional level to decrease transcription into RNA and the RNA level is decreased to be translated into lower level of protein than baseline, thereby decreasing the expression of the protein.

[85] As used herein, the terms "short interfering RNA", "siRNA" and "silencing RNA" can be used interchangeably and refer to a ribonucleic acid molecule that can downregulate, knockdown, or silence target gene expression. It can be a double-stranded nucleic acid molecule. siRNA binds to target mRNA mainly in the cytoplasm to down-regulate gene expression post-transcriptionally via the RNA interference (RNAi) mechanism.

[86] siRNAs may contain natural nucleotides or chemically modified nucleotides. The modifications can impart increased nuclease stability and/or increased cellular potency. Examples of chemical modifications include phosphorothioate backbone modification, 2'-deoxynucleotide, 2'-OCH_3-containing ribonucleotides, 2'-F-ribonucleotides, 2'-methoxyethyl ribonucleotides, combinations thereof and the like. The siRNA can have varying lengths (*e.g.*, 10-200 bps) and structures (*e.g.*, hairpins, single/double strands, bulges, nicks/gaps, mismatches) and are processed in cells to knock down target mRNA. A double-stranded siRNA can have the same number of nucleotides on each strand (blunt ends) or asymmetric ends (overhangs). An overhang of 1-2 nucleotides, for example, can be present on the sense and/or the antisense strand, as well as present on the 5'- and/or the 3'-ends of a given strand.

[87] The length of the siRNA molecule is typically about 10 to about 60, about 10 to about 50, about 15 to about 30, about 17 to about 29, about 18 to about 28, about 19 to about 27, about 20 to about 26, about 21 to about 25, and about 22 to about 24 base pairs, and typically about 15, about 16, about 17, about 18, about 19, about 20, about 21, about 23, about 25, about 30, about 40, or about 50 base pairs. In addition, the terms "small interfering RNA", "silencing RNA" and "siRNA" also contain nucleic acids other than the ribonucleotide, including, but not limited to, modified nucleotides or analogues.

[88] The term "equal length portion" refers to a portion of a sequence that is compared with an object sequence (*e.g.*, a continuous oligonucleotide sequence from the siRNA) and has equal length (equal number of bases) to the object sequence.

[89] The term "sequence specific mode" as used herein means a binding or hybridization way of two nucleic acid fragments according to their nucleotide sequence, *e.g.*, a Watson-Crick manner (such as A to T, A to U, and C to G) or any other manner allowing the formation of a duplex (such as Hoogsteen or reverse Hoogsteen base pairing).

[90] As used herein, the terms "isolated target site", "target site" and "isolated polynucleotide" can be used interchangeably, and herein means a nucleic acid target site to which a siRNA has complementarity or hybridizes to. For example, an isolated nucleic acid sequence of a target site can include a nucleic acid sequence to which a region of siRNAs has complementarity or hybridize to.

[91] As used herein, the term "complementary" refers to the capability of forming base pairs between two oligonucleotide strands. The base pairs are generally formed through hydrogen bonds between nucleotides in the antiparallel oligonucleotide strands. The bases of the complementary oligonucleotide strands can be paired in the Watson-Crick manner (such as A to T, A to U, and C to G) or in any other manner allowing the formation of a duplex (such as Hoogsteen or reverse Hoogsteen base pairing).

[92] Complementarity includes complete complementarity and incomplete complementarity. "Complete complementarity" or "100% complementarity" means that each nucleotide from the first oligonucleotide strand can form a hydrogen bond with a nucleotide at a corresponding position in the second oligonucleotide strand in the double-stranded region of the siRNA molecule, with no base pair being "mispaired". "Incomplete complementarity" or "mismatch" means that not all the nucleotide units of the two strands are bound with each other by hydrogen bonds. For example, for two oligonucleotide strands each of 20 nucleotides in length in the double-stranded region, if only two base pairs in this double-stranded region can be formed through hydrogen bonds, the oligonucleotide strands have a complementarity of 10%. In the same example, if 18 base pairs in this double-stranded region can be formed through hydrogen bonds, the oligonucleotide strands have a complementarity of 90%. Substantial complementarity refers to at least about 75%, about 79%, about 80%, about 85%, about 90%, about 95% or 99% complementarity.

[93] As used herein, "ODV" and "oligonucleotide delivery vehicle" are used interchangeably, which refer to an oligonucleotide molecule comprising a duplex or double-stranded RNA (*e.g.*, siRNA or saRNA) and an ACO which is covalently linked to the duplex RNA via a linker as described in more detail below.

[94] As used herein, "covalent linker", "linker" and "linking component" are used interchangeably, which refer to an organic moiety that connects two parts of a compound. For example, one or more of a single-stranded oligonucleotide (*e.g.*, ACO) and a dsRNA (*e.g.*, siRNA), two dsRNAs, etc. are covalently linked by, *e.g.*, a nucleic acid linker, a peptide linker, and the like and, includes disulfide linkers.

[95] As used herein, the term "synthetic" refers to the manner in which oligonucleotides are synthesized, including any means capable of synthesizing or chemically modifying RNA, such as chemical synthesis, *in vitro* transcription, vector expression, and the like.

[96] The terms "oligonucleotide modulator" and "oligonucleotide agent" can be used interchangeably and refer to an oligonucleotide-containing substance which at least comprises or consists of one or more siRNA of the invention and has the activity of modulating target gene expression or enhance the effect of the siRNA, and may further comprise other oligonucleotide moieties/components (such as ASO, or accessory oligonucleotide (ACO)) or non-oligonucleotide moieties/components conjugated, combined or mixed with the siRNA(s). In certain embodiments, the oligonucleotide modulator comprises an RNA (such as the siRNA of the invention), a DNA, a BNA, an LNA, a GNA or a PNA.

[97] As used herein, the term "LNA" refers to a locked nucleic acid in which the 2'-oxygen and 4'-carbon atoms are joined by an extra bridge. As used herein, the term "BNA" refers to a 2'-O and 4'-aminoethylene bridged nucleic acid that can contain a five-membered or six-membered bridged structure with an N-O linkage. As used herein, the term "PNA" refers to a nucleic acid mimic with a pseudopeptide backbone composed of N-(2-aminoethyl) glycine units with the nucleobases attached to the glycine nitrogen via carbonyl methylene linkers. As used herein, the term "GNA" also referred to as glycerol nucleic acid, is a nucleic acid similar to DNA or RNA but differing in the composition of its sugar-phosphodiester backbone, using propylene glycol in place of ribose or deoxyribose.

[98] In the present application, singular forms, such as "a" and "this", include plural objects, unless otherwise specified clearly in the context.

[99] Unless otherwise defined, all the technological and scientific terms used therein have the same meanings as those generally understood by those of ordinary skill in the art covering the present application.

siRNA

[100] Embodiments of the present application are based in part on the surprising discovery that an oligonucleotide agent (for example, siRNA, also referred to as "*SOD1* gene siRNA" or "*SOD1* siRNA" herein) is capable of inhibiting or downregulating the expression of a *SOD1* gene in a cell. The decrease in functional *SOD1* gene transcript following administration with an oligonucleotide agent of the present application can achieve a significant decrease or downregulation in the levels of *SOD1* mRNA and SOD1 protein in a cell or a mammal.

[101] In particular, the inventors discovered that the functional oligonucleotide agents capable of inhibiting expression of superoxide dismutase 1 (*SOD1*) comprising a small interfering RNA (siRNA), wherein the siRNA comprises a sense strand and an antisense strand forming a double strand, wherein the antisense strand comprises a nucleotide sequence comprising at least 10 contiguous nucleotides, with 0, 1, 2 or 3 mismatches, having at least 85% nucleotide sequence complementarity or homology to a portion of the nucleotide sequence of *SOD1* mRNA.

[102] As a beneficial consequence, a target sequence (*e.g.*, an isolated nucleic acid sequence comprising the target sequence), upon interacting with the siRNA, can inhibit/downregulate the *SOD1* mRNA transcript by at least 10%, for example, at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 93%, at least 96%, at least 99%, or about 100%, as compared to a baseline level of *SOD1* mRNA. In one embodiment, *SOD1* mRNA is decreased by at least 80%. Based at least in part on these discoveries, the present application features siRNA, compositions, and pharmaceutical compositions for inhibiting/downregulating the *SOD1* mRNA transcript by at least 10% as compared to baseline levels of *SOD1* mRNA. Also provided herein are methods for preventing or treating a disease or condition induced by over-expression of SOD1 protein, a *SOD1* gene mutation, and/or high or abnormal *SOD1* level in an individual comprising administering to the individual any of the siRNA, compositions, and/or pharmaceutical compositions described herein.

[103] In certain embodiments, the oligonucleotide sequence disclosed in the present application has at least 85%, at least 90%, or at least 95% homology or complementarity to a nucleotide sequence selected from SEQ ID NOs: 2-269. In certain embodiments, the sense strand of the oligonucleotide sequence disclosed in the present application has at least 85%, at least 90%, or at least 95% homology to a nucleotide sequence selected from SEQ ID NOs:2-269. In certain embodiments, the antisense strand of the oligonucleotide sequence disclosed in the present application has at least 85%, at least 90%, or at least 95% complementarity to a nucleotide sequence selected from SEQ ID NOs: 2-269.

[104] Embodiments of the present application are also based in part on the discovery that the *SOD1* mRNA inhibitory oligonucleotide agents comprise a siRNA having a sense strand that is at least 85%, at least 90%, or at least 95% homology to the nucleotide sequence selected from SEQ ID NOs: 270-537.

[105] In some other embodiments, the *SOD1* mRNA inhibitory oligonucleotide agents comprise a siRNA having an antisense strand that is at least 85%, at least 90%, or at least 95% homology to the nucleotide sequence selected from SEQ ID NOs: 538-805.

[106] In some embodiments, the *SOD1* mRNA inhibitory oligonucleotide agents comprise a siRNA, wherein the sense strand and the antisense strand of the siRNA have nucleotide sequences that is independently at least 85%, at least 90%, or at least 95% homology to the nucleotide sequence selected from SEQ ID NOs: 808-849, 867 and 868.

[107] The siRNAs of the oligonucleotide agent described herein include an RNA strand (the antisense strand) having a region which is 60 nucleotides or less in length, *i.e.*, 15-40 nucleotides in length, generally 19-25 nucleotides in length, which region is substantially complementary to at least part of an mRNA transcript of a *SOD1* gene. The use of these siRNAs enables the targeted degradation of mRNAs of genes that are implicated in pathologies associated with *SOD1* expression in mammals. Very low dosages of *SOD1* siRNAs in particular can specifically and efficiently mediate RNAi, resulting in significant inhibition of expression of a *SOD1* gene. Using cell-based assays, the present inventors have demonstrated that siRNAs targeting *SOD*1 can specifically and

efficiently mediate RNAi, resulting in significant inhibition of expression of a *SOD1* gene. Thus, methods and oligonucleotide agents including these siRNAs are useful for treating pathological processes that can be mediated by down regulating *SOD1*, such as in the treatment of a disorder that causes elevated *SOD1* levels, *e.g.*, amyotrophic lateral sclerosis (ALS). The following detailed description discloses how to make and use oligonucleotide agents containing siRNAs to inhibit the expression of a *SOD1* gene, as well as oligonucleotide agents and methods for treating diseases and disorders caused by the expression of this gene.

[108] In one aspect, an RNA interference agent includes a single-stranded RNA that interacts with a target RNA sequence to direct the cleavage of the target RNA. Without wishing to be bound by theory, long double-stranded RNA introduced into plants and invertebrate cells is broken down into siRNA by a Type III endonuclease known as Dicer (Sharp et al., Genes Dev. 2001, 15:485). Dicer, a ribonuclease-III-like enzyme, processes the dsRNA into 19-23 base pair short interfering RNAs with characteristic two base 3' overhangs (Bernstein, et al., (2001) Nature 409:363). The siRNAs are then incorporated into an RNA-induced silencing complex (RISC) where one or more helicases unwind the siRNA duplex, enabling the complementary antisense strand to guide target recognition (Nykanen, et al., (2001) Cell 107:309). Upon binding to the appropriate target mRNA, one or more endonucleases within the RISC cleaves the target to induce silencing (Elbashir, et al., (2001) Genes Dev. 15:188). Thus, in one aspect the invention relates to a single-stranded RNA that promotes the formation of a RISC complex to effect silencing of the target gene.

[109] In some embodiments, the continuous oligonucleotide sequence of the siRNA has five or less, *i.e.*, 5, 4, 3, 2, 1, or 0 nucleotide differences or mismatches relative to the equal length portion of *SOD1* mRNA. In some embodiments, the continuous oligonucleotide sequence of the sense strand of siRNA has three or less, *i.e.*, 3, 2, 1, or 0 nucleotide differences or mismatches relative to the equal length portion of *SOD1* mRNA. In some embodiments, the continuous oligonucleotide sequence of the antisense strand of siRNA has three or less, *i.e.*, 3, 2, 1, or 0 nucleotide differences or mismatches relative to the equal length portion of *SOD1* mRNA.

[110] In some embodiments, the *SOD1* mRNA disclosed herein does not contain a nucleotide mutation. In some embodiments, the *SOD1* mRNA disclosed herein contains at least one nucleotide mutation. In some embodiments, the *SOD1* mRNA disclosed herein contains at least one nucleotide mutation on the targeting site of the siRNA. In some embodiments, the *SOD1* mRNA disclosed herein contains at least one nucleotide mutation upper stream and/or downstream the targeting site of the siRNA.

[111] In some embodiments, the differences or mismatches are located in the middle or 3' terminus of the oligonucleotide sequence of the siRNA. Methods and principles of siRNA molecule design are well known to those skilled in the art and are described in detail in, for example, Place et. al., *Molecular Therapy–Nucleic Acids* (2012) 1, e15; and Li et.al., *PNAS*, 2006, vol. 103, no. 46, 17337–17342, which are herein incorporated by reference in their entireties.

[112] In some embodiments, the siRNA disclosed herein comprises a sense strand and an antisense strand. The sense strand and the antisense strand comprise complementary regions capable of

forming a double-stranded nucleic acid structure that decreases the *SOD1* transcript level in a cell via the RNAi mechanism. The RNAi mechanism (also known as RNA interference) used herein refers to a mechanism that a double-stranded nucleic acid structure is capable of downregulating target genes in a sequence-specific manner at the transcriptional level. The sense strand and the antisense strand of the siRNA can exist either on two different nucleic acid strands or on one nucleic acid strand (*e.g.*, a contiguous nucleic acid sequence). When the sense strand and the antisense strand are located on two different strands, at least one strand of the siRNA has a 3' overhang of 0 to 6 nucleotides in length, such that the overhangs of 0, 1, 2, 3, 4, 5 or 6 nucleotides in length, and in some cases, both strands have a 3' overhang of 2 or 3 nucleotides in length. The nucleotide of the overhang is, in some cases thymine deoxyribonucleotide (dT), or in some cases, natural overhangs which are nucleotides selected from or complementary to the corresponding position on the DNA target. When the sense strand and the antisense strand are located on one nucleic acid strand, in some cases, the siRNA is a hairpin single-stranded nucleic acid molecule, where the complementary regions of the sense strand and the antisense strand form a double-stranded nucleic acid structure with each other. In the siRNA disclosed herein, in some embodiments, the sense strand has a length ranging from 10 to 60 nucleotides. For example, in some embodiments, the sense strand and the antisense strand, independently comprise a length of 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34 or 35 nucleotides. In some embodiments, the antisense strand has a length ranging from 10 to 60 nucleotides. For example, in some embodiments, the sense strand and the antisense strand, independently comprise a length of 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34 or 35 nucleotides.

[113] In some embodiments, the antisense strand disclosed herein is capable of interacting with a target nucleic acid sequence of a mRNA of a *SOD1* gene in a sequence specific manner, meaning that the antisense strand is capable of undergoing hybridization to a target nucleic acid through hydrogen bonding. In some embodiments, an antisense strand has a nucleotide sequence that, when written in the 5' to 3' direction, comprises the reverse complement of the target portion of a target nucleic acid to which it is targeted. In certain such embodiments, an antisense strand has a nucleotide sequence that, when written in the 5' to 3' direction, comprises the reverse complement of the target portion in a fragment of a *SOD1* gene transcript.

ACO

[114] Although some prior research proved that siRNAs capable of inhibiting *SOD1* mRNA and decreasing SOD1 protein expression can be used to treat SOD1 protein-related diseases, *e.g.*, for amyotrophic lateral sclerosis (ALS) patients, the inventors found there are two unsolved issues, one is lack of potency of *SOD1* siRNA molecules and the other is lack of efficient delivery method to deliver the siRNA molecule to cells of a target organ or tissue, e.g., CNS.

[115] "Delivering into a cell," when referring to an siRNA, means efficiently uptake or absorption by the cell, as is understood by those skilled in the art. Absorption or uptake of an siRNA 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 siRNA can also be "introduced into a cell," wherein the cell is part of a living organism. In such an instance, introduction into the cell will

include the delivery to the organism. For example, for *in vivo* delivery, siRNA 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. Further approaches are described herein below which are not known in the art.

[116] When the siRNA agent is conjugated to a non-targeting single-stranded accessory oligonucleotide (ACO) as disclosed, bioavailability, biodistribution, and/or cellular uptake and *in vivo* potency of the siRNA were significantly improved as compared to an oligonucleotide agent without the ACO. Especially in some *in vivo* examples in the present application, the ACO of the oligonucleotide agent increased the biodistribution of siRNA within one, or two, or more target tissues as compared to an oligonucleotide agent without the ACO.

[117] Therefore, aspects of the present application further relate to an oligonucleotide agent capable of inhibiting the expression of superoxide dismutase 1 (*SOD1*) comprising a small interfering RNA (siRNA), and an ACO.

[118] In some embodiments, the oligonucleotide agent comprising one or more conjugated ACO enhance the biodistribution of the oligonucleotide agent in particular tissues of the oligonucleotide agent, and increase permeability of the oligonucleotide agent and passage through membranes, such as the blood brain barrier.

[119] In some embodiments, the ACO is an oligonucleotide comprising a 5' end and a 3' end.

[120] In some embodiments, the siRNA and the ACO are covalently linked, with or without one or more linking components, to form the oligonucleotide agent.

[121] In some embodiments, the length of the ACO comprises a nucleotide length ranging from 6 to 22 nucleotides, such as 6 nucleotides or more, 7 nucleotides or more, 8 nucleotides or more, 9 nucleotides or more, 10 nucleotides or more, 11 nucleotides or more, 12 nucleotides or more, 13 nucleotides or more, 14 nucleotides or more,15 nucleotides or more,16 nucleotides or more, 17 nucleotides or more, 18 nucleotides or more, 19 nucleotides or more, 20 nucleotides or more, 21 nucleotides or more, 22 nucleotides or more. In some embodiments, the length of the ACO is 6 to 18 contiguous oligonucleotides.

[122] In some embodiments, the length of the ACO can modulate the activity and/or biodistribution of the oligonucleotide agent within the target tissue or cell of interest. For example, the present inventors found that shorter ACOs demonstrated activity throughout the central nervous system, while longer ACOs demonstrated activity only in particular regions of the brain, such as the cerebellum.

[123] In another aspect of the present application, an oligonucleotide agent comprising a siRNA and a non-targeting ACO is provided, the ACO includes a single-stranded oligonucleotide sequence comprising a nucleotide sequence that is at least 60% (*e.g.*, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, at least 99% or 100%) identical to the nucleotide sequence selected from SEQ ID NOs: 865. In some embodiments, wherein the

ACO comprises a nucleotide sequence that is at least 90% identical to the nucleotide sequence selected from SEQ ID NO: 865.

[124] In some embodiments, the oligonucleotide agent of the present application comprises more than one ACO, for example, 2, 3, 4, 5, 6, 7, 9, 10 ACOs, covalently linked to a siRNA, with or without one or more linkers in between the ACOs and siRNA. The number of ACO can vary from 1 to 4, 2 to 10 linked to a siRNA via a multivalent linker, for example, a polymeric linker, in branch or liner form. In some embodiments, multiple ACOs are covalently linked to 2 or more siRNAs, for example, 2, 3, 4, 5, 6, 7, 9, 10 or more siRNAs, in one agent. PCT Application No. WO2023280190A1, the content of which is incorporated herein by reference, in its entirety, for all purposes, describes principles of ACO design and examples of ACO that are conjugated to the siRNAs to improve pharmacokinetics properties including accessory delivering of duplex RNAs to a cell.

Chemical modification

[125] In the siRNAs or ACOs disclosed herein, all nucleotides may be natural or non-chemically modified nucleotides, or at least one nucleotide is a chemically modified nucleotide. Non-limiting examples of the chemical modification include one or more of a combination of the following:

1) modification of a phosphodiester bond of nucleotides in the nucleotide sequence of the siRNA or ACO;

2) modification of 2'-OH of the ribose in the nucleotide sequence of the siRNA or ACO;

3) modification of a base in the nucleotide of the siRNA or ACO;

4) at least one nucleotide in the nucleotide sequence of the siRNA or ACO being a BNA, LNA, GNA or PNA, and

5) at least one nucleotide in the nucleotide sequence of the ACO being a deoxyribonucleotide (DNA).

[126] The chemical modification described herein is well-known to those skilled in the art, and the modification of the phosphodiester bond refers to the modification of oxygen in the phosphodiester bond, including phosphorothioate modification and boranophosphate modification. The modifications disclosed herein stabilize the siRNA structure, maintaining high specificity and high affinity for base pairing. The modifications disclosed herein also stabilize an ACO structure and maintain its delivering accessory properties including bioavailability, biodistribution, and/or cellular uptake of the oligonucleotide agent in various tissues prefrontal cortex, cerebellum, cerebrum, spinal cord (*e.g.*, cervical, thoracic, lumber), muscle, lung, eye, liver, and kidney.

[127] In some embodiments, the chemical modification is to substitute the phosphodiester bond with phosphorothioate (PS) bond on the backbone of the nucleotide sequence of the oligonucleotide agent disclosed herein. In some embodiments, the oligonucleotide agent disclosed herein comprises at least one PS backbone modification. In some embodiments, the ACO comprises at least one PS backbone modification. In some embodiments, the ACO comprises 6-17 PS backbone modifications.

[128] In some embodiments, the siRNA or ACO of the present application includes at least one chemically modified nucleotide which is modified at 2'-OH in pentose of a nucleotide, *i.e.*, the introduction of certain substituents at the hydroxyl position of the ribose, such as 2'-fluoro modification, 2'-oxymethyl modification, 2'-oxyethylidene methoxy modification, 2,4'-dinitrophenol modification, locked nucleic acid (LNA), 2'-amino modification or 2'-deoxy modification, *e.g.*, a 2'-deoxy-2'-fluoro modified nucleotide, a 2'-deoxy-modified nucleotide.

[129] In some embodiments, the siRNA or ACO of the present application includes at least one chemically modified nucleotide which is modified at the base of the nucleotide, *e.g.*, 5 '-bromouracil modification, 5'-iodouracil modification, N-methyluracil modification, or 2,6-diaminopurine modification.

[130] In some embodiments, the chemical modification of the siRNA or ACO is an addition of a (E)-vinylphosphonate moiety at the 5' end of the sense or antisense sequence. In some embodiments, the chemical modification of the at least one chemically modified nucleotide is an addition of a 5-methyl cytosine moiety at the 5' end of the sense or antisense sequence.

[131] In some embodiments, the siRNA or ACO of the present application includes at least one nucleotide in the nucleotide sequence of the siRNA/ACO being a chemically modified nucleic acid, *e.g.*, a locked nucleotide, an abasic nucleotide, a glycerol nucleic acid (GNA), a morpholino nucleotide, a phosphoramidate, and a non-natural base comprising nucleotide. In some embodiments, the siRNA disclosed herein includes an "endo-light" modification with 2'-O-methyl modified nucleotides and nucleotides comprising a 5'-phosphorothioate group.

[132] In some embodiments, the siRNA or ACO of the present application is chemically modified to enhance stability or other beneficial characteristics. The nucleic acids featured in the present application may be synthesized and/or modified by conventional methods, such as those described in "Current protocols in nucleic acid chemistry," Beaucage, S. L. *et al.* (Edrs.), John Wiley & Sons, Inc., New York, N.Y., USA, which is hereby incorporated herein by reference. Modifications include, for example, (a) end modifications, *e.g.*, 5' end modifications (phosphorylation, conjugation, inverted linkages, etc.) 3' end modifications (conjugation, DNA nucleotides, inverted linkages, etc.), (b) base modifications, *e.g.*, replacement with stabilizing bases, destabilizing bases, or bases that base pair with an expanded repertoire of partners, removal of bases (abasic nucleotides), or conjugated bases, (c) sugar modifications (*e.g.*, at the 2' position or 4' position) or replacement of the sugar, as well as (d) backbone modifications, including modification or replacement of the phosphodiester linkages. Specific examples of siRNA molecules that can be used in this present application include but are not limited to RNAs containing modified backbones or no natural internucleoside linkages. In some embodiments, RNAs having modified backbones include, among others, those that do not have a phosphorus atom in the backbone. In some embodiments, modified RNAs that do not have a phosphorus atom in their internucleoside backbone can also be oligonucleosides. In some embodiments, the modified oligonucleotide will have a phosphorus atom in its internucleoside backbone.

[133] Modified oligonucleotide 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.

[134] In some embodiments, the siRNA or the ACO is composed of one or more of RNA, DNA, BNA, LNA, GNA or PNA.

Covalent Linkage

[135] Aspects of the present application include an oligonucleotide agent comprising a double stranded targeting oligonucleotide (*i.e.*, siRNA) and an ACO that are covalently linked.

[136] In some embodiments, any of the oligonucleotides in the oligonucleotide agent of the present application includes a linking component. In some embodiments, a siRNA and a ACO are covalently linked by a linking component. In some embodiments, the siRNA and the ACO are linked with a covalent linker. Various combinations of strands can be linked, *e.g.*, the first and second dsRNA sense strands are covalently linked or, *e.g.*, the first and second dsRNA antisense strands are covalently linked.

[137] In some embodiments, the sense strand of the siRNA is covalently linked to the ACO. In some embodiments, the antisense strand of the siRNA is covalently linked to the ACO. In some embodiments, the ACO is covalently linked to the 3' end, or the 5' end, or both the 3' and 5' ends of the sense strand of the siRNA. In some embodiments, the ACO is covalently linked to the 3' end, or the 5' end, or both the 3' and 5' ends of the antisense strand of the siRNA. In some embodiments, more than one ACO is covalently linked to a siRNA. In some embodiments, 2-10 ACOs are covalently linked to the siRNA. In some embodiments, more than one siRNA is covalently linked to an ACO. In some embodiments, 2-10 siRNAs are covalently linked to an ACO.

[138] In some embodiments, the ACO is conjugated to a linking component. In some embodiments, the 5' end or the 3' end of the ACO is conjugated to a linking component. In some embodiments, the siRNA and the ACO are covalently linked by a linking component. In some embodiments, the sense strand or the antisense strand of the siRNA are covalently linked to the ACO by a linking component.

[139] Linkers typically comprise a direct bond or an atom such as oxygen or sulfur, a unit such as NR^1, C(O), C(O)O, $C(O)NR^1$, SO, SO_2, SO_2NH or a chain of atoms, such as substituted or unsubstituted alkyl, substituted or unsubstituted alkenyl, substituted or unsubstituted alkynyl, arylalkyl, arylalkenyl, arylalkynyl, heteroarylalkyl, heteroarylalkenyl, heteroarylalkynyl, heterocyclylalkyl, heterocyclylalkenyl, heterocyclylalkynyl, aryl, heteroaryl, heterocyclyl, cycloalkyl, cycloalkenyl, alkylarylalkyl, alkylarylalkenyl, alkylarylalkynyl, alkenylarylalkyl, alkenylarylalkenyl, alkenylarylalkynyl, alkynylarylalkyl, alkynylarylalkenyl, alkynylarylalkynyl, alkylheteroarylalkyl,

alkylheteroarylalkenyl, alkylheteroarylalkynyl, alkenylheteroarylalkyl, alkenylheteroarylalkenyl, alkenylheteroarylalkynyl, alkynylheteroarylalkyl, alkynylheteroarylalkenyl, alkynylheteroarylalkynyl, alkylheterocyclylalkyl, alkylheterocyclylalkenyl, alkylhererocyclylalkynyl, alkenylheterocyclylalkyl, alkenylheterocyclylalkenyl, alkenylheterocyclylalkynyl, alkynylheterocyclylalkyl, alkynylheterocyclylalkenyl, alkynylheterocyclylalkynyl, alkylaryl, alkenylaryl, alkynylaryl, alkylheteroaryl, alkenylheteroaryl, alkynylhereroaryl, where one or more methylenes can be interrupted or terminated by O, S, S(O), SO_2, $N(R')_2$, C(O), cleavable linking group, substituted or unsubstituted aryl, substituted or unsubstituted heteroaryl, substituted or unsubstituted heterocyclic; where R^1 is hydrogen, acyl, aliphatic or substituted aliphatic.

[140] Without limitations, various types of linker functionality can be included in the subject conjugates, including but not limited to cleavable linkers, and non-cleavable linkers, as well as reversible linkers and irreversible linkers.

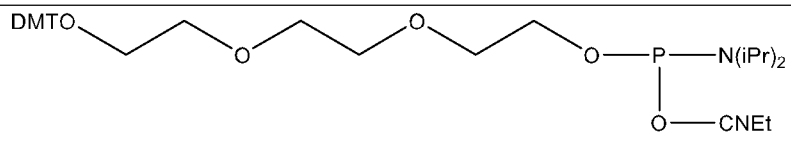
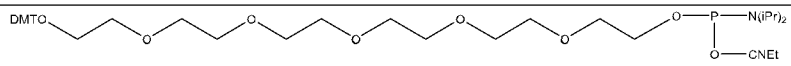
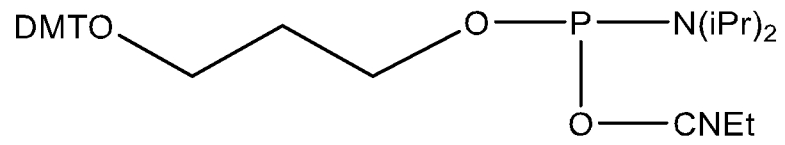
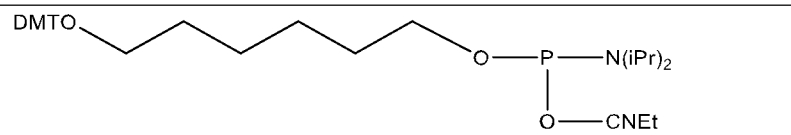
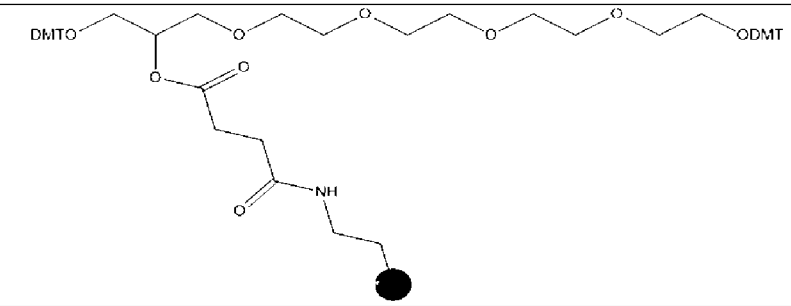
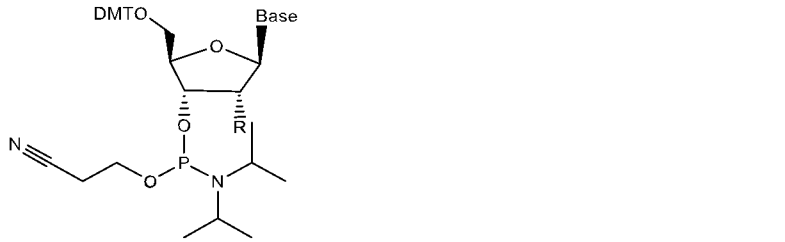
[141] In some embodiments, the linker is a cleavable linker. Cleavable linkers are those that rely on processes inside a target cell to liberate the two parts the linker is holding together, *e.g.*, the ACO and the dsRNA, as reduction in the cytoplasm, exposure to acidic conditions in a lysosome or endosome, or cleavage by specific enzymes (*e.g.*, proteases) within the cell. As such, cleavable linkers allow the dsRNA to be released in its original form after the conjugate has been internalized and processed inside a target cell. Cleavable linkers include, but are not limited to, those whose bonds can be cleaved by enzymes (*e.g.*, peptide linkers); reducing conditions (*e.g.*, disulfide linkers); or acidic conditions (*e.g.*, hydrazones and carbonates).

[142] In some embodiments, the linking component is selected from one or more of ethylene glycol chain, an alkyl chain, a peptide, RNA, DNA, carbohydrates, thiol linkage, a phosphodiester, a phosphorothioate, a phosphoramidate, an amide, and a carbamate. In some embodiments, the linking component includes, but is not limited to: Spacer phosphoramidite 18 (Phosphoramidous acid, N,N-bis(1-methylethyl)-, 19,19-bis(4-methoxyphenyl)-19-phenyl-3,6,9,12,15,18-hexaoxanonadec-1-yl 2-cyanoethyl ester); Spacer-9 (3-[2-[2-[2-[bis(4-methoxyphenyl)-phenylmethoxy]ethoxy]ethoxy]ethoxy-[di(propan-2-yl)amino]phosphanyl]oxypropanenitrile); Spacer phosphoramidite C3 (6-(4,4'-Dimethoxytrityl)hexyl-1-[(2-cyanoethyl)-(N,N-diisopropyl)]-phosphoramidite); Spacer-C6 Phosphoramidite (6-(4,4'-Dimethoxytrityl)hexyl-1-[(2-cyanoethyl)-(N,N-diisopropyl)]- phosphoramidite); and Divalent linker (DIO) 16-((bis(4-methoxyphenyl)(phenyl)methoxy)methyl)-1,1-bis(4-methoxyphenyl)-18-oxo-1-phenyl-2,5,8,11,14,17-hexaoxahenicosan-CPG. In some embodiments, the linking component comprises a compound structure shown in Table 15.

[143] In some embodiments, the linking component is Spacer phosphoramidite 18 (Phosphoramidous acid, N,N-bis(1-methylethyl)-, 19,19-bis(4-methoxyphenyl)-19-phenyl-3,6,9,12,15,18-hexaoxanonadec-1-yl 2-cyanoethyl ester).

[144] Table 15. Linkers used in the oligonucleotide agent

Name	Formula	Structure

Spacer-9	$C_{36}H_{49}N_2O_7P$	
Spacer-18	$C_{42}H_{61}N_2O_{10}P$	
Spacer-C3	$C_{33}H_{43}N_2O_5P$	
Spacer-C6	$C_{36}H_{49}N_2O_5P$	
Divalent linker (DIO)	/	
Nucleotide	/	
Note: R, -H or -OH or 2'-O-methyl (Ome), or 2'-O-methoxy-ethyl (MOE), or 2'-fluoro (F), or other 2' chemical modifications. / represents not shown.		

[145] In some embodiments, the siRNA and the ACO are covalently linked by a phosphodiester bond. In some embodiments, the siRNA and the ACO are covalently linked by a phosphorothioate bond.

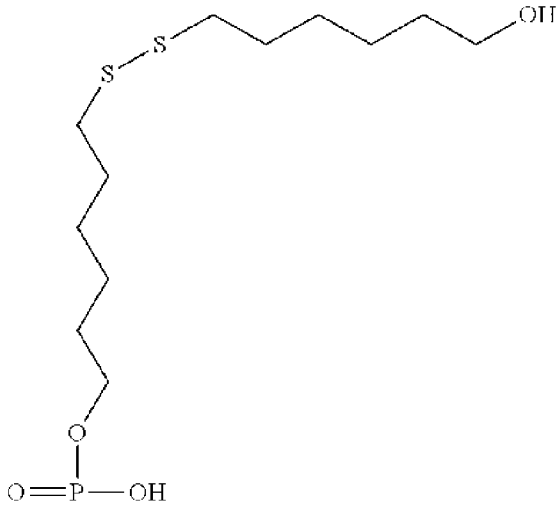
[146] In some embodiments, the siRNA comprises a sense strand that is covalently linked to the ACO. In some embodiments, the siRNA comprises an antisense strand that is covalently linked to the ACO.

[147] In some embodiments, the siRNA and the ACO are covalently linked by one or more nucleotides.

[148] Non-limiting examples of covalent linkers can be found in U.S. Patent Application Publication No.: 20200332292, which is hereby incorporated by reference in its entirety. The covalent linker can join the siRNA and the ACO.

[149] In some embodiments, the covalent linker includes RNA and/or DNA and/or a peptide. The linker can be single stranded, double stranded, partially single stranded, or partially double stranded. In some embodiments the linker includes a disulfide bond. The linker can be cleavable or non-cleavable.

[150] In some embodiments, the covalent linker includes a disulfide bond, optionally a bis-hexyl-disulfide linker. In one embodiment, the disulfide linker is



$C_{12}H_{26}O_4PS_2$
Exact Mass: 329.1010
Mol. Wt.: 329.4362

[151] In some embodiments, the covalent linker includes a peptide bond, *e.g.*, include amino acids. In one embodiment, the covalent linker is a 1-10 amino acid long linker, preferably comprising 4-5 amino acids, optionally X-Gly-Phe-Gly-Y wherein X and Y represent any amino acid.

[152] In some embodiments, the covalent linker includes HEG, a hexaethylenglycol linker.

ODV-siRNA oligonucleotide agent

[153] In some embodiments, the oligonucleotide agent decreases the expression of a *SOD1* gene or SOD1 protein. Administration of the oligonucleotide agent to a patient treats or delays the onset of ALS, such as familial or sporadic ALS or Leu Lou Gehrig's disease. In some embodiments, the described oligonucleotide agent decreases the amount of SOD1 protein by, for example, downregulating *SOD1* transcript level or decrease the amount of full-length *SOD1* mRNA. In some embodiments, *SOD1* mRNA is decreased by at least 10% (*e.g.*, at least 15%, at least 20%, at least 25%, at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, or at least 95%). In some embodiments, *SOD1* mRNA is decreased by at least 80%. In some embodiments, SOD1 protein is decreased in an amount sufficient to attenuate the symptoms associated with an ALS. In some embodiments, SOD1 protein is decreased by at least 10% (*e.g.*, at least 15%, at least 20%, at least 25%, at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least

60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, or at least 95%). In some embodiments, SOD1 protein is decreased by at least 80%.

[154] In some embodiments, the oligonucleotide agent that decreases the expression of the *SOD1* gene or SOD1 protein is a siRNA-ACO conjugate (or ODV-siRNA). The *SOD1* siRNA-ACO conjugate decreases or downregulates the expression of an *SOD1* gene in a cell in which the *SOD1* gene is abnormally or over expressed.

[155] In typical embodiments, a first strand of the *SOD1* siRNA in the oligonucleotide agent comprises a segment that has at least 75% sequence identity or sequence complementarity to a 6-60 nucleotide fragments of a select target region of the *SOD1* gene thereby effecting deactivation or downregulation of expression of the gene.

[156] In some embodiments, the oligonucleotide agent has a nucleotide sequence that is at least 60% (*e.g.*, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, at least 99% or 100%) identical to the nucleotide sequences of siSOD1-047M3-AC1 whose antisense strand has a nucleotide sequence of SEQ ID NO: 834 that has complementarity with a fragment the of the ODV structured sense strand of SEQ ID NO: 850.

[157] In some embodiments, the oligonucleotide agent has a nucleotide sequence that is at least 60% (*e.g.*, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, at least 99% or 100%) identical to the nucleotide sequences of siSOD1-005M3-AC1 whose antisense strand has a nucleotide sequence of SEQ ID NO: 842 that has complementarity with a fragment of the ODV structured sense strand of SEQ ID NO: 851.

[158] In some embodiments, the oligonucleotide agent has a nucleotide sequence that is at least 60% (*e.g.*, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, at least 99% or 100%) identical to the nucleotide sequences of siCON1-AC1^{VP} whose antisense strand has a nucleotide sequence of SEQ ID NO: 858 that has complementarity with a fragment the of the ODV structured sense strand of SEQ ID NO: 852.

[159] In some embodiments, the oligonucleotide agent has a nucleotide sequence that is at least 60% (*e.g.*, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, at least 99% or 100%) identical to the nucleotide sequences of siCON2-AC1^{VP} whose antisense strand has a nucleotide sequence of SEQ ID NO: 859 that has complementarity with a fragment the of the ODV structured sense strand of SEQ ID NO: 853.

[160] In some embodiments, the oligonucleotide agent has a nucleotide sequence that is at least 60% (*e.g.*, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, at least 99% or 100%) identical to the nucleotide sequences of siCON3-AC1^{VP} whose antisense strand has a nucleotide sequence of SEQ ID NO: 860 that has complementarity with a fragment the of the ODV structured sense strand of SEQ ID NO: 854.

[161] In some embodiments, the oligonucleotide agent has a nucleotide sequence that is at least 60% (*e.g.*, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, at least 99% or 100%) identical to the nucleotide sequences of siSOD1-063M3-AC1^{VP}

whose antisense strand has a nucleotide sequence of SEQ ID NO: 861 that has complementarity with a fragment of the ODV structured sense strand of SEQ ID NO: 855.

[162] In some embodiments, the oligonucleotide agent has a nucleotide sequence that is at least 60% (*e.g.*, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, at least 99% or 100%) identical to the nucleotide sequences of siSOD1-047M3-AC1^{VP} whose antisense strand has a nucleotide sequence of SEQ ID NO: 848 that has complementarity with a fragment the of the ODV structured sense strand of SEQ ID NO: 850.

[163] In some embodiments, the oligonucleotide agent has a nucleotide sequence that is at least 60% (*e.g.*, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, at least 99% or 100%) identical to the nucleotide sequences of siSOD1-104M3-AC1^{VP} whose antisense strand has a nucleotide sequence of SEQ ID NO: 862 that has complementarity with a fragment the of the ODV structured sense strand of SEQ ID NO: 856.

[164] In some embodiments, the oligonucleotide agent has a nucleotide sequence that is at least 60% (*e.g.*, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, at least 99% or 100%) identical to the nucleotide sequences of siSOD1-005M3-AC1^{VP} whose antisense strand has a nucleotide sequence of SEQ ID NO: 849 that has complementarity with a fragment the of the ODV structured sense strand of SEQ ID NO: 852.

[165] In some embodiments, the oligonucleotide agent has a nucleotide sequence that is at least 60% (*e.g.*, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 97%, at least 99% or 100%) identical to the nucleotide sequences of siSOD1-258M3-AC1VP whose antisense strand has a nucleotide sequence of SEQ ID NO: 863 that has complementarity with a fragment the of the ODV structured sense strand of SEQ ID NO: 857.

[166] In addition, to facilitate entry of the siRNA into a cell, chemical conjugation groups other than the ACO disclosure herein may be introduced at the ends of the sense or antisense strands of the siRNA on the basis of the above modifications to facilitate action through a cell membrane composed of lipid bilayers and mRNA regions within the nuclear membrane and nucleus.

[167] In some embodiments, siRNAs disclosed in the present application are covalently attached to one or more conjugate groups. In some embodiments, conjugate groups modify one or more properties of the attached oligonucleotide, including but not limited to pharmacodynamics, pharmacokinetics, stability, binding, absorption, tissue distribution, cellular distribution, cellular uptake, charge and clearance. In some embodiments, conjugate groups impart a new property on the attached oligonucleotide, *e.g.*, fluorophores or reporter groups that enable detection of the oligonucleotide. Certain conjugate groups and conjugate moieties have been described previously, for example: cholesterol moiety (Letsinger *et al.*, *Proc. Natl. Acad. Sci. USA*, 1989, 86, 6553-6556), cholic acid (Manoharan et al., *Bioorg. Med. Chem. Lett.,* 1994, 4, 1053-1060), a thioether, *e.g.*, hexyl-S-tritylthiol (Manoharan *et al.*, *Ann. N.Y. Acad. Sci.,* 1992, 660, 306-309; Manoharan *et al.*, *Bioorg. Med. Chem. Lett.,* 1993, 3, 2765-2770), a thiocholesterol (Oberhauser *et al.*, *Nucl. Acids Res.,* 1992, 20, 533-538), an aliphatic chain, *e.g.*, do-decan-diol or undecyl residues (Saison-Behmoaras et al., *EMBO* 1, 1991, 10, 1111-1118; 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 1,2-di-O-hexadecyl-rac-glycero-3-H-phosphonate (Manoharan *et al.*, *Tetrahedron Lett.,* 1995, 36, 3651-3654; Shea *et al.*, *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 a palmityl moiety (Mishra *et al.*, *Biochim. Biophys. Acta,* 1995, 1264, 229-237), an octadecylamine or hexylamino-carbonyl-oxycholesterol moiety (Crooke *et al.*, *J. Pharmacol. Exp. Ther.,* 1996, 277, 923-937), a tocopherol group (Nishina *et al.*, *Molecular Therapy Nucleic Acids,* 2015, 4, e220; and Nishina *et al.*, *Molecular Therapy,* 2008, 16, 734-740), or a GalNAc cluster (*e.g.*, WO2014/179620).

[168] In some embodiments, the siRNA of the present application relates to the sense strand or the antisense strand of the siRNA that is conjugated to one or more conjugation groups selected from: intercalators, reporter molecules, polyamines, polyamides, peptides, carbohydrates, vitamin moieties, polyethylene glycols, thioethers, polyethers, cholesterols, thiocholesterols, cholic acid moieties, folate, lipids, phospholipids, biotin, phenazine, phenanthridine, anthraquinone, adamantane, acridine, fluoresceins, rhodamines, coumarins, fluorophores, and dyes.

[169] In some embodiments, a conjugate group comprises an active drug substance, for example, aspirin, warfarin, phenylbutazone, ibuprofen, suprofen, fen-bufen, ketoprofen, (S)-(+)-pranoprofen, carprofen, dansylsarcosine, 2,3,5-triiodobenzoic acid, fingolimod, flufenamic acid, folinic acid, a benzothiadiazide, chlorothiazide, a diazepine, indo-methicin, a barbiturate, a cephalosporin, a sulfa drug, an antidiabetic, an antibacterial or an antibiotic.

[170] In some embodiments, the siRNA of the present application is conjugated to one or more conjugation groups selected from: a lipid, a fatty acid, a fluorophore, a ligand, a saccharide, a peptide, and an antibody.

[171] In some embodiments, the siRNA of the present application relates to the sense strand or the antisense strand of the siRNA that is conjugated to one or more conjugation groups selected from a cell-penetrating peptide, polyethylene glycol, an alkaloid, a tryptamine, a benzimidazole, a quinolone, an amino acid, a cholesterol, glucose and N-acetylgalactosamine.

[172] In some embodiments, the siRNA conjugated to one or more conjugation groups disclosed in the embodiments is directly contacted, transferred, delivered or administrated to a cell or a subject.

Cell comprising siRNA

[173] After contacting a cell, the oligonucleotide agent disclosed herein can effectively inhibit or downregulate the expression of *SOD1* gene in a cell, for example downregulate the expression by at least 10% (*e.g.*, as compared to baseline level of *SOD1* transcript).

[174] In some embodiments, the present application relates to a cell comprising the oligonucleotide agent disclosed herein. In some embodiments, the cell is a mammalian cell. In some embodiments, the cell is a human cell, such as a human cell in various tissues including prefrontal cortex, cerebellum, spinal cord (*e.g.*, cervical, thoracic, lumber), muscle, liver, and kidney.

[175] The cell disclosed herein may be *in vitro*, or *ex vivo*, such as a cell line or a cell strain, or may exist in a mammalian body, such as a human body. The human body disclosed herein is a subject suffering from a disease or symptom caused by a *SOD1* gene mutation, abnormal *SOD1* mRNA level, and/or overexpression of SOD1 protein in CNS.

[176] In some embodiments, the cell is from a CNS tissue of a subject suffering from ALS. In some embodiments, the cell is from a subject suffering from ALS. In some embodiments, the cell is from a subject suffering from Alzheimer's disease (AD), Parkinson's disease (PD), and Down's syndrome (DS).

Composition comprising siRNA

[177] Another aspect of the present application provides a composition or pharmaceutical composition capable of downregulated the level of *SOD1* mRNA transcript by the mechanism of action (MoA) of RNA interference, comprising the oligonucleotide agent disclosed herein, to treat or prevent onset of a *SOD1* related disease (particularly ALS).

[178] In some embodiments, the present application relates to a composition or pharmaceutical composition comprising the siRNA of the present application.

[179] In some embodiments, the present application relates to a composition or pharmaceutical composition comprising siRNA and the ACO as described herein. In some embodiments, the present application relates to a composition or pharmaceutical composition comprising the siRNA and the ACO covalently linked by a linking component as described herein.

[180] In one embodiment, the pharmaceutically acceptable carrier includes one or more of an aqueous carrier, liposome or LNP, polymer, micelle, colloid, metal nanoparticle, non-metallic nanoparticle, bioconjugates (*e.g.*, GalNAc), and polypeptide. In one embodiment, the aqueous carrier may be, for example, RNase-free water, or RNase-free buffer. The composition may contain 1-150 nM, for example 1-100 nM, for example 1-50 nM, for example 1-20 nM, for example 10-100 nM, 10-50 nM, 20-50 nM, 20-100 nM, for example 50 nM of the oligonucleotide agent or nucleic acid encoding full-length or partial of the oligonucleotide agent according to the present application.

[181] In some embodiments, the composition comprises 1-150 nM of the oligonucleotide agent of the present application.

[182] Another embodiment provides pharmaceutical compositions or medicaments comprising the oligonucleotide agent of the present application and a therapeutically inert carrier, diluent or pharmaceutically acceptable excipient, as well as methods of using the oligonucleotide agent of the present application to prepare such compositions and medicaments.

[183] A typical formulation is prepared by mixing an agent of the present application and a carrier or excipient. Suitable carriers and excipients are well known to those skilled in the art and are described in detail in, *e.g.*, Ansel H. C. et al., Ansel's Pharmaceutical Dosage Forms and Drug Delivery Systems (2004) Lippincott, Williams & Wilkins, Philadelphia; Gennaro A. R. et al., Remington: The Science and Practice of Pharmacy (2000) Lippincott, Williams & Wilkins,

Philadelphia; and Rowe R. C, Handbook of Pharmaceutical Excipients (2005) Pharmaceutical Press, Chicago. The formulations may also include one or more buffers, stabilizing agents, surfactants, wetting agents, lubricating agents, emulsifiers, suspending agents, preservatives, antioxidants, opaquing agents, glidants, processing aids, colorants, sweeteners, perfuming agents, flavoring agents, diluents and other known additives to provide an elegant presentation of the drug (*i.e.*, an agent of the present application or pharmaceutical composition thereof) or aid in the manufacturing of the pharmaceutical product (*i.e.*, medicament).

[184] Compositions of the present application are formulated, dosed, and administered in a fashion consistent with good medical practice. Factors for consideration in this context include the particular disorder being treated, the particular mammal being treated, the clinical condition of the individual patient, the cause of the disorder, the site of delivery of the agent, the method of administration, the scheduling of administration, and other factors known to medical practitioners.

[185] For the oligonucleotide agent compositions of the present application, the delivery can be optionally through parenteral infusions including intrathecal, intramuscular, intravenous, intraarterial, intraperitoneal, intravesical, intracerebroventricular, intravitreal or subcutaneous administration; or through oral administration, intranasal administration, inhaled administration, vaginal administration, or rectal administration.

[186] In another aspect, the application provides use of the oligonucleotide agent, according to any one of the embodiments described herein, or a composition according to any one of the embodiments described herein, in the manufacture of a medicament for the treatment of gene or protein-related condition in an individual. The use according to certain embodiments, the condition can include a *SOD1* related condition that comprises ALS, AD, PD and/or DS. Also provided is the use according to certain embodiments wherein the individual is a mammal, preferably a human.

Kits

[187] In another aspect, any of the compositions described herein can be provided in one or more kits, optionally including instructions for use of the compositions. That is, the kit can include a description of use of an oligonucleotide agent or composition or pharmaceutical composition in any method described herein. A "kit," as used herein, typically defines a package, assembly, or container (such as an insulated container) including one or more of the components or embodiments of the application, and/or other components associated with the application, for example, as previously described. Any of the antes or components of the kit may be provided in liquid form (*e.g.*, in solution), or in solid form (*e.g.*, a dried powder, frozen, etc.).

[188] In some cases, the kit includes one or more components, which may be within the same or in two or more receptacles, and/or in any combination thereof. The receptacle is able to contain a liquid, and non-limiting examples include bottles, vials, jars, tubes, flasks, beakers, or the like. In some cases, the receptacle is spill-proof (when closed, liquid cannot exit the receptacle, regardless of orientation of the receptacle).

[189] Examples of other compositions or components associated with the agents, compounds and methods described herein include, but are not limited to: diluents, salts, buffers, chelating agents, preservatives, drying agents, antimicrobials, needles, syringes, packaging materials, tubes, bottles, flasks, beakers, and the like, for example, for using, modifying, assembling, storing, packaging, preparing, mixing, diluting, and/or preserving the components for a particular use. In embodiments where liquid forms of any of the components are used, the liquid form may be concentrated or ready to use.

[190] In additional embodiments, a kit can include instructions or instructions to a website or other source in any form that are provided for using the kit in connection with the components and/or methods described herein. For instance, the instructions may include instructions for the use, modification, mixing, diluting, preserving, assembly, storage, packaging, and/or preparation of the components and/or other components associated with the kit. In some cases, the instructions may also include instructions for the delivery of the components, for example, for shipping or storage at room temperature, sub-zero temperatures, cryogenic temperatures, etc. The instructions may be provided in any form that is useful to the user of the kit, such as written or oral (*e.g.*, telephonic), digital, optical, visual (*e.g.*, videotape, DVD, etc.) and/or electronic communications (including Internet or web-based communications), provided in any manner.

Method of use

[191] Another aspect of the present application relates to the oligonucleotide agents of the present application being used in therapeutic approaches to treating diseases such as ALS.

[192] By non-limiting embodiments, the present application provides a method of decreasing the transcript level of a *SOD1* gene or SOD1 protein, comprising administering to a subject a pharmaceutical composition disclosed herein.

[193] In some embodiments, the present application relates to a method for treating or delaying the onset or progression of Amyotrophic lateral sclerosis (ALS) in a subject, the method comprising: administering to the subject a pharmaceutical composition disclosed herein. In some embodiments, the subject has sporadic ALS (sALS). In some embodiments, the subject has familial ALS (fALS). In some embodiments, the pharmaceutical composition decreases the transcript of the *SOD1* gene or SOD1 protein.

[194] In some embodiments, the ACO of the oligonucleotide agent improves the stability, bioavailability, biodistribution, and/or cellular uptake of the siRNA as compared to an oligonucleotide agent without the ACO.

[195] In some embodiments, the ACO of the oligonucleotide agent increases the biodistribution of siRNA within one or more target tissues as compared to an oligonucleotide agent without the ACO.

[196] In some embodiments, the ACO of the oligonucleotide agent increases the biodistribution of siRNA within two or more target tissues as compared to an oligonucleotide agent without the ACO.

[197] In some embodiments, one or more target tissues is selected from: prefrontal cortex, cerebellum, cerebrum, spinal cord, muscle, lung, eye, liver, and kidney.

[198] In some embodiments, the oligonucleotide agent of the present application achieves a decrease in full-length SOD1 protein that is less than the amount achieved by administration of the same amount of double stranded oligonucleotide such as a siRNA substance without an ODV structure used individually, with higher potency, reduced toxicity, or unwanted side effects. In some embodiments, the oligonucleotide agent of the present application achieves a decrease in full-length SOD1 protein that is less than the additive effect of treatment with the same amount of the siRNA used individually.

[199] Specifically, the oligonucleotide agent of the present application inhibits/down-regulates the SOD1 mRNA transcript by at least 10% (*e.g.*, at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or about 100% as compared to baseline *SOD1* mRNA transcript). In some embodiments, upon administering the oligonucleotide agent disclosed in the embodiments, *e.g.*, to a cell or a subject, the *SOD1* mRNA transcript is inhibited/downregulated by at least 50%, 60%, 70%, 77%, 79%, 81%, 84%, 85%, and 88% at 10 nM treatment compared to baseline *SOD1* mRNA transcript in control group) in an *in vitro* cell line. In some embodiments, an oligonucleotide agent inhibits or downregulates the *SOD1* mRNA transcript by about 80%.

[200] In some embodiments, the expression of *SOD1* gene is inhibited/downregulated by administering the oligonucleotide agent disclosed in the embodiments to a cell at a concentration of at least 0.01 nM, *e.g.*, 0.02 nM, 0.05 nM, 0.08 nM, 0.1 nM, 0.2 nM, 0.3 nM, 0.4 nM, 0.5 nM, 0.6 nM, 0.8 nM, 1 nM, 5 nM, 10 nM, 25 nM, 50 nM, 75 nM, 100 nM, or 150 nM. In some embodiments, the *SOD1* gene coded protein (SOD1 protein) is inhibited/downregulated by administering the oligonucleotide agent disclosed in the embodiments, *e.g.*, to a cell or a subject. The knockdown of the SOD1 protein by at least at least 10% (*e.g.*, at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or about 100%, as compared to baseline expression of the SOD1 protein). In some embodiments, an oligonucleotide agent inhibits or downregulates the expression of the SOD1 protein by about 80%. In some embodiments, the SOD1 protein is inhibited/down-regulated by administering the oligonucleotide agent disclosed in the embodiments to a cell at a concentration of at least 0.01 nM, *e.g.*, 0.02 nM, 0.05 nM, 0.08 nM, 0.1 nM, 0.2 nM, 0.3 nM, 0.4 nM, 0.5 nM, 0.6 nM, 0.8 nM, 1 nM, 2 nM, 3 nM, 4nM, 5 nM, 10 nM, 25 nM, 50 nM, 75 nM, 100 nM, or 150 nM.

[201] In some embodiments, the oligonucleotide agents disclosed in the embodiments have a dose-dependent knockdown activity in cells. In some embodiments, the oligonucleotide agents knockdown the *SOD1* mRNA transcript in cells with a IC_{50} of less than 10nM, 5nM, 4nM, 3nM, 2nM, 1nM, 0.8nM, 0.6 nM, 0.5nM, 0.4nM, 0.3nM, 0.2nM. 0.1nM, 0.08nM, 0.06nM, 0.04nM, 0.02nM, 0.01nM, 0.008nM, or 0.005nM.

[202] Another aspect of the present application relates to a method for preventing or treating a disorder or condition induced by over-expression of SOD1 protein, a *SOD1* gene mutation, and/or

high *SOD1* mRNA levels in an individual comprising: administering an effective amount of the siRNA, the oligonucleotide agent, or the composition comprising the oligonucleotide agent disclosed herein to the individual. In some embodiments, the effective amount of the siRNA disclosed herein can be a concentration ranging from 0.01 nM to 50 nM, *e.g.*, 0.01 nM, 0.02 nM, 0.05 nM, 0.08 nM, 0.1 nM, 0.2 nM, 0.3 nM, 0.4 nM, 0.5 nM, 0.6 nM, 0.8 nM, 1 nM, 5 nM, 10 nM, 25 nM, 50 nM, 75 nM, 100 nM, or 150 nM. In some embodiments, the disorder or condition is ALS. In some embodiments, the individual is a mammal. In some embodiments, the individual is a human.

[203] In any of the embodiments provided herein, such cells may be *ex vivo*, such as cell lines, and the like, or may be present in mammalian bodies, such as humans. In some embodiments, the human is a subject or individual suffering from a SOD1 protein related condition or ALS, AD, PD, or DS.

[204] Another aspect of the present application relates administering an effective mount of the oligonucleotide agent or the composition to an individual using administration pathway as described herein. In some embodiments, the administration pathway is selected from one or more of: parenteral infusions, oral administration, intranasal administration, inhaled administration, vaginal administration, and rectal administration. In some embodiments, the administration pathway is selected from one or more of: intrathecal, intramuscular, intravenous, intra-arterial, intraperitoneal, intravesical, intracerebroventricular, intravitreal and subcutaneous administrations.

Dose regiments and route of administration

[205] Aspects of the present application relate to a pharmaceutical composition comprising the oligonucleotide agent of the present application. In some embodiments, the pharmaceutical composition comprises the oligonucleotide agent of the present application and a pharmaceutically acceptable carrier, a therapeutically inert carrier, diluent or pharmaceutically acceptable excipient. The pharmaceutical composition disclosed herein is to be developed into a medicament preventing or treating the SOD1 protein related condition or ALS.

[206] Aspects of the present application also relate to methods of using the oligonucleotide agents of the present application to prepare such compositions.

[207] Another aspect of the present application relates to use of the oligonucleotide agent of the present application in manufacturing the pharmaceutical composition disclosed herein.

[208] Another aspect of the present application relates to use of the oligonucleotide agent, according to any one of the embodiments described herein, or a composition according to any one of the embodiments described herein, in the manufacture of a medicament for the prevention or treatment of gene or protein-related symptom induced by the over-expression of SOD1 protein, a *SOD1* gene mutation, and/or high SOD1 protein levels in an individual. For the use according to certain embodiments, the condition can include a SOD1 protein-mutation-related disorder or condition that comprises ALS. For the use according to certain embodiments, the symptom induced by over-expression of abnormal SOD1 protein is ALS. Also related is the use according to certain embodiments wherein the individual is a mammal, for example a human.

[209] The dosage at which the oligonucleotide agents or compositions of the present application can be administered can vary within wide limits and will be fitted to the individual requirements in each case. In some embodiments, a first dose of a pharmaceutical composition according to the present application is administered when the subject is less than one week old, less than one month old, less than 3 months old, less than 6 months old, less than one-year-old, less than 2 years old, less than 15 years old, or older than 15 years old.

[210] The single dose of the oligonucleotide agent can be a single dose ranging from 0.01 mg/kg to 1000 mg/kg for example, about 0.01, 0.02, 0.05, 0.1, 0.2, 0.5, 1, 2, 2.5, 5, 7.5, 10, 12.5, 15, 17.5, 20, 25, 30, 40, 50, 75, 100, 120, 150, 200, 250, 300, 400, 500, 750, or 1000 mg/kg. The doses described herein may contain two or more of any of the oligonucleotide agent sequences described herein.

[211] In some embodiments, the proposed dose frequency is approximate. For example, in some embodiments if the proposed dose frequency is a dose at day 1 and a second dose at day 29, an ALS patient may receive a second dose 25, 26, 27, 28, 29, 30, 31, 32, 33, or 34 days after receipt of the first dose. In some embodiments, if the proposed dose frequency is a dose at day 1 and a second dose at day 15, an ALS patient may receive a second dose 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 days after receipt of the first dose. In some embodiments, if the proposed dose frequency is a dose at day 1 and a second dose at day 85, an ALS patient may receive a second dose 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, or 90 days after receipt of the first dose.

[212] In some embodiments, the dose and/or the volume of the injection will be adjusted based on the subject's age, the subject's body weight, and/or other factors that may require adjustment of the parameters of the injection.

[213] In some embodiments, pharmaceutical compositions comprise a co-solvent system. Certain of such co-solvent systems comprise, for example, benzyl alcohol, a nonpolar surfactant, a water-miscible organic polymer, and an aqueous phase. In some embodiments, such co-solvent systems are used for hydrophobic compounds. A non-limiting example of such a co-solvent system is the VPD co-solvent system, which is a solution of absolute ethanol comprising 3% w/v benzyl alcohol, 8% w/v of the nonpolar surfactant Polysorbate 80™ and 65% w/v polyethylene glycol 300. The proportions of such co-solvent systems may vary considerably without significantly altering their solubility and toxicity characteristics. Furthermore, the identity of co-solvent components may be varied: for example, other surfactants may be used instead of Polysorbate 80™; the fraction size of polyethylene glycol may be varied; other biocompatible polymers may replace polyethylene glycol, *e.g.*, polyvinyl pyrrolidone; and other sugars or polysaccharides may substitute for dextrose.

[214] Examples of other compositions or components associated with the oligonucleotide agent, compositions, pharmaceutical compositions, and methods described herein include, but are not limited to: diluents, salts, buffers, chelating agents, preservatives, drying agents, antimicrobials, needles, syringes, packaging materials, tubes, bottles, flasks, beakers, and the like, for example, for using, modifying, assembling, storing, packaging, preparing, mixing, diluting, and/or preserving the components for a particular use. In embodiments where liquid forms of any of the components are used, the liquid form may be concentrated or ready to use.

[215] In some embodiments, lipid moieties used in nucleic acid therapies can be applied in the present application for delivery of the oligonucleotide agent molecules disclosed herein. In such methods, the nucleic acid (*e.g.*, one or more oligonucleotide agents described herein) is introduced into preformed liposomes or lipoplexes made of mixtures of cationic lipids and neutral lipids. In certain methods, oligonucleotide agent complexes with mono- or poly-cationic lipids are formed without the presence of a neutral lipid. In some embodiments, a lipid moiety is selected to increase distribution of a pharmaceutical agent to a particular cell or tissue. In some embodiments, a lipid moiety is selected to increase distribution of a pharmaceutical agent to fat tissue. In some embodiments, a lipid moiety is selected to increase distribution of a pharmaceutical agent to muscle tissue.

[216] In some embodiments, pharmaceutical compositions comprise a delivery system. Examples of delivery systems include, but are not limited to, liposomes and emulsions. Certain delivery systems are useful for preparing certain pharmaceutical compositions including those comprising hydrophobic compounds. In some embodiments, certain organic solvents such as dimethylsulfoxide are used.

[217] In some embodiments, pharmaceutical compositions comprise one or more tissue-specific delivery molecules designed to deliver the one or more pharmaceutical agents of the present application to specific tissues or cell types. For example, in some embodiments, pharmaceutical compositions include liposomes coated with a tissue-specific antibody.

[218] In some embodiments, the oligonucleotide agent can be delivered or administered via a vector. Any vectors that may be used for gene delivery may be used. In some embodiments, a viral vector may be used. Non-limiting examples of viral vectors that may be used in the present application include, but are not limited to, human immunodeficiency virus; HSV, herpes simplex virus; MMSV, Moloney murine sarcoma virus; MSCV, murine stem cell virus; SFV, Semliki Forest virus; SIN, Sindbis virus; VEE, Venezuelan equine encephalitis virus; VSV, vesicular stomatitis virus; VV, vaccinia virus; AAV, adeno-associated virus; adenovirus; lentivirus; and retrovirus.

[219] In some embodiments, the vector is a recombinant AAV vector (rAAV). AAV vectors are DNA viruses of relatively small size that can integrate, in a stable and site-specific manner, into the genome of the cells that they infect. They are able to infect a wide spectrum of cells without inducing any effects on cellular growth, morphology or differentiation, and they do not appear to be involved in human pathologies. The AAV genome has been cloned, sequenced and characterized. It encompasses approximately 4700 bases and contains an inverted terminal repeat (ITR) region of approximately 145 bases at each end, which serves as an origin of replication for the virus. The remainder of the genome is divided into two essential regions that carry the encapsidation functions: the left-hand part of the genome, that contains the rep gene involved in viral replication and expression of the viral genes; and the right-hand part of the genome, that contains the cap gene encoding the capsid proteins of the virus.

[220] Preparations, pharmaceutical compositions, or medicaments of the present disclosure are formulated, dosed, and administered in a fashion consistent with good medical practice. Factors for

consideration in this context include the particular disorder being treated, the particular mammal being treated, the clinical condition of the individual subject, the cause of the disorder, the site of delivery of the agent, the method of administration, the scheduling of administration, and other factors known to medical practitioners.

[221] For the preparations, pharmaceutical compositions, or medicaments of the present disclosure, the delivery can be optionally through parenteral infusions including intrathecal, intramuscular, intravenous, intra-arterial, intraperitoneal, intravesical, intracerebroventricular, intravitreal or subcutaneous administration; or through oral administration, intranasal administration, inhaled administration, vaginal administration, or rectal administration.

[222] A typical formulation of the oligonucleotide modulator in the present disclosure is prepared by mixing a siRNA of the present disclosure and a carrier or excipient. Suitable carriers and excipients are well known to those skilled in the art and are described in detail in, *e.g.*, Ansel H. C. et al., Ansel's Pharmaceutical Dosage Forms and Drug Delivery Systems (2004) Lippincott, Williams & Wilkins, Philadelphia; Gennaro A. R. et al., Remington: The Science and Practice of Pharmacy (2000) Lippincott, Williams & Wilkins, Philadelphia; and Rowe R. C, Handbook of Pharmaceutical Excipients (2005) Pharmaceutical Press, Chicago. The formulations may also include one or more buffers, stabilizing agents, surfactants, wetting agents, lubricating agents, emulsifiers, suspending agents, preservatives, antioxidants, opaquing agents, glidants, processing aids, colorants, sweeteners, perfuming agents, flavoring agents, diluents and other known additives to provide an elegant presentation of the drug (*i.e.*, a siRNA of the present disclosure or pharmaceutical composition thereof) or aid in the manufacturing of the pharmaceutical product (*i.e.*, medicament).

EXAMPLES

[223] The following examples are set forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how to make and use the present invention and are not intended to limit the scope of what the inventors regard as their invention nor are they intended to represent that the experiments below are all or the only experiments performed. Efforts have been made to ensure accuracy with respect to numbers used (*e.g.*, amounts, temperature, etc.) but some experimental errors and deviations should be accounted for. Unless indicated otherwise, parts are parts by weight, molecular weight is weight average molecular weight, temperature is in degrees Celsius, and pressure is at or near atmospheric. Standard abbreviations may be used, *e.g.*, bp, base pair(s); kb, kilobase(s); pl, picoliter(s); s or sec, second(s); min, minute(s); h or hr, hour(s); aa, amino acid(s); nt, nucleotide(s); i.m., intramuscular(ly); i.p., intraperitoneal(ly); s.c., subcutaneous(ly); i.c.v. or ICV, intracerebroventricular and the like.

Example 1. Development of siRNA drug candidates for knockdown of human *SOD1*

[224] The sequence for human *SOD1* transcript (NM_000454.5) was retrieved from the NCBI nucleotide database. It includes a 465 bp open reading frame (ORF) located between nucleotides 78 and 542, which served as the template for siRNA design (**Table 1**).

Table 1. *SOD1* cDNA sequence for siRNA design

***SOD1* sequence (5'-3')**	**Size (nt)**	**SEQ ID NO**
atggcgacgaaggccgtgtgcgtgctgaagggcgacggcccagtgcagggcatcatcaatttcgagcaga aggaaagtaatggaccagtgaaggtgtggggaagcattaaaggactgactgaaggcctgcatggattccat gttcatgagtttggagataatacagcaggctgtaccagtgcaggtcctcactttaatcctctatccagaaaacac ggtgggccaaaggatgaagagaggcatgttggagacttgggcaatgtgactgctgacaaagatgctgtggc cgatgtgtctattgaagattctgtgatctcactctcaggagaccattgcatcattggccgcacactggtggtcca tgaaaaagcagatgacttgggcaaaggtggaaatgaagaaagtacaaagacaggaaacgctggaagtcgt ttggcttgtggtgtaattgggatcgcccaataa	465	1

[225] A total of 268 siRNA duplexes were designed and synthesized at 21 nucleotides (nt) in length with no more than 4 repetitive nucleotides in a row and GC content between 35-65%. Their target sites and cognate siRNA strand sequences are listed in **Table 2**. Knockdown activity of each siRNA was assessed in HEK293A cells using high throughput RT-qPCR at both 0.1 and 10 nM concentrations. Data was ranked according to mean knockdown activity in which 121 siRNAs reduce *SOD1* by more than 90% at 10 nM (**Figure 1A**). As an indicator of potency, 69 and 15 siRNAs reduced *SOD1* levels at 0.1 nM treatments by over 50% and 75%, respectively. 25 performing siRNAs were subjected to an additional round of screening at 6 concentrations (*i.e.*, 0.0064, 0.032, 0.16, 0.8, 4 and 20 nM) in HEK293A cells to demonstrate dose dependent activity in which propidium iodide (PI) was integrated into sample preparation to monitor variation in nucleic acid content as an indicator of untoward cytotoxicity (**Figure 2**). Shown in **Figure 1B** is data for the top 5 siRNAs (*i.e.*, siSOD1-063, 047, 104, 005, and 258) with the most potent knockdown activity which also show an absence of overt cytotoxicity (*i.e.*, <20% reduction in PI staining).

[226] Dose response curves were subsequently generated for each of the 5 siRNA candidates to validate potency in model cell lines representative of neuronal disease including SK-N-AS (**Figure 1C**) and T98G (**Figure 3**) cells. As summarized in **Table 3**, *in vitro* potency for each duplex was in the low picomolar range for both cell lines. Untoward cytotoxicity was also evaluated 72 hours after treatment at concentrations well above 200X the extrapolated IC_{50} values. As shown in **Figure 4A-B**, only siSOD1-047 and 005 had no detectable impact on apoptosis or cell number in either SK-N-AS or T98G cells, whereas the remaining candidates (*i.e.*, siSOD1-063, 104, and 258) had dose-dependent responses with regards to caspase 3/7 activity in T98G cells that inversely correlated with cell viability. While SK-N-AS cells appeared more tolerant to treatment, a similar pattern was observed for cell viability.

[227] Several medicinal chemistry patterns referred to as M1, M2, M3, or M4 representing different duplex lengths (*i.e.*, 20, 21, 22, and 23 nt, respectively) comprised of phosphorothionate (PS) backbone modifications at select positions with 2'-O-methylation (2'Ome) or 2'-fluoro (2'F) substitutions at every nucleotide were applied to each lead candidate and screened for target mRNA knockdown activity. As shown in **Figure 1D**, M3 variants (*i.e.*, siSOD1-063M3, 047M3, 104M3, 005M3, and 258M3) generally had better knockdown activity compared to the other chemically modified siRNAs at 0.1 nM treatment concentrations in SK-N-AS cells. A near identical pattern was

also observed in T98G cells (**Figure 5).** To further characterize potency, dose response curves were generated for M3-modified duplexes (*i.e.,* siSOD1-063M3, 047M3, 104M3, 005M3, 258M3, and 270M3) in both SK-N-AS (**Figure 1E**) and T98G (**Figure 6**) cell lines. As summarized in **Table 3,** *in vitro* potency was generally well-retained following chemical modification.

[228] Accessory oligonucleotide (ACO) conjugation was developed to impart self-delivery properties similar to ASOs by sharing medicinal chemistry normally not tolerated by canonical siRNAs. As such, the sense strand of each M3 variant (*i.e.*, siSOD1-063M3, 047M3, 104M3, 005M3, and 258M3) was synthesized covalently linked to a 14-nucleotide ACO (referred to AC1) via a short linker (L9, *i.e.*, Spacer-9 linker), wherein the ACO that possessed PS backbone substitutions and 2'-O-methoxyethyl (2'MOE) modifications at every position within the ACO (**Figure 7A**). As shown in **Figure 7B**, AC1 treatment alone had no detectable knockdown of *SOD1* at 0.25 and 2.5 nM concentrations *in vitro*, whereas activity was only perceived when conjugated to siRNA. Additional dose response analysis noted a ~10X loss in siRNA potency as a consequence of AC1 conjugation (*i.e.*, siSOD1-005M3-AC1) compared to only chemically modified duplex (*i.e.*, siSOD1-005M3) (**Figure 7C**). However, potency was restored upon modification with 5'-(*E*)-vinylphosphonate (5'VP) at the 5' terminus of the guide strand (*i.e.*, siSOD1-005M3-AC1^{VP}) (**Figure 7D**). In comparison to an ASO resembling Tofersen (an antisense oligonucleotide ASO under clinical investigation that has shown therapeutic benefit in the treatment of ALS by suppressing mutant SOD1 mRNA levels; cf. T. Miller et al., New England Journal of Medicine 383, 109-119 (2020)) in both sequence and chemistry (*i.e.*, ASO^{SOD1}, SEQ ID NO: 864), *SOD1* knockdown with either siRNA-ACO variant (*i.e.*, siSOD1-005M3-AC1 or siSOD1-005M3-AC1^{VP}) was more potent than ASO^{SOD1} regardless of 5'VP modification (**Figure 7D**).

[229] siRNA-ACO conjugates were subsequently synthesized using M3 chemistry and 5'VP modification (*i.e.*, siSOD1-063M3-AC1^{VP}, 047M3-AC1^{VP}, 104M3-AC1^{VP}, 005M3-AC1^{VP}, 258M3-AC1^{VP}, and 270M3-AC1^{VP}) for downstream screening *in vivo*. Prior to treatment, dose response curves were generated in both SK-N-AS and T98G cells to validate activity of each siRNA-ACO candidate in suppressing mutant *SOD1* mRNA levels (**Figures 8A-8B**). As summarized in **Table 3**, *in vitro* potencies were generally well conserved in comparison to their non-conjugate forms. Apoptosis and cell viability were also quantified 72 hours after treatments to measure any changes in cytotoxicity as a consequence of chemical modification and ACO conjugation. As shown in **Figures 9A-9B**, siSOD1-047M3-AC1^{VP} and 005M3-AC1^{VP} remained generally unaffected with nominal impact on cell health in both SK-N-AS and T98G cells, whereas siSOD1-104M3-AC1^{VP} retained signs of untoward cytotoxicity and the remaining candidates (*i.e.*, siSOD1-063M3-AC1^{VP} and 258M3-AC1^{VP}) noted an improvement in *in vitro* safety (*i.e.*, reduction in caspase 3/7 activity) compared to their non-modified forms (**Figure 4A-B**).

Table 3. *In vitro* potency of drug candidates

siRNA	IC_{50} (pM)*	
	SK-N-AS	**T98G**
siSOD1-063	**4.7 ± 1.4**	**7.9 ± 6.1**
siSOD1-047	**5.1 ± 1.3**	**8.5 ± 3.6**

siSOD1-104	**5.3 ± 1.7**	**8.5 ± 3.9**
siSOD1-005	**3.4 ± 1.1**	**5.4 ± 2.5**
siSOD1-258	**19.7 ± 10.0**	**27.3 ± 15.7**
siSOD1-063M3	**9.3 ± 3.4**	**13.6 ± 2.9**
siSOD1-047M3	**4.9 ± 1.5**	**6.9 ± 1.9**
siSOD1-104M3	**18.8 ± 7.6**	**15.5 ± 4.8**
siSOD1-005M3	**10.4 ± 7.8**	**9.9 ± 2.2**
siSOD1-258M3	**17.8 ± 7.7**	**11.2 ± 3.1**
siSOD1-270M3	/	**27.5 ± 8.9**
siSOD1-063M3-AC1^{VP}	**7.1 ± 2.3**	**9.4 ± 6.3**
siSOD1-047M3-AC1^{VP}	**5.3 ± 2.5**	**4.8 ± 1.5**
siSOD1-104M3-AC1^{VP}	**21.6 ± 10.9**	**15.68 ± 7.1**
siSOD1-005M3-AC1^{VP}	**17.3 ± 6.6**	**12.1 ± 7.7**
siSOD1-258M3-AC1^{VP}	**16.7 ± 3.7**	**14.3 ± 7.1**
siSOD1-270M3-AC1^{VP}	/	**11.6 ± 6.5**

* IC_{50} vales ± SD, the concentration of siRNAs where *SOD1* gene is knocked down by half; "/", means not tested.

Example 2. *In vivo* selection of siRNA-ACO drug candidates

[230] Mice hemizygous for *hSOD1*^{G93A} transgene express a mutant form of human *SOD1* and exhibit disease phenotypes similar to ALS including progressive loss of motor function and abbreviated life span via neuronal degradation (P. Weydt et al., *Neuroreport* 14, 1051-1054 (2003); P. H. Tu, et al., *Proc Natl Acad Sci USA* 93, 3155-60 (1996)). To test knockdown activity *in vivo*, *h*SOD1^{G93A} mice were treated via ICV injection at equimolar quantities (*i.e.*, 20 nmole/dose) with siRNA-ACOs (*i.e.*, siSOD1-047M3-AC1^{VP} or siSOD1-005M3-AC1^{VP}) in comparison to non-conjugate controls (*i.e.*, siSOD1-047M3^{VP} or siSOD1-005M3^{VP}) to demonstrate the effect AC1 conjugation imparts on knockdown activity *in vivo*. All siRNA-ACOs were formulated in aCSF in which treatment alone served as a vehicle control to establish baseline expression, while siCON2-AC1^{VP} functioned as a negative control for siRNA-ACO activity. As shown in **Figure 10**, both siRNA-ACO duplexes provided greater knockdown activity comparative to their non-conjugate cognates in all tissues of the brain (*i.e.*, frontal cortex, cerebellum, and cerebrum) and spinal cord (*i.e.*, cervical, thoracic, and lumbar spine) with minimal activity in the periphery (*i.e.*, liver). Knockdown durability was also characterized for siSOD1-005M3-AC1^{VP} relative to its non-5'VP control (*i.e.*, siSOD1-005M3-AC1) with comparison to ASO^{SOD1}. Taken together, the combination of both AC1 conjugation and 5'VP modification provided our siRNAs with enhanced activity needed for a durable response *in vivo*.

Example 3. Single dose treatment via ICV injection with siRNA-ACO delays disease progression and prolongs survival in *hSOD1*^{G93A} mice

[231] Adult *h*SOD1^{G93A} mice were treated via ICV injection with siRNA-ACO candidates (*i.e.*, siSOD1-047M3-AC1^{VP} or siSOD1-005M3-AC1^{VP}) at 50, 100, 200, or 400 mg/dose on PND 85 or 60, respectively. Drug concentrations and hSOD1 expression levels were quantified in a subset of animals on day 14 after treatment in CNS tissue (*i.e.*, cerebellum, cerebrum, and spinal cord). As shown in **Figure 11A-B**, both activity and tissue accumulation of siSOD1-047M3-AC1^{VP} and siSOD1-005M3-AC1^{VP} were dose-dependent in which *SOD1* knockdown inversely correlated with increasing concentrations of siRNA-ACO within the CNS tissues. Drug concentrations projected to elicit an ED_{50} (median effective dose) response in each tissue are summarized in **Table 4**.

Table 4. ED_{50} and tissue concentrations of siRNA-ACO to trigger median response

siRNA-ACO	CNS Tissue	ED_{50} (mg)	Conc. (mg/g)*
siSOD1-047M3-AC1^{VP}	Cerebellum	99.8	1.22
	Cerebrum	80.9	0.174
	Spinal Cord	151	0.082
siSOD1-005M3-AC1^{VP}	Cerebellum	38.8	*ND*
	Cerebrum	26.4	0.079
	Spinal Cord	25	0.045

* mass (mg) of siRNA-ACO per gram (g) of tissue.

ND: could not be determined

[232] In remaining animals, changes in body weight were plotted to monitor growth rate and disease progression. As shown in **Figure 12A**, all groups treated with either siSOD1-047M3-AC1^{VP} or siSOD1-005M3-AC1^{VP} continued to gain weight in comparison to aCSF treatment. In addition, disease-related weight loss was delayed in a dose-dependent manner as noted by time needed for growth rates to return to starting weight (dotted line). Disease progression was confirmed in each animal when a 10% loss in peak body weight was recorded. Plotting data via Kaplan-Meier curves indicated when animals in each treatment group transitioned to progressive disease (**Figure 12B**). Data was collected until animals inevitably succumbed to their disease in which survival curves were also generated (**Figure 12C**). To summarize, both siSOD1-047M3-AC1^{VP} (**Table 5**) and siSOD1-005M3-AC1^{VP} (**Table 6**) treatment delayed disease progression and extended animal survival in which the highest dose (*i.e.*, 400 mg) prolonged life by 70 and 111.5 days compared to vehicle controls, respectively.

Table 5. Median age for disease onset and animal survival following single dose siSOD1-047M3-AC1^{VP} on PND 85

Treatment	**aCSF**	**siSOD1-047M3-AC1^{VP}**			
Dose (µg)	**-**	**50**	**100**	**200**	**400**
Animals No.	9	4	7	4	8
Disease Onset					
Median Age	143	148	180*	181.5†	206*

(Days)					
Survival					
Median Age (Days)	160	157.5	195†	200	230*

*P < 0.001 compared to aCSF group
†P < 0.01 compared to aCSF group

Table 6. Median age for disease onset and animal survival following single dose siSOD1-005M3-AC1^{VP} on PND 60

Treatment	**aCSF**	**siSOD1-005M3-AC1^{VP}**			
Dose (µg)	**-**	**50**	**100**	**200**	**400**
Animals No.	8	7	8	8	8
Disease Onset					
Median Age (Days)	146.5	175*	173*	188*	225*
Survival					
Median Age (Days)	147.5	199*	197.5*	215.5*	259*

*P < 0.001 compared to aCSF group

Example 4. Pathogenic mutation in target sites of siRNA-ACO impact lead selection

[233] *In silico* analysis of pathogenic single nucleotide polymorphisms (SNPs) located within the target site of siSOD1-005M3-AC1^{VP} revealed 5 total SNPs in which 4 were in the region complementary to its "seed" sequence (**Figure 13A**). Mismatches in this region are known to inhibit siRNA activity, which could eliminate a projected ~8.9-12.4% of the global ALS^{SOD1} patients from its treatment pool (O. Abel et al., *Hum Mutat* 33, 1345–51 (2012); https://alsod.ac.uk/, Amyotrophic Lateral Sclerosis online Database - ALSoD). Conversely, siSOD1-047M3-AC1^{VP} has only 2 reported pathogenic SNPs within its target site (*i.e.*, P.E22G and P.F21C) comprising approximately 4.04% and 2.70% of the global ALS^{SOD1} population, respectively (**Figure 13B**). Luciferase reporter constructs (*i.e.*, pLuc^{SOD1}, pLuc$^{P.E22G}$, and pLuc$^{P.F21C}$) containing either consensus sequence perfectly complementary to siSOD1-047M3-AC1^{VP} guide strand or one of pathogenic mutations (*i.e.*, P.E22G and P.F21C) were co-transfected into HEK293A cells along with siRNA-ACO. Knockdown of luciferase activity was specific to siSOD1-047M3-AC1^{VP} as transfection with a scramble control (*i.e.*, siCON2-AC1^{VP}) did not reduce reporter expression (**Figure 13C**). Dose response data indicated that the P.E22G mutation does not have any significant impact on siSOD1-047M3-AC1^{VP} knockdown activity/potency compared to consensus target site sequence, while P.F21C was partially resilient to treatment demonstrating incomplete knockdown and inferior potency (**Figure 13D).**

Example 5. siRNA-ACO delays disease progression, prolongs survival and improves motor function of *hSOD1^{G93A}* mice via IT injection

[234] Based on perceived patient populations, siSOD1-047M3-AC1^{VP} was selected for further *in vivo* analysis. Male and female *h*SOD1^{G93A} mice were treated via IT injection with two sequential doses of siSOD1-047M3-AC1^{VP} on PND 68 and 100 at 75, 150, or 300 µg/dose. A non-specific siRNA-ACO (*i.e.*, siCON3-AC1^{VP}) served as a negative control for therapeutic efficacy. Animal weight was monitored with comparison to wild-type animals (*i.e.*, WT) in which all siSOD1-047M3-AC1^{VP} treatments provided similar benefit to male mice, whereas weight gain in females appeared more noticeably dose-dependent (**Figure 14A**). Both disease progression (*i.e.*, 10% loss in peak body weight) and animal survival were also plotted in which siSOD1-047M3-AC1^{VP} treatments delayed advance disease and extended survival in both male and female mice (**Figure 14B-C**). **Table 7** summarizes median days for disease progression and survival following siRNA-ACO treatment via IT injection in male and female populations. Overall, siCON3-AC1^{VP} provided no therapeutic benefit in comparison to vehicle control, while equivalent doses of siSOD1-047M3-AC1^{VP} (*i.e.*, 150 mg) extended animal survival in males and females by 61 and 29 days, respectively.

Table 7. Median age of onset and survival in male and female mice following siSOD1-047M3-AC1^{VP} treatment via IT injection

Treatment	**aCSF**	**siCON3-AC1^{VP}**	**ASO^{SOD1}**	**siSOD1-047M3-AC1^{VP}**		
Dose (µg)	**-**	**150**	**150**	**75**	**150**	**300**
Animal Number						
Male (♂)	10	10	9	10	8	10
Female (♀)	6	10	9	10	10	9
Disease Onset (days)						
Male (♂)	152	146	185*	185*	204.5†	182†
Female (♀)	152	150.5	167†	176*	183.5*	206*
Survival (days)						
Male (♂)	163.5	161	191*	207*	224.5†	196.5*
Female (♀)	173	173	189	198.5*	202*	227*

*P < 0.001 compared to aCSF group

†P < 0.01 compared to aCSF group

[235] Neuromuscular performance was also evaluated in groups comprised of both male and female mice. Distance traveled via open field roaming (**Figure 15A**), rotarod test, (**Figure 15B**), and grip strength (**Figure 15C**) all showed siSOD1-047M3-AC1^{VP} treatment greatly improved motor function in *hSOD1^{G93A}* mice, which was generally well sustained until the end of study. Comparing rotarod performance of each individual animal at an early timepoint prior to measurable weight loss (*i.e.*, PND 90) to their respective last timepoints further indicates siSOD1-047M3-AC1^{VP} treatment retained or improved neuromuscular performance in a majority of animals compared to controls or ASO^{SOD1} (**Figure 16**). The latency of rotarod performance of each individual animal is summarized in Table 8.

Table 8. The latency time of rotarod performance of each individual animal.

Postnatal day (PND)	Latency time (second)															
	aCSF (n=8)								siCON3-AC1^{VP} (150 μg, n=8)							
90	224	120	129	124	166	148	214	128	182	165	168	75	92	237	217	86
110	118	87	119	132	132	149	150	34	80	119	71	122	119	180	105	205
131	141	130	107	108	136	95	123	74	126	82	105	132	121	162	118	132
137	103	64	76	91	111	93	98	87	128	72	/	115	112	300	120	/
144	64	17	43	63	78	55	81	60	41	56	/	60	80	221	92	/
151	/	31	42	43	46	42	24	44	/	40	/	55	36	72	38	/
158	/	8	27	18	23	23	14	24	/	14	/	47	21	48	22	/
165	/	/	20	/	5	10	6	7	/	/	/	17	14	13	6	/
172	/	/	16	/	/	47	/	10	/	/	/	/	/	/	/	/

Postnatal day (PND)	Latency time (second)													
	ASO^{SOD1} (150 μg, n=7)							siSOD1-047M3-AC1^{VP} (75 μg, n=7)						
90	127	178	182	79	302	194	132	277	79	237	155	66	136	104
110	171	173	174	147	302	152	63	230	230	206	87	85	143	144
131	182	206	253	162	257	160	132	216	255	217	197	168	222	300
137	204	244	300	194	300	127	187	234	300	279	300	223	222	300
144	208	245	216	204	285	116	197	217	295	272	250	190	245	312
151	214	250	236	214	240	78	185	180	300	286	257	174	213	300
158	211	259	255	220	223	53	202	163	300	285	191	174	172	300
165	251	232	191	252	216	305	176	117	300	304	217	134	104	300
172	160	257	137	300	227	13	135	105	300	236	211	133	98	300
179	185	300	3	300		14	109	35	300	187	238	119	93	300
186	225	300	/	/	/	/	83	/	300	207	217	139	48	300
193	216	273	/	/	/	/	28	/	300	191	/	/	41	300
200	/	/	/	/	/	/	/	/	300	143	/	/	18	300

Postnatal day (PND)	Latency time (second)													
	siSOD1-047M3-AC1^{VP} (150 μg, n=7)							siSOD1-047M3-AC1^{VP} (300 μg, n=6)						
90	105	101	301	156	109	235	179	74	70	90	169	222	143	
110	196	246	300	249	162	271	300	80	220	112	135	271	275	

131	238	205	300	278	193	259	300	300	191	131	224	300	252
137	274	293	300	276	300	270	300	266	237	219	300	300	300
144	289	300	300	272	179	221	300	302	300	300	300	300	300
151	300	300	300	272	191	181	300	300	274	267	300	300	300
158	300	300	300	236	182	138	300	254	202	269	300	253	296
165	259	300	300	209	138	97	300	297	210	252	300	316	273
172	248	300	293	204	129	32	300	289	218	219	300	300	276
179	293	300	238	157	74	4	300	292	163	222	300	269	224
186	300	300	248	113	46	/	300	289	199	239	300	300	188
193	300	300	146	/	25	/	/	298	138	222	/	300	163
200	300	300	17	/	/	/	/	242	147	240	/	300	194
207	252	207	/	/	/	/	/	249	143	190	/	300	143
214	300	273	/	/	/	/	/	297	232	204	/	300	133
221	191	300	/	/	/	/	/	300	37	128	/	109	136
228	/	/	/	/	/	/	/	/	/	50	/	/	97

Note: "/" represents not shown.

[236] Motor function was also scored for all animals prior to open field, rotarod, and/or grip strength tests using the ALS Therapy Development Institute (ALS TDI) neuroscore (NS) system. As shown in **Figure 15D**, all mice developed abnormal splay (*i.e.*, NS2) by approximately PND 130 in both aCSF and siCON3-AC1^{VP} control groups that continued to increase in severity over time (*i.e.*, ≥NS3). Conversely, mean scores for all siSOD1-047M3-AC1^{VP} doses never surpassed NS2 at any time point during the course of the study particularly for the highest dose group (*i.e.*, 300 mg) in which mean NS remained predominantly flat at around NS1. ASO^{SOD1} treatment also demonstrated an improved NS comparative to vehicle and siCON3-AC1^{VP} controls, however, by PND 150, NS began to increase at a slope similar to controls despite ASO^{SOD1} having ~3X molecular excess than that of siSOD1-047M3-AC1^{VP} at 150 mg dose.

[237] In summary, siRNA-ACO conjugates provided *SOD1* siRNAs with pharmacological properties necessary for clinical development including delivery to the CNS and favorable tissue biodistribution with potent and durable activity. Local delivery via ICV or IT injection into *hSOD1*^{G93A} mice delayed disease onset /progression and extended animal survival with superior efficacy compared to an ASO compound resembling Tofersen in sequence and chemistry. These results clearly indicate that siRNA represents a vast improvement over current clinical therapeutic modalities (*i.e.*, Tofersen) to target and knockdown *SOD1* for treatment of ALS.

Example 6. Knockdown activity of siRNA on the expression of *SOD1* mRNA in HeLa and SK-N-AS cells

[238] To assess the knockdown activity of siRNAs, the indicated siRNAs (*i.e.*, RD-15757, RD-18972, RD-12500, RD-18973, RD-18948 and RD-18949) were directly added into culture medium containing HeLa cell at 1500 nM for 3 days. Cells without any treatment served as a mock control and cells treated with RD-11566 served as a duplex control. As shown in **FIG.17**, compared to RD-11566, all the siRNA caused varying degree of reduction in *SOD1* mRNA level (ranging from 1 to 32%) with RD-12500 causing the greatest reduction (32%).

[239] To further assess dose-dependent knockdown activity of siRNAs, the indicated siRNAs (*i.e.*, RD-12926, RD-15757, RD-12500, RD-18947, RD-18948, RD-18949, RD-18946, RD-18972 and RD-18973) were transfected into SK-N-AS cells at indicated concentrations (*i.e.*, 0.0002, 0.001, 0.0039, 0.0156, 0.0625, 0.25, 1 and 4 nM) for 24 hours. *SOD1* mRNA levels as quantified by two-step RT-qPCR were shown in **FIGs.18A** and **18B**. EC_{50} values were extrapolated to define potency in context to maximal activity for each of the tested siRNAs that demonstrated dose-dependent knockdown of *SOD1* mRNA. The resulting EC_{50} values following siRNA treatment in SK-N-AS cells are summarized in Table 9.

Table 9. EC_{50} values following siRNA treatment in SK-N-AS cells

siRNA	EC_{50} (pM)
RD-12926	0.1832
RD-15757	0.1420
RD-12500	0.1194

RD-18947	0.9290
RD-18948	0.1678
RD-18949	0.3360
RD-18946	4480.0
RD-18972	0.6933
RD-18973	0.6786

MATERIALS AND METHODS

High-throughput screening of siRNAs targeting human *SOD1*

Open reading frame (ORF) from human *SOD1* (*hSOD1*) cDNA sequence (NM_000454.5) served as the template for siRNA design via in-house algorithm. A total of 268 duplexes were synthesized at 19-nucleotides in length without medicinal chemistry (**Table 2**). Plating and transfection of HEK293A cells was performed in 96-well plates each containing 32 siRNAs and 8 quality control treatments at 2 concentrations (*i.e.*, 0.1 and 10 nM) in duplicate. Cells were cultured for 24 hours, and lysis was automated via the Fluent System 780 liquid handling system (Tecan, Hombrechtikon, The Switzerland) using an optimized formula containing propidium iodide (PI) based on the Cell-Lysis (CL) buffer for one-step RT-qPCR as previous described (K. Shatzkes et al, *Sci Rep* 4, 4659 (2015)). Integration of PI in sample preparation served to monitor variation in cell number (*e.g.*, untoward cytotoxicity) by staining total nucleic acid content in crude lysates. Staining was quantified via optical density (OD) at 535 nm excitation and 615 nm emission wavelengths on the Infinite M200 Pro microplate reader (Tecan). Samples were subsequently transferred to 384-well plates for analysis by RT-qPCR on the 480 Real-Time PCR system (Roche, Basel, Switzerland) using the One-Step TB Green PrimeScript RT-PCR Kit II (Takara, Kyoto, Japan). Preparation of PCR reactions was automated by the Echo 525 Acoustic Liquid Handler (Beckman Coulter, Brea, CA, USA). A secondary screen was subsequently performed only on the top 30 performing siRNAs at 6 concentrations (*i.e.*, 0.0064, 0.032, 0.16, 0.8, 4 and 20 nM). All samples were amplified in triplicate.

siRNA synthesis

[240] Oligonucleotide sequences were synthesized in-house at Ractigen Therapeutics (Rudong, China) on solid-phase support using a HJ-12 synthesizer (Highgene-Tech Automation, Beijing, China) and subsequently purified via RP-HPLC using an acetonitrile gradient over a UniPS column (NanoMicro Technology, Suzhou, China). Each sequence was reconstituted in sterile water via buffer exchange. Equal molar quantities of each strand were annealed into their corresponding duplexes by briefly heating strand mixtures and cooling to room temperature. Resolution of a single band via gel electrophoresis at predicted molecular weights was used to qualify duplex formation. ESI-MS was used to confirm duplex identity, while overall purity was analyzed via SEC-HPLC using a XBridge Protein BEH SEC 125 A column (Waters Corporation, Milford, MA, USA). Endotoxin levels in each batch were quantified using the end-point Chromogenic Endotoxin Quant Kit (Bioendo, Xiamen, China) via proenzyme Factor C. All control duplexes and chemically modified sequences are listed in Table 10.

Table 10. Chemically modified siRNAs and controls

siRNA	SEQ ID NO	Sense (5'-3')	SEQ ID NO	Antisense (5'-3')
siCON/Neg Con	806	ACUACUGAGUGACAGUAGAtt	807	UCUACUGUCACUCAGUAGUtt
siSOD1-063M1	808	mG*fA*mGfCmAfGmAfAfGfGfAfAmAfGmUfA*mA*fU	828	mA*fU*mUfAmCfUmUfUmCfCmUfUmCfUmGfCmUfC*mG*fA
siSOD1-063M2	809	fC*mG*fAmGfCmAfGmAfAfGfGmAfAmAfGmUfA*mA*fU	829	mA*fU*mUfAmCfUmUfUmCfCmUfUmCfUmGfCmUfCmG*fA*mA
siSOD1-063M3	810	mU*fC*mGfAmGfCmAfGfAfAfGfGmAfAmAfGmUfA*mA*fU	830	mA*fU*mUfAmCfUmUfUmCfCmUfUmCfUmGfCmUfCmGfA*mA*fA
siSOD1-063M4	811	fU*mU*fCmGfAmGfCmAfGfAfAmGfGmAfAmAfGmUfA*mA*fU	831	mA*fU*mUfAmCfUmUfUmCfCmUfUmCfUmGfCmUfCmGfAmA*fA*mU
siSOD1-047M1	812	mG*fG*mGfCmAfUmCfAfUfCfAfAmUfUmUfC*mG*fA	832	mU*fC*mGfAmAfAmUfUmGfAmUfGmAfUmGfCmCfC*mU*fG
siSOD1-047M2	813	fA*mG*fGmGfCmAfUmCfAfUfCmAfAmUfUmUfC*mG*fA	833	mU*fC*mGfAmAfAmUfUmGfAmUfGmAfUmGfCmCfCmU*fG*mC
siSOD1-047M3	814	mC*fA*mGfGmGfCmAfUfCfAfUfCmAfAmUfUmUfC*mG*fA	834	mU*fC*mGfAmAfAmUfUmGfAmUfGmAfUmGfCmCfCmUfG*mC*fA
siSOD1-047M4	815	fG*mC*fAmGfGmGfCmAfUfCfAmUfCmAfAmUfUmUfC*mG*fA	835	mU*fC*mGfAmAfAmUfUmGfAmUfGmAfUmGfCmCfCmUfGmC*fA*mC
siSOD1-104M1	816	mC*fA*mUfUmAfAmAfGfGfAfCfUmGfAmCfU*mG*fA	836	mU*fC*mAfGmUfCmAfGmUfCmCfUmUfUmAfAmUfG*mC*fU
siSOD1-104M2	817	fG*mC*fAmUfUmAfAmAfGfGfAmCfUmGfAmCfU*mG*fA	837	mU*fC*mAfGmUfCmAfGmUfCmCfUmUfUmAfAmUfGmC*fU*mU
siSOD1-104M3	818	mA*fG*mCfAmUfUmAfAfAfGfGfAmCfUmGfAmCfU*mG*fA	838	mU*fC*mAfGmUfCmAfGmUfCmCfUmUfUmAfAmUfGmCfU*mU*fC
siSOD1-104M4	819	fA*mA*fGmCfAmUfUmAfAfAfGmGfAmCfUmGfAmCfU*mG*fA	839	mU*fC*mAfGmUfCmAfGmUfCmCfUmUfUmAfAmUfGmCfUmU*fC*mC
siSOD1-005M1	820	mG*fA*mCfGmAfAmGfGfCfCfGfUmGfUmGfC*mG*fU	840	mA*fC*mGfCmAfCmAfCmGfGmCfCmUfUmCfGmUfC*mG*fC
siSOD1-005M2	821	fC*mG*fAmCfGmAfAmGfGfCfCmGfUmGfUmGfC*mG*fU	841	mA*fC*mGfCmAfCmAfCmGfGmCfCmUfUmCfGmUfCmG*fC*mC
siSOD1-005M3	822	mG*fC*mGfAmCfGmAfAfGfGfCfCmGfUmGfUmGfC*mG*fU	842	mA*fC*mGfCmAfCmAfCmGfGmCfCmUfUmCfGmUfCmGfC*mC*fA
siSOD1-005M4	823	fG*mG*fCmGfAmCfGmAfAfGfGmCfCmGfUmGfUmGfC*mG*fU	843	mA*fC*mGfCmAfCmAfCmGfGmCfCmUfUmCfGmUfCmGfCmC*fA*mU
siSOD1-258M1	824	mA*fA*mUfGmUfGmAfCfUfGfCfUmGfAmCfA*mA*fA	844	mU*fU*mUfGmUfCmAfGmCfAmGfUmCfAmCfAmUfU*mG*fC
siSOD1-258M2	825	fC*mA*fAmUfGmUfGmAfCfUfGmCfUmGfAmCfA*mA*fA	845	mU*fU*mUfGmUfCmAfGmCfAmGfUmCfAmCfAmUfU

				mG*fC*mC
siSOD1-258M3	826	mG*fC*mAfAmUfGmUfGfAfCfUfGmCfUmGfAmCfA*mA*fA	846	mU*fU*mUfGmUfCmAfGmCfAmGfUmCfAmCfAmUfUmGfC*mC*fC
siSOD1-258M4	827	fG*mG*fCmAfAmUfGmUfGfAfCmUfGmCfUmGfAmCfA*mA*fA	847	mU*fU*mUfGmUfCmAfGmCfAmGfUmCfAmCfAmUfUmGfCmC*fC*mA
siSOD1-270M3	814	mC*fA*mGfGmGfCmAfUfCfAfUfCmAfAmUfUmUfC*mG*fA	866	VpmU*fC*mGfAmAfAmUfUmGfUmUfGmAfUmGfCmCfCmUfG*mC*fA
siSOD1-047M3^{VP}	814	mC*fA*mGfGmGfCmAfUfCfAfUfCmAfAmUfUmUfC*mG*fA	848	VpmU*fC*mGfAmAfAmUfUmGfAmUfGmAfUmGfCmCfCmUfG*mC*fA
siSOD1-005M3^{VP}	822	mG*fC*mGfAmCfGmAfAfGfGfCfCmGfUmGfUmGfC*mG*fU	849	VpmA*fC*mGfCmAfCmAfCmGfGmCfCmUfUmCfGmUfCmGfC*mC*fA
siSOD1-047M3-AC1	850	mC*fA*mGfGmGfCmAfUfCfAfUfCmAfAmUfUmUfC*mG*fA-L9-meU*meU*meG*meU*meA*meU*meU*meC*meU*meA*meU*meG*meU*meU	834	mU*fC*mGfAmAfAmUfUmGfAmUfGmAfUmGfCmCfCmUfG*mC*fA
siSOD1-005M3-AC1	851	mG*fC*mGfAmCfGmAfAfGfGfCfCmGfUmGfUmGfC*mG*fU-L9-meU*meU*meG*meU*meA*meU*meU*meC*meU*meA*meU*meG*meU*meU	842	mA*fC*mGfCmAfCmAfCmGfGmCfCmUfUmCfGmUfCmGfC*mC*fA
siCON1-AC1^{VP}	852	fC*mG*fGmUfGmGfAmGfGfCfCfUfGmGfCmGfCmA*fA*mU-L9-meU*meU*meG*meU*meA*meU*meU*meC*meU*meA*meU*meG*meU*meU	858	VpmG*fC*mCfAmCfCmUfCmCfGmGfAmCfCmGfCmGfUmUfA*mC*fA
siCON2-AC1^{VP}	853	mA*fG*mUfUmCfGmUfGfUfCfAfUmCfCmAfGmAfG*mA*fU-L9-meU*meU*meG*meU*meA*meU*meU*meC*meU*meA*meU*meG*meU*meU	859	VpmA*fU*mCfUmCfUmGfGmAfUmGfAmCfAmCfGmAfAmCfU*mU*fG
siCON3-AC1^{VP}	854	mG*fU*mAfCmUfUmUfUfGfUfGfUmAfGmUfAmCfA*mA*fA-L9-meU*meU*meG*meU*meA*meU*meU*meC*meU*meA*meU*meG*meU*meU	860	VpmU*fU*mUfGmUfAmCfUmAfCmAfCmAfAmAfAmGfUmAfC*mU*fG
siSOD1-063M3-AC1^{VP}	855	mU*fC*mGfAmGfCmAfGfAfAfGfGmAfAmAfGmUfA*mA*fU-L9-meU*meU*meG*meU*meA*meU*meU*meC*meU*meA*meU*meG*meU*meU	861	VpmA*fU*mUfAmCfUmUfUmCfCmUfUmCfUmGfCmUfCmGfA*mA*fA
siSOD1-047M3-AC1^{VP}	850	mC*fA*mGfGmGfCmAfUfCfAfUfCmAfAmUfUmUfC*mG*fA-L9-meU*meU*meG*meU*meA*meU*meU*meC*meU*meA*meU*meG*meU*meU	848	VpmU*fC*mGfAmAfAmUfUmGfAmUfGmAfUmGfCmCfCmUfG*mC*fA

siSOD1-104M3-AC1^{VP}	856	mA*fG*mCfAmUfUmAfAfAfGfGfAmCfUmGfAmCfU*mG*fA-L9-meU*meU*meG*meU*meA*meU*meU*meC*meU*meA*meU*meG*meU*meU	862	VpmU*fC*mAfGmUfCmAfGmUfCmCfUmUfUmAfAmUfGmCfU*mU*fC
siSOD1-005M3-AC1^{VP}	852	mG*fC*mGfAmCfGmAfAfGfGfCfCmGfUmGfUmGfC*mG*fU-L9-meU*meU*meG*meU*meA*meU*meU*meC*meU*meA*meU*meG*meU*meU	849	VpmA*fC*mGfCmAfCmAfCmGfGmCfCmUfUmCfGmUfCmGfC*mC*fA
siSOD1-258M3-AC1^{VP}	857	mG*fC*mAfAmUfGmUfGfAfCfUfGmCfUmGfAmCfA*mA*fA-L9-meU*meU*meG*meU*meA*meU*meU*meC*meU*meA*meU*meG*meU*meU	863	VpmU*fU*mUfGmUfCmAfGmCfAmGfUmCfAmCfAmUfUmGfC*mC*fC
siSOD1-270M3-AC1^{VP}	850	mC*fA*mGfGmGfCmAfUfCfAfUfCmAfAmUfUmUfC*mG*fA-L9-meU*meU*meG*meU*meA*meU*meU*meC*meU*meA*meU*meG*meU*meU	866	VpmU*fC*mGfAmAfAmUfUmGfUmUfGmAfUmGfCmCfCmUfG*mC*fA
RD-12926	867	CAGGGCAUCAUCAAUUUCGA	868	UCGAAAUUGAUGAUGCCCUGCA
RD-15757	814	mC*fA*mGfGmGfCmAfUfCfAfUfCmAfAmUfUmUfC*mG*fA	848	VpmU*fC*mGfAmAfAmUfUmGfAmUfGmAfUmGfCmCfCmUfG*mC*fA
RD-12500	850	mC*fA*mGfGmGfCmAfUfCfAfUfCmAfAmUfUmUfC*mG*fA-L9-meU*meU*meG*meU*meA*meU*meU*meC*meU*meA*meU*meG*meU*meU	848	VpmU*fC*mGfAmAfAmUfUmGfAmUfGmAfUmGfCmCfCmUfG*mC*fA
RD-18947	869	GCAUCAUCAAUUUCGA	870	UCGAAAUUGAUGAUGCCCUGU
RD-18948	871	mG*mC*mAfUmCfAmUfCmAfAmUmUmUfC*mG*mA-DIO	872	VmU*fC*mGfAfAfAmUfUmGfAmUfGmAfU*mG*fC*mC*mC*mU*fG*mU
RD-18949	873	mG*mC*mAmUmCfAfUfCmAfAmUmUmUmC*mG*mA-DIO	874	VmU*fC*mGmAmAfAmUmUmGmAmUmGmAfU*mG*fC*mC*mC*mU*fG*mU
RD-18946	867	CAGGGCAUCAUCAAUUUCGA	875	UCGAAAUUGUUGAUGCCCUGCA
RD-18972	814	mC*fA*mGfGmGfCmAfUfCfAfUfCmAfAmUfUmUfC*mG*fA	866	VpmU*fC*mGfAmAfAmUfUmGfUmUfGmAfUmGfCmCfCmUfG*mC*fA

RD-18973	850	mC*fA*mGfGmGfCmAfUfCfAfUfCmAfAmUfUmUfC*mG*fA-L9-meU*meU*meG*meU*meA*meU*meU*meC*meU*meA*meU*meG*meU*meU	866	VpmU*fC*mGfAmAfAmUfUmGfUmUfGmAfUmGfCmCfCmUfG*mC*fA
RD-11566	882	mG*fA*mCfUmAfCmUfGmAfGfUfGmAfCmAfGmUfA*mG*fA	883	VpmU*fC*mUfAmCfUmGfUmCfAmCfUmCfAmGfUmAfGmUfC*mG*fU
ASO/ACO		**Sequence (5'-3')**		
ASO^{SOD1}	864	meC*meAmeG*meGmeA*t*a*c*a*t*t*t*c*t*a*meCmeA*meGmeC*meT		
AC1	865	L9-meU*meU*meG*meU*meA*meU*meU*meC*meU*meA*meU*meG*meU*meU		

Note: f: 2'-fluoro, m: 2'-O-methyl, *: phosphorothioate, Vp or V: 5'-(E)-vinylphosphonate, L9: Spacer-9 linker, me: 2'-O-methoxy-ethyl, meC: 2'-O-methoxyethyl-5-methylcytosine, meU: 2'-O-methoxyethyl-5-methyl-uridine, c: 5-methyl-2'-deoxycytidine, lower case: DNA, DIO: a 3′ divalent linker (*i.e.*, a linker that attaches the 3′ end of a first sense strand to the 3′ end of a second sense strand that is identical to the first sense strand) purchased from Suzhou Biosyntech Co., Ltd (Kunshan, Suzhou, China).

Cell culture and treatment

[241] HEK293A cells (Cobioer, Nanjing, China, Cat# CBP60436) and SK-N-AS (Procell, Wuhan, China, Cat# CL-0621) cells were maintained in DMEM medium supplemented with 10% bovine calf serum (Sigma-Aldrich), penicillin (100 U/ml, Gibco) and streptomycin (100 mg/ml, Gibco). T98G cells (Cobioer, Cat# CBP60301) were maintained in MEM medium supplemented with 10% FBS, 1% NEAA, sodium pyruvate (1 mM), penicillin (100 U/ml) and streptomycin (100 ug/ml). Human cervical carcinoma cell HeLa (ATCC) cells were cultured in modified RPMI 1640 medium (Gibco, Thermo Fisher Scientific, Carlsbad, CA) supplemented with 10% bovine calf serum and 1% penicillin/streptomycin. All cell lines were cultured in a humidified atmosphere of 5% CO_2 at 37°C. Transfections were carried out using Lipofectamine RNAiMax (ThermoFisher, Waltham, MA, USA) in growth media without antibiotics according to the manufacture's protocol.

RT-qPCR

One-step reverse transcription-quantitative polymerase chain reaction (one-step RT-qPCR)

[242] At the end of transfection, medium was discarded, and cells were washed with 150 µL of PBS once per well. After discarding the PBS, 100 µL of cell lysis was added into each well and incubated at room temperature for 5 min. 0.5 µL of the cell lysis was taken from each well and analyzed by RT-qPCR using One Step TB GreenTM PrimeScripTM RT-PCR kit II (Takara, RR086A) in a Roche Lightcycler 480 real-time PCR machine. PCR reactions were prepared using Echo 525 Acoustic Liquid Handler (Beckman Coulter). Each transfection sample was amplified in 3 repeat wells. PCR reaction conditions are shown in Table 11.

Table 11. PCR reaction preparation

Reagent	Volume (µL)
2 × One Step TB Green RT-PCR buffer 4	2.5
PrimeScript™ 1 step enzyme Mix 2	0.2
Forward and Reverse Primers Mix (5 µM)	0.4
dH_2O without RNase	1.6
Crude lysate (RNA)	0.4
Total volume	5.1

[243] The reaction conditions were as follows: reverse transcription reaction (stage 1): 42°C for 5 min, 95°C for 10 sec; PCR reaction (stage 2): 95°C for 5 sec, 59°C for 20 sec, 72°C for 10 sec; 40 cycles of amplification; and melting curve (stage 3). Human *SOD1* gene was amplified as target genes. Human *TBP* or mouse *Tbp* was served as reference genes and also amplified as internal controls for RNA loading. Primer sequences are listed in Table 12.

Table 12. Primer sequences for RT-qPCR assay

Primer	Gene	SEQ ID No.	Sequence (5'-3')	Product size (bp)

SOD1 F	Human SOD1	876	AAGCATTAAAGGACTGACTGAAGG	154
SOD1 R		877	CAAGTCTCCAACATGCCTCTC	
TBP F	Human TBP	878	TGCTCACCCACCAACAATTTAG	139
TBP R		879	TCTGCTCTGACTTTAGCACCTG	
Tbp F	Mouse Tbp	880	CCGTGAATCTTGGCTGTAAACT	116
Tbp R		881	TGTCCGTGGCTCTCTTATTC	

Two-step RT-qPCR

[244] Animal tissue frozen in RNALater (Sigma-Aldrich, St. Louis, MO, USA) was homogenized in Total RNA Isolation Reagent (Biosharp, Hefei, China) using a Bioprep-24 Homogenizer (Allsheng, Hangzhou, China). Chloroform was added to the homogenate in which the aqueous phase was removed and mixed with isopropanol. Total RNA was extracted from the tissue prep using the RNeasy RNA kit (Qiagen) according to the manufacture's protocol. RNA from cell culture was extracted using the Auto-Pure 96A (Allsheng) nucleic acid extraction system. Reverse transcription (RT) reactions were performed with 1 μg total RNA using the PrimeScript RT kit with gDNA Eraser (Takara, Shlga, Japan). The resulting cDNA was amplified in triplicate on the Roche LightCycler 480 Multiwell Plate 384 (Roche, ref: 4729749001, US) using SYBR Premix Ex Taq II (Takara, Shlga, Japan) in conjunction with primer sets specific to human *SOD1* (*hSOD1*) and an internal control for either human (*i.e.*, *TBP*) or mouse (*i.e.*, m*Tbp*) samples. Melting curves were performed after amplification to confirm primer specificity. Reaction conditions were as follows: reverse transcription reaction (stage 1): 42°C for 5 min, 95°C for 10 sec; PCR reaction (stage 2): 95°C for 5 sec, 60°C for 30 sec, 72°C for 10 sec; 40 cycles of amplification; Melting curve (stage 3). PCR reaction conditions are shown in Table 13 and Table 14. Primer sequences are listed in Table 12.

Table 13. RT reaction

Reaction-1 (Takara, RR047A)	Volume (μL)
5×gDNA Eraser Buffer	2
gDNA Eraser	1
Total RNA (1 μg) + RNase Free dH_2O	7
Total Volume	10
42°C 5 min, store at 4°C	
Reagent-2 (Takara, RR047A)	
5 × PrimeScript Buffer2	4
PrimeScript RT Enzyme Mix I	1
RT Prime Mix	1
RNase free dH_2O	4
Reaction-1	10
Total Volume	20
37°C 15 min, 85°C 5 sec, store at 4°C	

Table 14. RT-qPCR reaction

Reagent (Takara, RR820A)	Volume (μL)
SYBR Premix Ex Taq II (2 ×)	5
PCR Primer (forward + reverse) 5 μM	1
cDNA (RT product)	4
Total	10

[245] To calculate the expression level (*Erel*) of *SOD1* mRNA in an siRNA-transfected sample relative to control treatment (Mock), the averaged Ct values of the target gene and the internal reference gene were substituted into Formula 1,

$$E_{rel} = 2^{(CtTm-CtTs)} / 2^{(CtRm-CtRs)}$$

(Formula 1)

wherein CtT_m was the Ct value of the target gene from the mock-treated sample; CtT_s was the Ct value of the target gene from the siRNA-treated sample; CtR_m was the Ct value of the internal reference gene from the mock-treated sample; CtR_s was the Ct value of the internal reference gene from the siRNA-treated sample.

Caspase 3/7 activity assay

[246] Caspase 3/7 activity was quantified in cell culture by using the Caspase-Glo 3/7 assay system (Promega, Madison, WI, USA). Briefly, a luminogenic substrate was added directly to culture media and incubated for 20 mins at 37°C. Luminescence was subsequently measured on an Infinite M200 Pro microplate reader (Tecan). Relative Caspase 3/7 activity was calculated by subtracting background signal of blank from the luminescence values in each well and normalizing data to non-treated (Mock) controls.

Cell viability assay

[247] In vitro cell viability was measured using the CCK-8 assay (Dojindo, Mashiki-machi, Japan) according to the manufacture's protocol. Briefly, fresh media containing WST-8 substrate was added to each well of the tissue culture plate and incubated for at least 1 hour at 37°C. Absorbance was measured at 450 nm on an Infinite M200 Pro microplate reader (Tecan). Relative viability is calculated by subtracting background absorbance of the blank control from the OD values in each well and normalizing data to non-treated (Mock) controls.

Luciferase reporter constructs and knockdown assessment

[248] Target sequence containing either P.E22G or P.F21C mutant SNPs were cloned into the multiple cloning site (MCS) of luciferase reporter vector pmirGLO (Promega) between the NheI and SalI restriction enzyme (RE) sites downstream of the firefly luciferase gene (luc2) to generate constructs $pLuc^{P.E22G}$ and $pLuc^{P.F21C}$, respectively. A control reporter construct (*i.e.*, $pLuc^{SOD1}$) was also created containing consensus h*SOD1* sequence perfectly complementary to siSOD1-047M3-$AC1^{VP}$ guide strand. All constructs were subcloned in DH5a bacteria (Tolobio, Shanghai, China)

and colonies were selected for DNA sequencing to confirm insertion of target sequence. Exemplary colonies were scaled up for plasmid isolation via midiPrep (Qiagen, Hilden, Germany). HEK293A cells were plated in 96-well cell culture plates at 30,000 cells/well in absence of antibiotics. Cells were co-transfected with one of reporter plasmids (*i.e.*, $pLuc^{P.E22G}$, $pLuc^{P.F21C}$, or $pLuc^{SOD1}$) at 100 ng/well in combination with siSOD1-047M3-$AC1^{VP}$ or scramble control at the indicated concentrations using 0.3 µL of Lipofectamine 2000 (ThermoFisher). Wells treated in absence of test article (0 nM) served as non-treated controls. Cells were cultured for 24 hours, and luciferase activity was quantified using the Dual-Glo Luciferase Assay System (Promega) according to the manufacture's protocol. Briefly, cells were lysed in 50 µL Passive lysis buffer (Promega) in which 20 µL of lysate was mixed with 20 µL of Dual-Glo Luciferase Reagent and incubated for 10 mins at room temperature. Luminescence was subsequently measured on an Infinite 200 Pro microplate reader (Tecan) to quantify luciferase activity. Following measurements, 20 µL of Dual-Glo Stop & Glo Reagent (Promega) was added to each well and incubated for an additional 10 mins at room temperature. Luminescence was again measured to quantify *Renilla* activity, which served to normalize luciferase reporter results. Data was calculated as the ratio of reporter luminescence to *Renilla* luminescence relative to the ratio of non-treated controls. Percent knockdown (% KD) was calculated as $1-(Ratio_{siRNA}/Ratio_{non\text{-}treated}) *100$. $Ratio_{siRNA}$ = Firefly luciferase activity of siRNA / Renilla luciferase activity of siRNA, $Ratio_{non\text{-}treated}$ = Firefly luciferase activity of non-treated group / Renilla luciferase activity of non-treated group.

Animal handling and grouping

[249] Parental transgenic *h*$SOD1^{G93A}$ mice (Strain ID #004435) were purchased from The Jackson Laboratory (Bar Harbor, ME, USA) and imported into China via Nantong University (Nantong City, Jiangsu Province, China). Mice were delivered to the animal facility at 6 weeks of age and subsequently bred domestically at Nantong University who supplied the animals for this study. All animal procedures were approved by the IACUC at Nantong University. Formulations for animal treatments were prepared fresh prior to use by dissolving allotments of lyophilized oligonucleotide into aCSF to create stock solutions for dilution to the intended treatment concentrations. Animals were randomly allocated into study groups based on body weight and sex. Any animals in poor health or with obvious abnormalities were omitted from the experiments. Randomization was analyzed using Ordinary one-way ANOVA via GraphPad Prism version 8.3.0 Windows (GraphPad Software, San Diego, CA, USA). Female *h*$SOD1^{G93A}$ mice typical weighed approximately 20-25% less than their male liter mates.

Intracerebroventricular (ICV) injection

[250] Avertin (1.2%) was prepared fresh and sterilized via a 0.2-micron filter. Mice were dosed at 0.30-0.35 ml per 10 g body weight via intraperitoneal (IP) injection in a stereotaxic apparatus to rapidly induce anesthesia for up to 30 minutes. An approximate 11.5 mm incision was made in the animal's scalp and a 25-gauge needle attached to a Hamilton syringe containing the appropriate siRNA formulation was placed at bregma level. The needle was moved to the appropriate

anterior/posterior and medial/lateral coordinates (0.2 mm anterior/posterior and 1 mm to the right medial/lateral). A total of 10 μL was injected into the lateral ventricle at an approximate rate of 1 μl/s. Following treatment, the needle was slowly withdrawn, and the wound sutured close.

Intrathecal (IT) injection

[251] Anesthesia was administered via 3.0% isoflurane in an induction chamber for continuous 10 mins. Hair was shaved around the injection site at the base of the tail and cleaned with 75% ethanol. The space between the L5-L6 spinous processes was identified and a 30-gauge needle attached to a microliter syringe containing the appropriate drug formulations was slowly inserted into the intradural space until a tail flick was observed. The needle position was subsequently secured in which 10 μL total volume of solution was injected over the course of 1 min.

Quantification of siRNA-ACO in animal tissues

[252] Tissue lysate was prepared in lysis buffer (0.5% CA-630, 1mM EDTA, 150 mM NaCl) using a Bioprep-24 Homogenizer (Allsheng). Samples were subsequently heated to 95°C to inactivate sample proteins. Serial dilution of non-treated lysate spiked with siRNA-ACO was used to generate 8-point standard curves. Reverse transcription (RT) reactions were performed using the PrimeScript RT reagent kit (Takara) in conjugation with custom stem-loop primers specific to siRNA guide strands. Each sample was amplified in triplicate on the 480 Real-Time PCR system (Roche) using SYBR Premix Ex Taq II (Takara) reaction mix with primer sets specific to guide strand cDNA. Melting curves were performed after amplification to confirm primer specificity. Absolute quantities of siRNA were extrapolated by linear regression using the appropriate standard curves. Tissue concentrations were calculated as the ratio of absolute siRNA mass (ng) relative to the total weight (g) of tissue sample prepped for lysis.

Clinical observation and endpoint criteria

[253] Animals were observed after injection for up to 4 hours and daily thereafter until endpoint. Body weight was determined before test substance administration and at recorded intervals thereafter. Animals with weight loss >20% relative to their initial mass at day of treatment or a neuroscore of NS4 met endpoint criteria.

Neurological scoring

[254] For animals treated via IT injection, mice were evaluated for signs of motor deficit using the ALS Therapy Development Institute (ALS TDI) neuroscore (NS) system, which was developed to provide unbiased assessment of disease progression based on hindlimb dysfunction common to *h*SOD1^{G93A} mice (T. Hatzipetros, et al., Journal of Visualized Experiments (2015), https:/doi.org/10.3791/53257). NS was assigned based on the following 4-point scale: 0 if no signs of motor dysfunction (*i.e.*, pre-symptomatic), 1 if hindlimb tremors are evident when suspended by

tail (*i.e.*, first symptoms), 2 if gait abnormalities are present (*i.e.*, onset of paresis), 3 if dragging at least 1 hindlimb (*i.e.*, partial paralysis), and 4 if inability to right itself within 10 seconds (*i.e.*, endpoint paralysis).

Open field test

[255] Each mouse was placed in the corner of an open field apparatus (50 cm length × 50 cm width × 50 cm height) during daylight hours and allowed to freely roam for 15 minutes. An overhead camera recorded the travel path of each animal. Video footage was analyzed by automated tracking software Samart 3.0 (Bioseb, Vitrolles, France) to calculate total distance traveled.

Rotarod analysis

[256] Animals were trained for 3 days prior to data acquisition. Mice were placed on a motionless rotarod apparatus (XinRuan Information Technology, Shanghai, China) with a swivel bar 60 mm in diameter. Rotational speed was accelerated from 0-30 rpm over the course of 300 seconds. Latency time was recorded as the amount of time it took for each animal to fall off the swivel bar. Each animal was tested in triplicate in which the longest value represents latency time.

Grip strength test

[257] Mice were lowered onto a grid plate in which its forepaws and hind paws were allowed to grasp the grid. The tail was gently pulled, and maximal muscle strength was measured on the XR501 grip strength meter (XinRuan Information Technology) in units of mass until the animal relinquished its grasp. Each animal was tested in triplicate in which the mean value represents grip strength.

Statistical analysis

[258] Data analytics were performed using GraphPad Prism version 8.3.0 Windows. Dose response curves and IC_{50} values were extrapolated using non-linear regression via 4-parameter concentration-inhibition model. Where specified, mean values were compared using Tukey's multiple comparison test to determine statistical differences between different dose response curves. Drug quantities in tissue in relationship to knockdown activity including extrapolation of ED_{50} values were performed using non-linear regression via 3-parameter concentration-response model. Time-stratified data (*i.e.*, peak weight analysis and animal survival) was plotted via Kaplan-Meier graphs in which statistical significance was verified using the Mantel-Cox test.

[259] While preferred embodiments of the present invention have been shown and described herein, it will be obvious to those skilled in the art that such embodiments are provided by way of example only. It is not intended that the invention be limited by the specific examples provided within the specification. While the invention has been described with reference to the aforementioned

specification, the descriptions and illustrations of the embodiments herein are not meant to be construed in a limiting sense. Numerous variations, changes, and substitutions will now occur to those skilled in the art without departing from the invention. Furthermore, it should be understood that all aspects of the invention are not limited to the specific depictions, configurations or relative proportions set forth herein which depend upon a variety of conditions and variables. It should be understood that various alternatives to the embodiments of the invention described herein may be employed in practicing the invention. It is therefore contemplated that the invention shall also cover any such alternatives, modifications, variations or equivalents. It is intended that the following claims define the scope of the invention and that methods and structures within the scope of these claims and their equivalents be covered thereby.

Table 2. *SOD1* target sites and cognate siRNA strand sequences

siRNA name	SEQ ID NO	Target sequence (5'-3')	SEQ ID NO	Sense (5'-3')	SEQ ID NO	Antisense (5'-3')
siSOD1-5	2	cgacgaaggccgtgtgcgt	270	CGACGAAGGCCGUGUGCGUTT	538	ACGCACACGGCCUUCGUCGTT
siSOD1-8	3	cgaaggccgtgtgcgtgct	271	CGAAGGCCGUGUGCGUGCUTT	539	AGCACGCACACGGCCUUCGTT
siSOD1-10	4	aaggccgtgtgcgtgctga	272	AAGGCCGUGUGCGUGCUGATT	540	UCAGCACGCACACGGCCUUTT
siSOD1-11	5	aggccgtgtgcgtgctgaa	273	AGGCCGUGUGCGUGCUGAATT	541	UUCAGCACGCACACGGCCUTT
siSOD1-17	6	tgtgcgtgctgaagggcga	274	UGUGCGUGCUGAAGGGCGATT	542	UCGCCCUUCAGCACGCACATT
siSOD1-35	7	acggcccagtgcagggcat	275	ACGGCCCAGUGCAGGGCAUTT	543	AUGCCCUGCACUGGGCCGUTT
siSOD1-37	8	ggcccagtgcagggcatca	276	GGCCCAGUGCAGGGCAUCATT	544	UGAUGCCCUGCACUGGGCCTT
siSOD1-38	9	gcccagtgcagggcatcat	277	GCCCAGUGCAGGGCAUCAUTT	545	AUGAUGCCCUGCACUGGGCTT
siSOD1-40	10	ccagtgcagggcatcatca	278	CCAGUGCAGGGCAUCAUCATT	546	UGAUGAUGCCCUGCACUGGTT
siSOD1-41	11	cagtgcagggcatcatcaa	279	CAGUGCAGGGCAUCAUCAATT	547	UUGAUGAUGCCCUGCACUGTT
siSOD1-42	12	agtgcagggcatcatcaat	280	AGUGCAGGGCAUCAUCAAUTT	548	AUUGAUGAUGCCCUGCACUTT
siSOD1-43	13	gtgcagggcatcatcaatt	281	GUGCAGGGCAUCAUCAAUUTT	549	AAUUGAUGAUGCCCUGCACTT
siSOD1-44	14	tgcagggcatcatcaattt	282	UGCAGGGCAUCAUCAAUUUTT	550	AAAUUGAUGAUGCCCUGCATT
siSOD1-45	15	gcagggcatcatcaatttc	283	GCAGGGCAUCAUCAAUUUCTT	551	GAAAUUGAUGAUGCCCUGCTT
siSOD1-46	16	cagggcatcatcaatttcg	284	CAGGGCAUCAUCAAUUUCGTT	552	CGAAAUUGAUGAUGCCCUGTT
siSOD1-47	17	agggcatcatcaatttcga	285	AGGGCAUCAUCAAUUUCGATT	553	UCGAAAUUGAUGAUGCCCUTT
siSOD1-50	18	gcatcatcaatttcgagca	286	GCAUCAUCAAUUUCGAGCATT	554	UGCUCGAAAUUGAUGAUGCTT
siSOD1-51	19	catcatcaatttcgagcag	287	CAUCAUCAAUUUCGAGCAGTT	555	CUGCUCGAAAUUGAUGAUGTT
siSOD1-52	20	atcatcaatttcgagcaga	288	AUCAUCAAUUUCGAGCAGATT	556	UCUGCUCGAAAUUGAUGAUTT
siSOD1-53	21	tcatcaatttcgagcagaa	289	UCAUCAAUUUCGAGCAGAATT	557	UUCUGCUCGAAAUUGAUGATT
siSOD1-56	22	tcaatttcgagcagaagga	290	UCAAUUUCGAGCAGAAGGATT	558	UCCUUCUGCUCGAAAUUGATT
siSOD1-57	23	caatttcgagcagaaggaa	291	CAAUUUCGAGCAGAAGGAATT	559	UUCCUUCUGCUCGAAAUUGTT
siSOD1-58	24	aatttcgagcagaaggaaa	292	AAUUUCGAGCAGAAGGAAATT	560	UUUCCUUCUGCUCGAAAUUTT
siSOD1-59	25	atttcgagcagaaggaaag	293	AUUUCGAGCAGAAGGAAAGTT	561	CUUUCCUUCUGCUCGAAAUTT
siSOD1-60	26	tttcgagcagaaggaaagt	294	UUUCGAGCAGAAGGAAAGUTT	562	ACUUUCCUUCUGCUCGAAATT
siSOD1-61	27	ttcgagcagaaggaaagta	295	UUCGAGCAGAAGGAAAGUATT	563	UACUUUCCUUCUGCUCGAATT
siSOD1-62	28	tcgagcagaaggaaagtaa	296	UCGAGCAGAAGGAAAGUAATT	564	UUACUUUCCUUCUGCUCGATT

siSOD1-63	29	cgagcagaaggaaagtaat	297	CGAGCAGAAGGAAAGUAAUTT	565	AUUACUUUCCUUCUGCUCGTT
siSOD1-64	30	gagcagaaggaaagtaatg	298	GAGCAGAAGGAAAGUAAUGTT	566	CAUUACUUUCCUUCUGCUCTT
siSOD1-66	31	gcagaaggaaagtaatgga	299	GCAGAAGGAAAGUAAUGGATT	567	UCCAUUACUUUCCUUCUGCTT
siSOD1-69	32	gaaggaaagtaatggacca	300	GAAGGAAAGUAAUGGACCATT	568	UGGUCCAUUACUUUCCUUCTT
siSOD1-72	33	ggaaagtaatggaccagtg	301	GGAAAGUAAUGGACCAGUGTT	569	CACUGGUCCAUUACUUUCCTT
siSOD1-73	34	gaaagtaatggaccagtga	302	GAAAGUAAUGGACCAGUGATT	570	UCACUGGUCCAUUACUUUCTT
siSOD1-74	35	aaagtaatggaccagtgaa	303	AAAGUAAUGGACCAGUGAATT	571	UUCACUGGUCCAUUACUUUTT
siSOD1-75	36	aagtaatggaccagtgaag	304	AAGUAAUGGACCAGUGAAGTT	572	CUUCACUGGUCCAUUACUUTT
siSOD1-77	37	gtaatggaccagtgaaggt	305	GUAAUGGACCAGUGAAGGUTT	573	ACCUUCACUGGUCCAUUACTT
siSOD1-79	38	aatggaccagtgaaggtgt	306	AAUGGACCAGUGAAGGUGUTT	574	ACACCUUCACUGGUCCAUUTT
siSOD1-80	39	atggaccagtgaaggtgtg	307	AUGGACCAGUGAAGGUGUGTT	575	CACACCUUCACUGGUCCAUTT
siSOD1-85	40	ccagtgaaggtgtgggaa	308	CCAGUGAAGGUGUGUGGGAATT	576	UUCCCCACACCUUCACUGGTT
siSOD1-88	41	gtgaaggtgtgggaagca	309	GUGAAGGUGUGUGGGGAAGCATT	577	UGCUUCCCCACACCUUCACTT
siSOD1-89	42	tgaaggtgtgggaagcat	310	UGAAGGUGUGUGGGGAAGCAUTT	578	AUGCUUCCCCACACCUUCATT
siSOD1-90	43	gaaggtgtgggaagcatt	311	GAAGGUGUGUGGGGAAGCAUUTT	579	AAUGCUUCCCCACACCUUCTT
siSOD1-91	44	aaggtgtgggaagcatta	312	AAGGUGUGUGGGGAAGCAUUATT	580	UAAUGCUUCCCCACACCUUTT
siSOD1-92	45	aggtgtgggaagcattaa	313	AGGUGUGUGGGGAAGCAUUAATT	581	UUAAUGCUUCCCCACACCUTT
siSOD1-93	46	ggtgtgggaagcattaaa	314	GGUGUGUGGGGAAGCAUUAAATT	582	UUUAAUGCUUCCCCACACCTT
siSOD1-94	47	gtgtgggaagcattaaag	315	GUGUGUGGGGAAGCAUUAAAGTT	583	CUUUAAUGCUUCCCCACACTT
siSOD1-95	48	tgtgggaagcattaaagg	316	UGUGGGGAAGCAUUAAAGGTT	584	CCUUUAAUGCUUCCCCACATT
siSOD1-96	49	gtgggaagcattaaagga	317	GUGGGGAAGCAUUAAAGGATT	585	UCCUUUAAUGCUUCCCCACTT
siSOD1-98	50	ggggaagcattaaaggact	318	GGGGAAGCAUUAAAGGACUTT	586	AGUCCUUUAAUGCUUCCCCTT
siSOD1-99	51	gggaagcattaaaggactg	319	GGGAAGCAUUAAAGGACUGTT	587	CAGUCCUUUAAUGCUUCCCTT
siSOD1-100	52	ggaagcattaaaggactga	320	GGAAGCAUUAAAGGACUGATT	588	UCAGUCCUUUAAUGCUUCCTT
siSOD1-102	53	aagcattaaaggactgact	321	AAGCAUUAAAGGACUGACUTT	589	AGUCAGUCCUUUAAUGCUUTT
siSOD1-104	54	gcattaaaggactgactga	322	GCAUUAAAGGACUGACUGATT	590	UCAGUCAGUCCUUUAAUGCTT
siSOD1-105	55	cattaaaggactgactgaa	323	CAUUAAAGGACUGACUGAATT	591	UUCAGUCAGUCCUUUAAUGTT
siSOD1-106	56	attaaaggactgactgaag	324	AUUAAAGGACUGACUGAAGTT	592	CUUCAGUCAGUCCUUUAAUTT
siSOD1-107	57	ttaaaggactgactgaagg	325	UUAAAGGACUGACUGAAGGTT	593	CCUUCAGUCAGUCCUUUAATT
siSOD1-108	58	taaaggactgactgaaggc	326	UAAAGGACUGACUGAAGGCTT	594	GCCUUCAGUCAGUCCUUUATT
siSOD1-114	59	actgactgaaggcctgcat	327	ACUGACUGAAGGCCUGCAUTT	595	AUGCAGGCCUUCAGUCAGUTT
siSOD1-117	60	gactgaaggcctgcatgga	328	GACUGAAGGCCUGCAUGGATT	596	UCCAUGCAGGCCUUCAGUCTT

siSOD1-118	61	actgaaggcctgcatggat	329	ACUGAAGGcCUGCAUGGAUTT	597	AUCCAUGCAGGCCUUCAGUTT
siSOD1-119	62	ctgaaggcctgcatggatt	330	CUGAAGGCCUGCAUGGAUUTT	598	AAUCCAUGCAGGCCUUCAGTT
siSOD1-120	63	tgaaggcctgcatggattc	331	UGAAGGCCUGCAUGGAUUCTT	599	GAAUCCAUGCAGGCCUUCATT
siSOD1-122	64	aaggcctgcatggattcca	332	AAGGCCUGCAUGGAUUCCATT	600	UGGAAUCCAUGCAGGCCUUTT
siSOD1-123	65	aggcctgcatggattccat	333	AGGCCUGCAUGGAUUCCAUTT	601	AUGGAAUCCAUGCAGGCCUTT
siSOD1-125	66	gcctgcatggattccatgt	334	GCCUGCAUGGAUUCCAUGUTT	602	ACAUGGAAUCCAUGCAGGCTT
siSOD1-126	67	cctgcatggattccatgtt	335	CCUGCAUGGAUUCCAUGUUTT	603	AACAUGGAAUCCAUGCAGGTT
siSOD1-127	68	ctgcatggattccatgttc	336	CUGCAUGGAUUCCAUGUUCTT	604	GAACAUGGAAUCCAUGCAGTT
siSOD1-128	69	tgcatggattccatgttca	337	UGCAUGGAUUCCAUGUUCATT	605	UGAACAUGGAAUCCAUGCATT
siSOD1-129	70	gcatggattccatgttcat	338	GCAUGGAUUCCAUGUUCAUTT	606	AUGAACAUGGAAUCCAUGCTT
siSOD1-131	71	atggattccatgttcatga	339	AUGGAUUCCAUGUUCAUGATT	607	UCAUGAACAUGGAAUCCAUTT
siSOD1-132	72	tggattccatgttcatgag	340	UGGAUUCCAUGUUCAUGAGTT	608	CUCAUGAACAUGGAAUCCATT
siSOD1-133	73	ggattccatgttcatgagt	341	GGAUUCCAUGUUCAUGAGUTT	609	ACUCAUGAACAUGGAAUCCTT
siSOD1-134	74	gattccatgttcatgagtt	342	GAUUCCAUGUUCAUGAGUUTT	610	AACUCAUGAACAUGGAAUCTT
siSOD1-137	75	tccatgttcatgagtttgg	343	UCCAUGUUCAUGAGUUUGGTT	611	CCAAACUCAUGAACAUGGATT
siSOD1-138	76	ccatgttcatgagtttgga	344	CCAUGUUCAUGAGUUUGGATT	612	UCCAAACUCAUGAACAUGGTT
siSOD1-140	77	atgttcatgagtttggaga	345	AUGUUCAUGAGUUUGGAGATT	613	UCUCCAAACUCAUGAACAUTT
siSOD1-141	78	tgttcatgagtttggagat	346	UGUUCAUGAGUUUGGAGAUTT	614	AUCUCCAAACUCAUGAACATT
siSOD1-142	79	gttcatgagtttggagata	347	GUUCAUGAGUUUGGAGAUATT	615	UAUCUCCAAACUCAUGAACTT
siSOD1-148	80	gagtttggagataatacag	348	GAGUUUGGAGAUAAUACAGTT	616	CUGUAUUAUCUCCAAACUCTT
siSOD1-150	81	gtttggagataatacagca	349	GUUUGGAGAUAAUACAGCATT	617	UGCUGUAUUAUCUCCAAACTT
siSOD1-154	82	ggagataatacagcaggct	350	GGAGAUAAUACAGCAGGCUTT	618	AGCCUGCUGUAUUAUCUCCTT
siSOD1-156	83	agataatacagcaggctgt	351	AGAUAAUACAGCAGGCUGUTT	619	ACAGCCUGCUGUAUUAUCUTT
siSOD1-157	84	gataatacagcaggctgta	352	GAUAAUACAGCAGGCUGUATT	620	UACAGCCUGCUGUAUUAUCTT
siSOD1-158	85	ataatacagcaggctgtac	353	AUAAUACAGCAGGCUGUACTT	621	GUACAGCCUGCUGUAUUAUTT
siSOD1-159	86	taatacagcaggctgtacc	354	UAAUACAGCAGGCUGUACCTT	622	GGUACAGCCUGCUGUAUUATT
siSOD1-160	87	aatacagcaggctgtacca	355	AAUACAGCAGGCUGUACCATT	623	UGGUACAGCCUGCUGUAUUTT
siSOD1-162	88	tacagcaggctgtaccagt	356	UACAGCAGGCUGUACCAGUTT	624	ACUGGUACAGCCUGCUGUATT
siSOD1-163	89	acagcaggctgtaccagtg	357	ACAGCAGGCUGUACCAGUGTT	625	CACUGGUACAGCCUGCUGUTT
siSOD1-165	90	agcaggctgtaccagtgca	358	AGCAGGCUGUACCAGUGCATT	626	UGCACUGGUACAGCCUGCUTT
siSOD1-171	91	ctgtaccagtgcaggtcct	359	CUGUACCAGUGCAGGUCCUTT	627	AGGACCUGCACUGGUACAGTT
siSOD1-173	92	gtaccagtgcaggtcctca	360	GUACCAGUGCAGGUCCUCATT	628	UGAGGACCUGCACUGGUACTT

siSOD1-175	93	accagtgcaggtcctcact	361	ACCAGUGCAGGUCCUCACUTT	629	AGUGAGGACCUGCACUGGUTT
siSOD1-176	94	ccagtgcaggtcctcactt	362	CCAGUGCAGGUCCUCACUUTT	630	AAGUGAGGACCUGCACUGGTT
siSOD1-177	95	cagtgcaggtcctcacttt	363	CAGUGCAGGUCCUCACUUUTT	631	AAAGUGAGGACCUGCACUGTT
siSOD1-178	96	agtgcaggtcctcactta	364	AGUGCAGGUCCUCACUUUATT	632	UAAAGUGAGGACCUGCACUTT
siSOD1-179	97	gtgcaggtcctcacttaa	365	GUGCAGGUCCUCACUUUAATT	633	UUAAAGUGAGGACCUGCACTT
siSOD1-180	98	tgcaggtcctcactttaat	366	UGCAGGUCCUCACUUUAAUTT	634	AUUAAAGUGAGGACCUGCATT
siSOD1-181	99	gcaggtcctcactttaatc	367	GCAGGUCCUCACUUUAAUCTT	635	GAUUAAAGUGAGGACCUGCTT
siSOD1-182	100	caggtcctcactttaatcc	368	CAGGUCCUCACUUUAAUCCTT	636	GGAUUAAAGUGAGGACCUGTT
siSOD1-183	101	aggtcctcactttaatcct	369	AGGUCCUCACUUUAAUCCUTT	637	AGGAUUAAAGUGAGGACCUTT
siSOD1-184	102	ggtcctcactttaatcctc	370	GGUCCUCACUUUAAUCCUCTT	638	GAGGAUUAAAGUGAGGACCTT
siSOD1-185	103	gtcctcactttaatcctct	371	GUCCUCACUUUAAUCCUCUTT	639	AGAGGAUUAAAGUGAGGACTT
siSOD1-186	104	tcctcactttaatcctcta	372	UCCUCACUUUAAUCCUCUATT	640	UAGAGGAUUAAAGUGAGGATT
siSOD1-187	105	cctcactttaatcctctat	373	CCUCACUUUAAUCCUCUAUTT	641	AUAGAGGAUUAAAGUGAGGTT
siSOD1-188	106	ctcactttaatcctctatc	374	CUCACUUUAAUCCUCUAUCTT	642	GAUAGAGGAUUAAAGUGAGTT
siSOD1-189	107	tcactttaatcctctatcc	375	UCACUUUAAUCCUCUAUCCTT	643	GGAUAGAGGAUUAAAGUGATT
siSOD1-190	108	cactttaatcctctatcca	376	CACUUUAAUCCUCUAUCCATT	644	UGGAUAGAGGAUUAAAGUGTT
siSOD1-192	109	ctttaatcctctatccaga	377	CUUUAAUCCUCUAUCCAGATT	645	UCUGGAUAGAGGAUUAAAGTT
siSOD1-196	110	aatcctctatccagaaaac	378	AAUCCUCUAUCCAGAAAACTT	646	GUUUUCUGGAUAGAGGAUUTT
siSOD1-197	111	atcctctatccagaaaaca	379	AUCCUCUAUCCAGAAAACATT	647	UGUUUUCUGGAUAGAGGAUTT
siSOD1-198	112	tcctctatccagaaaacac	380	UCCUCUAUCCAGAAAACACTT	648	GUGUUUUCUGGAUAGAGGATT
siSOD1-201	113	tctatccagaaaacacggt	381	UCUAUCCAGAAAACACGGUTT	649	ACCGUGUUUUCUGGAUAGATT
siSOD1-208	114	agaaaacacggtgggccaa	382	AGAAAACACGGUGGGCCAATT	650	UUGGCCCACCGUGUUUUCUTT
siSOD1-209	115	gaaaacacggtgggccaaa	383	GAAAACACGGUGGGCCAAATT	651	UUUGGCCCACCGUGUUUUCTT
siSOD1-210	116	aaaacacggtgggccaaag	384	AAAACACGGUGGGCCAAAGTT	652	CUUUGGCCCACCGUGUUUUTT
siSOD1-211	117	aaacacggtgggccaaagg	385	AAACACGGUGGGCCAAAGGTT	653	CCUUUGGCCCACCGUGUUUTT
siSOD1-212	118	aacacggtgggccaaagga	386	AACACGGUGGGCCAAAGGATT	654	UCCUUUGGCCCACCGUGUUTT
siSOD1-213	119	acacggtgggccaaaggat	387	ACACGGUGGGCCAAAGGAUTT	655	AUCCUUUGGCCCACCGUGUTT
siSOD1-215	120	acggtgggccaaaggatga	388	ACGGUGGGCCAAAGGAUGATT	656	UCAUCCUUUGGCCCACCGUTT
siSOD1-216	121	cggtgggccaaaggatgaa	389	CGGUGGGCCAAAGGAUGAATT	657	UUCAUCCUUUGGCCCACCGTT
siSOD1-218	122	gtgggccaaaggatgaaga	390	GUGGGCCAAAGGAUGAAGATT	658	UCUUCAUCCUUUGGCCCACTT
siSOD1-219	123	tgggccaaaggatgaagag	391	UGGGCCAAAGGAUGAAGAGTT	659	CUCUUCAUCCUUUGGCCCATT
siSOD1-220	124	gggccaaaggatgaagaga	392	GGGCCAAAGGAUGAAGAGATT	660	UCUCUUCAUCCUUUGGCCCTT

siSOD1-222	125	gccaaaggatgaagagagg	393	GCCAAAGGAUGAAGAGAGGTT	661	CCUCUCUUCAUCCUUUGGCTT
siSOD1-224	126	caaaggatgaagagaggca	394	CAAAGGAUGAAGAGAGGCATT	662	UGCCUCUCUUCAUCCUUUGTT
siSOD1-225	127	aaaggatgaagagaggcat	395	AAAGGAUGAAGAGAGGCAUTT	663	AUGCCUCUCUUCAUCCUUUTT
siSOD1-227	128	aggatgaagagaggcatgt	396	AGGAUGAAGAGAGGCAUGUTT	664	ACAUGCCUCUCUUCAUCCUTT
siSOD1-228	129	ggatgaagagaggcatgtt	397	GGAUGAAGAGAGGCAUGUUTT	665	AACAUGCCUCUCUUCAUCCTT
siSOD1-229	130	gatgaagagaggcatgttg	398	GAUGAAGAGAGGCAUGUUGTT	666	CAACAUGCCUCUCUUCAUCTT
siSOD1-231	131	tgaagagaggcatgttgga	399	UGAAGAGAGGCAUGUUGGATT	667	UCCAACAUGCCUCUCUUCATT
siSOD1-233	132	aagagaggcatgttggaga	400	AAGAGAGGCAUGUUGGAGATT	668	UCUCCAACAUGCCUCUCUUTT
siSOD1-234	133	agagaggcatgttggagac	401	AGAGAGGCAUGUUGGAGACTT	669	GUCUCCAACAUGCCUCUCUTT
siSOD1-235	134	gagaggcatgttggagact	402	GAGAGGCAUGUUGGAGACUTT	670	AGUCUCCAACAUGCCUCUCTT
siSOD1-236	135	agaggcatgttggagactt	403	AGAGGCAUGUUGGAGACUUTT	671	AAGUCUCCAACAUGCCUCUTT
siSOD1-237	136	gaggcatgttggagacttg	404	GAGGCAUGUUGGAGACUUGTT	672	CAAGUCUCCAACAUGCCUCTT
siSOD1-241	137	catgttggagacttggcca	405	CAUGUUGGAGACUUGGGCATT	673	UGCCCAAGUCUCCAACAUGTT
siSOD1-242	138	atgttggagacttgggcaa	406	AUGUUGGAGACUUGGGCAATT	674	UUGCCCAAGUCUCCAACAUTT
siSOD1-243	139	tgttggagacttgggcaat	407	UGUUGGAGACUUGGGCAAUTT	675	AUUGCCCAAGUCUCCAACATT
siSOD1-244	140	gttggagacttgggcaatg	408	GUUGGAGACUUGGGCAAUGTT	676	CAUUGCCCAAGUCUCCAACTT
siSOD1-245	141	ttggagacttgggcaatgt	409	UUGGAGACUUGGGCAAUGUTT	677	ACAUUGCCCAAGUCUCCAATT
siSOD1-246	142	tggagacttgggcaatgtg	410	UGGAGACUUGGGCAAUGUGTT	678	CACAUUGCCCAAGUCUCCATT
siSOD1-247	143	ggagacttgggcaatgtga	411	GGAGACUUGGGCAAUGUGATT	679	UCACAUUGCCCAAGUCUCCTT
siSOD1-249	144	agacttgggcaatgtgact	412	AGACUUGGGCAAUGUGACUTT	680	AGUCACAUUGCCCAAGUCUTT
siSOD1-252	145	cttgggcaatgtgactgct	413	CUUGGGCAAUGUGACUGCUTT	681	AGCAGUCACAUUGCCCAAGTT
siSOD1-254	146	tgggcaatgtgactgctga	414	UGGGCAAUGUGACUGCUGATT	682	UCAGCAGUCACAUUGCCCATT
siSOD1-256	147	ggcaatgtgactgctgaca	415	GGCAAUGUGACUGCUGACATT	683	UGUCAGCAGUCACAUUGCCTT
siSOD1-257	148	gcaatgtgactgctgacaa	416	GCAAUGUGACUGCUGACAATT	684	UUGUCAGCAGUCACAUUGCTT
siSOD1-258	149	caatgtgactgctgacaaa	417	CAAUGUGACUGCUGACAAATT	685	UUUGUCAGCAGUCACAUUGTT
siSOD1-260	150	atgtgactgctgacaaaga	418	AUGUGACUGCUGACAAAGATT	686	UCUUUGUCAGCAGUCACAUTT
siSOD1-261	151	tgtgactgctgacaaagat	419	UGUGACUGCUGACAAAGAUTT	687	AUCUUUGUCAGCAGUCACATT
siSOD1-262	152	gtgactgctgacaaagatg	420	GUGACUGCUGACAAAGAUGTT	688	CAUCUUUGUCAGCAGUCACTT
siSOD1-263	153	tgactgctgacaaagatgc	421	UGACUGCUGACAAAGAUGCTT	689	GCAUCUUUGUCAGCAGUCATT
siSOD1-264	154	gactgctgacaaagatgct	422	GACUGCUGACAAAGAUGCUTT	690	AGCAUCUUUGUCAGCAGUCTT
siSOD1-266	155	ctgctgacaaagatgctgt	423	CUGCUGACAAAGAUGCUGUTT	691	ACAGCAUCUUUGUCAGCAGTT
siSOD1-270	156	tgacaaagatgctgtggcc	424	UGACAAAGAUGCUGUGGCCTT	692	GGCCACAGCAUCUUUGUCATT

siSOD1-272	157	acaaagatgctgtggccga	425	ACAAAGAUGCUGUGGCCGATT	693	UCGGCCACAGCAUCUUUGUTT
siSOD1-273	158	caaagatgctgtggccgat	426	CAAAGAUGCUGUGGCCGAUTT	694	AUCGGCCACAGCAUCUUUGTT
siSOD1-275	159	aagatgctgtggccgatgt	427	AAGAUGCUGUGGCCGAUGUTT	695	ACAUCGGCCACAGCAUCUUTT
siSOD1-276	160	agatgctgtggccgatgtg	428	AGAUGCUGUGGCCGAUGUGTT	696	CACAUCGGCCACAGCAUCUTT
siSOD1-279	161	tgctgtggccgatgtgtct	429	UGCUGUGGCCGAUGUGUCUTT	697	AGACACAUCGGCCACAGCATT
siSOD1-280	162	gctgtggccgatgtgtcta	430	GCUGUGGCCGAUGUGUCUATT	698	UAGACACAUCGGCCACAGCTT
siSOD1-281	163	ctgtggccgatgtgtctat	431	CUGUGGCCGAUGUGUCUAUTT	699	AUAGACACAUCGGCCACAGTT
siSOD1-282	164	tgtggccgatgtgtctatt	432	UGUGGCCGAUGUGUCUAUUTT	700	AAUAGACACAUCGGCCACATT
siSOD1-283	165	gtggccgatgtgtctattg	433	GUGGCCGAUGUGUCUAUUGTT	701	CAAUAGACACAUCGGCCACTT
siSOD1-284	166	tggccgatgtgtctattga	434	UGGCCGAUGUGUCUAUUGATT	702	UCAAUAGACACAUCGGCCATT
siSOD1-285	167	ggccgatgtgtctattgaa	435	GGCCGAUGUGUCUAUUGAATT	703	UUCAAUAGACACAUCGGCCTT
siSOD1-286	168	gccgatgtgtctattgaag	436	GCCGAUGUGUCUAUUGAAGTT	704	CUUCAAUAGACACAUCGGCTT
siSOD1-287	169	ccgatgtgtctattgaaga	437	CCGAUGUGUCUAUUGAAGATT	705	UCUUCAAUAGACACAUCGGTT
siSOD1-288	170	cgatgtgtctattgaagat	438	CGAUGUGUCUAUUGAAGAUTT	706	AUCUUCAAUAGACACAUCGTT
siSOD1-299	171	ttgaagattctgtgatctc	439	UUGAAGAUUCUGUGAUCUCTT	707	GAGAUCACAGAAUCUUCAATT
siSOD1-300	172	tgaagattctgtgatctca	440	UGAAGAUUCUGUGAUCUCATT	708	UGAGAUCACAGAAUCUUCATT
siSOD1-301	173	gaagattctgtgatctcac	441	GAAGAUUCUGUGAUCUCACTT	709	GUGAGAUCACAGAAUCUUCTT
siSOD1-302	174	aagattctgtgatctcact	442	AAGAUUCUGUGAUCUCACUTT	710	AGUGAGAUCACAGAAUCUUTT
siSOD1-303	175	agattctgtgatctcactc	443	AGAUUCUGUGAUCUCACUCTT	711	GAGUGAGAUCACAGAAUCUTT
siSOD1-304	176	gattctgtgatctcactct	444	GAUUCUGUGAUCUCACUCUTT	712	AGAGUGAGAUCACAGAAUCTT
siSOD1-305	177	attctgtgatctcactctc	445	AUUCUGUGAUCUCACUCUCTT	713	GAGAGUGAGAUCACAGAAUTT
siSOD1-306	178	ttctgtgatctcactctca	446	UUCUGUGAUCUCACUCUCATT	714	UGAGAGUGAGAUCACAGAATT
siSOD1-307	179	tctgtgatctcactctcag	447	UCUGUGAUCUCACUCUCAGTT	715	CUGAGAGUGAGAUCACAGATT
siSOD1-309	180	tgtgatctcactctcagga	448	UGUGAUCUCACUCUCAGGATT	716	UCCUGAGAGUGAGAUCACATT
siSOD1-311	181	tgatctcactctcaggaga	449	UGAUCUCACUCUCAGGAGATT	717	UCUCCUGAGAGUGAGAUCATT
siSOD1-314	182	tctcactctcaggagacca	450	UCUCACUCUCAGGAGACCATT	718	UGGUCUCCUGAGAGUGAGATT
siSOD1-315	183	ctcactctcaggagaccat	451	CUCACUCUCAGGAGACCAUTT	719	AUGGUCUCCUGAGAGUGAGTT
siSOD1-316	184	tcactctcaggagaccatt	452	UCACUCUCAGGAGACCAUUTT	720	AAUGGUCUCCUGAGAGUGATT
siSOD1-317	185	cactctcaggagaccattg	453	CACUCUCAGGAGACCAUUGTT	721	CAAUGGUCUCCUGAGAGUGTT
siSOD1-318	186	actctcaggagaccattgc	454	ACUCUCAGGAGACCAUUGCTT	722	GCAAUGGUCUCCUGAGAGUTT
siSOD1-319	187	ctctcaggagaccattgca	455	CUCUCAGGAGACCAUUGCATT	723	UGCAAUGGUCUCCUGAGAGTT
siSOD1-320	188	tctcaggagaccattgcat	456	UCUCAGGAGACCAUUGCAUTT	724	AUGCAAUGGUCUCCUGAGATT

siSOD1-322	189	tcaggagaccattgcatca	457	UCAGGAGACCAUUGCAUCATT	725	UGAUGCAAUGGUCUCCUGATT
siSOD1-323	190	caggagaccattgcatcat	458	CAGGAGACCAUUGCAUCAUTT	726	AUGAUGCAAUGGUCUCCUGTT
siSOD1-324	191	aggagaccattgcatcatt	459	AGGAGACCAUUGCAUCAUUTT	727	AAUGAUGCAAUGGUCUCCUTT
siSOD1-325	192	ggagaccattgcatcattg	460	GGAGACCAUUGCAUCAUUGTT	728	CAAUGAUGCAAUGGUCUCCTT
siSOD1-326	193	gagaccattgcatcattgg	461	GAGACCAUUGCAUCAUUGGTT	729	CCAAUGAUGCAAUGGUCUCTT
siSOD1-327	194	agaccattgcatcattggc	462	AGACCAUUGCAUCAUUGGCTT	730	GCCAAUGAUGCAAUGGUCUTT
siSOD1-333	195	ttgcatcattggccgcaca	463	UUGCAUCAUUGGCCGCACATT	731	UGUGCGGCCAAUGAUGCAATT
siSOD1-335	196	gcatcattggccgcacact	464	GCAUCAUUGGCCGCACACUTT	732	AGUGUGCGGCCAAUGAUGCTT
siSOD1-338	197	tcattggccgcacactggt	465	UCAUUGGCCGCACACUGGUTT	733	ACCAGUGUGCGGCCAAUGATT
siSOD1-344	198	gccgcacactggtggtcca	466	GCCGCACACUGGUGGUCCATT	734	UGGACCACCAGUGUGCGGCTT
siSOD1-347	199	gcacactggtggtccatga	467	GCACACUGGUGGUCCAUGATT	735	UCAUGGACCACCAGUGUGCTT
siSOD1-348	200	cacactggtggtccatgaa	468	CACACUGGUGGUCCAUGAATT	736	UUCAUGGACCACCAGUGUGTT
siSOD1-349	201	acactggtggtccatgaaa	469	ACACUGGUGGUCCAUGAAATT	737	UUUCAUGGACCACCAGUGUTT
siSOD1-350	202	cactggtggtccatgaaaa	470	CACUGGUGGUCCAUGAAAATT	738	UUUUCAUGGACCACCAGUGTT
siSOD1-351	203	actggtggtccatgaaaaa	471	ACUGGUGGUCCAUGAAAAATT	739	UUUUUCAUGGACCACCAGUTT
siSOD1-352	204	ctggtggtccatgaaaaag	472	CUGGUGGUCCAUGAAAAAGTT	740	CUUUUUCAUGGACCACCAGTT
siSOD1-353	205	tggtggtccatgaaaaagc	473	UGGUGGUCCAUGAAAAAGCTT	741	GCUUUUUCAUGGACCACCATT
siSOD1-354	206	ggtggtccatgaaaaagca	474	GGUGGUCCAUGAAAAAGCATT	742	UGCUUUUUCAUGGACCACCTT
siSOD1-356	207	tggtccatgaaaaagcaga	475	UGGUCCAUGAAAAAGCAGATT	743	UCUGCUUUUUCAUGGACCATT
siSOD1-357	208	ggtccatgaaaaagcagat	476	GGUCCAUGAAAAAGCAGAUTT	744	AUCUGCUUUUUCAUGGACCTT
siSOD1-359	209	tccatgaaaaagcagatga	477	UCCAUGAAAAAGCAGAUGATT	745	UCAUCUGCUUUUUCAUGGATT
siSOD1-360	210	ccatgaaaaagcagatgac	478	CCAUGAAAAAGCAGAUGACTT	746	GUCAUCUGCUUUUUCAUGGTT
siSOD1-361	211	catgaaaaagcagatgact	479	CAUGAAAAAGCAGAUGACUTT	747	AGUCAUCUGCUUUUUCAUGTT
siSOD1-363	212	tgaaaaagcagatgacttg	480	UGAAAAAGCAGAUGACUUGTT	748	CAAGUCAUCUGCUUUUUCATT
siSOD1-365	213	aaaaagcagatgacttggg	481	AAAAAGCAGAUGACUUGGGTT	749	CCCAAGUCAUCUGCUUUUUTT
siSOD1-366	214	aaaagcagatgacttgggc	482	AAAAGCAGAUGACUUGGGCTT	750	GCCCAAGUCAUCUGCUUUUTT
siSOD1-367	215	aaagcagatgacttgggca	483	AAAGCAGAUGACUUGGGCATT	751	UGCCCAAGUCAUCUGCUUUTT
siSOD1-368	216	aagcagatgacttgggcaa	484	AAGCAGAUGACUUGGGCAATT	752	UUGCCCAAGUCAUCUGCUUTT
siSOD1-369	217	agcagatgacttgggcaaa	485	AGCAGAUGACUUGGGCAAATT	753	UUUGCCCAAGUCAUCUGCUTT
siSOD1-370	218	gcagatgacttgggcaaag	486	GCAGAUGACUUGGGCAAAGTT	754	CUUUGCCCAAGUCAUCUGCTT
siSOD1-371	219	cagatgacttgggcaaagg	487	CAGAUGACUUGGGCAAAGGTT	755	CCUUUGCCCAAGUCAUCUGTT
siSOD1-372	220	agatgacttgggcaaaggt	488	AGAUGACUUGGGCAAAGGUTT	756	ACCUUUGCCCAAGUCAUCUTT

siSOD1-375	221	tgacttgggcaaaggtgga	489	UGACUUGGGCAAAGGUGGATT	757	UCCACCUUUGCCCAAGUCATT
siSOD1-376	222	gacttgggcaaaggtggaa	490	GACUUGGGCAAAGGUGGAATT	758	UUCCACCUUUGCCCAAGUCTT
siSOD1-377	223	acttgggcaaaggtggaaa	491	ACUUGGGCAAAGGUGGAAATT	759	UUUCCACCUUUGCCCAAGUTT
siSOD1-378	224	cttgggcaaaggtggaaat	492	CUUGGGCAAAGGUGGAAAUTT	760	AUUUCCACCUUUGCCCAAGTT
siSOD1-379	225	ttgggcaaaggtggaaatg	493	UUGGGCAAAGGUGGAAAUGTT	761	CAUUUCCACCUUUGCCCAATT
siSOD1-380	226	tgggcaaaggtggaaatga	494	UGGGCAAAGGUGGAAAUGATT	762	UCAUUUCCACCUUUGCCCATT
siSOD1-381	227	gggcaaaggtggaaatgaa	495	GGGCAAAGGUGGAAAUGAATT	763	UUCAUUUCCACCUUUGCCCTT
siSOD1-382	228	ggcaaaggtggaaatgaag	496	GGCAAAGGUGGAAAUGAAGTT	764	CUUCAUUUCCACCUUUGCCTT
siSOD1-383	229	gcaaaggtggaaatgaaga	497	GCAAAGGUGGAAAUGAAGATT	765	UCUUCAUUUCCACCUUUGCTT
siSOD1-384	230	caaaggtggaaatgaagaa	498	CAAAGGUGGAAAUGAAGAATT	766	UUCUUCAUUUCCACCUUUGTT
siSOD1-386	231	aaggtggaaatgaagaaag	499	AAGGUGGAAAUGAAGAAAGTT	767	CUUUCUUCAUUUCCACCUUTT
siSOD1-387	232	aggtggaaatgaagaaagt	500	AGGUGGAAAUGAAGAAAGUTT	768	ACUUUCUUCAUUUCCACCUTT
siSOD1-388	233	ggtggaaatgaagaaagta	501	GGUGGAAAUGAAGAAAGUATT	769	UACUUUCUUCAUUUCCACCTT
siSOD1-389	234	gtggaaatgaagaaagtac	502	GUGGAAAUGAAGAAAGUACTT	770	GUACUUUCUUCAUUUCCACTT
siSOD1-397	235	gaagaaagtacaaagacag	503	GAAGAAAGUACAAAGACAGTT	771	CUGUCUUUGUACUUUCUUCTT
siSOD1-399	236	agaaagtacaaagacagga	504	AGAAAGUACAAAGACAGGATT	772	UCCUGUCUUUGUACUUUCUTT
siSOD1-400	237	gaaagtacaaagacaggaa	505	GAAAGUACAAAGACAGGAATT	773	UUCCUGUCUUUGUACUUUCTT
siSOD1-402	238	aagtacaaagacaggaaac	506	AAGUACAAAGACAGGAAACTT	774	GUUUCCUGUCUUUGUACUUTT
siSOD1-405	239	tacaaagacaggaaacgct	507	UACAAAGACAGGAAACGCUTT	775	AGCGUUUCCUGUCUUUGUATT
siSOD1-408	240	aaagacaggaaacgctgga	508	AAAGACAGGAAACGCUGGATT	776	UCCAGCGUUUCCUGUCUUUTT
siSOD1-409	241	aagacaggaaacgctggaa	509	AAGACAGGAAACGCUGGAATT	777	UUCCAGCGUUUCCUGUCUUTT
siSOD1-410	242	agacaggaaacgctggaag	510	AGACAGGAAACGCUGGAAGTT	778	CUUCCAGCGUUUCCUGUCUTT
siSOD1-411	243	gacaggaaacgctggaagt	511	GACAGGAAACGCUGGAAGUTT	779	ACUUCCAGCGUUUCCUGUCTT
siSOD1-412	244	acaggaaacgctggaagtc	512	ACAGGAAACGCUGGAAGUCTT	780	GACUUCCAGCGUUUCCUGUTT
siSOD1-414	245	aggaaacgctggaagtcgt	513	AGGAAACGCUGGAAGUCGUTT	781	ACGACUUCCAGCGUUUCCUTT
siSOD1-415	246	ggaaacgctggaagtcgtt	514	GGAAACGCUGGAAGUCGUUTT	782	AACGACUUCCAGCGUUUCCTT
siSOD1-416	247	gaaacgctggaagtcgttt	515	GAAACGCUGGAAGUCGUUUTT	783	AAACGACUUCCAGCGUUUCTT
siSOD1-417	248	aaacgctggaagtcgtttg	516	AAACGCUGGAAGUCGUUUGTT	784	CAAACGACUUCCAGCGUUUTT
siSOD1-418	249	aacgctggaagtcgtttgg	517	AACGCUGGAAGUCGUUUGGTT	785	CCAAACGACUUCCAGCGUUTT
siSOD1-420	250	cgctggaagtcgtttggct	518	CGCUGGAAGUCGUUUGGCUTT	786	AGCCAAACGACUUCCAGCGTT
siSOD1-421	251	gctggaagtcgtttggctt	519	GCUGGAAGUCGUUUGGCUUTT	787	AAGCCAAACGACUUCCAGCTT
siSOD1-423	252	tggaagtcgtttggcttgt	520	UGGAAGUCGUUUGGCUUGUTT	788	ACAAGCCAAACGACUUCCATT

siSOD1-424	253	ggaagtcgtttggcttgtg	521	GGAAGUCGUUUGGCUUGUGTT	789	CACAAGCCAAACGACUUCCTT
siSOD1-425	254	gaagtcgtttggcttgtgg	522	GAAGUCGUUUGGCUUGUGGTT	790	CCACAAGCCAAACGACUUCTT
siSOD1-426	255	aagtcgtttggcttgtggt	523	AAGUCGUUUGGCUUGUGGUTT	791	ACCACAAGCCAAACGACUUTT
siSOD1-429	256	tcgtttggcttgtggtgta	524	UCGUUUGGCUUGUGGUGUATT	792	UACACCACAAGCCAAACGATT
siSOD1-430	257	cgtttggcttgtggtgtaa	525	CGUUUGGCUUGUGGUGUAATT	793	UUACACCACAAGCCAAACGTT
siSOD1-431	258	gtttggcttgtggtgtaat	526	GUUUGGCUUGUGGUGUAAUTT	794	AUUACACCACAAGCCAAACTT
siSOD1-432	259	tttggcttgtggtgtaatt	527	UUUGGCUUGUGGUGUAAUUTT	795	AAUUACACCACAAGCCAAATT
siSOD1-433	260	ttggcttgtggtgtaattg	528	UUGGCUUGUGGUGUAAUUGTT	796	CAAUUACACCACAAGCCAATT
siSOD1-434	261	tggcttgtggtgtaattgg	529	UGGCUUGUGGUGUAAUUGGTT	797	CCAAUUACACCACAAGCCATT
siSOD1-435	262	ggcttgtggtgtaattggg	530	GGCUUGUGGUGUAAUUGGGTT	798	CCCAAUUACACCACAAGCCTT
siSOD1-436	263	gcttgtggtgtaattggga	531	GCUUGUGGUGUAAUUGGGATT	799	UCCCAAUUACACCACAAGCTT
siSOD1-437	264	cttgtggtgtaattgggat	532	CUUGUGGUGUAAUUGGGAUTT	800	AUCCCAAUUACACCACAAGTT
siSOD1-443	265	gtgtaattgggatcgccca	533	GUGUAAUUGGGAUCGCCCATT	801	UGGGCGAUCCCAAUUACACTT
siSOD1-444	266	tgtaattgggatcgcccaa	534	UGUAAUUGGGAUCGCCCAATT	802	UUGGGCGAUCCCAAUUACATT
siSOD1-445	267	gtaattgggatcgcccaat	535	GUAAUUGGGAUCGCCCAAUTT	803	AUUGGGCGAUCCCAAUUACTT
siSOD1-446	268	taattgggatcgcccaata	536	UAAUUGGGAUCGCCCAAUATT	804	UAUUGGGCGAUCCCAAUUATT
siSOD1-447	269	aattgggatcgcccaataa	537	AAUUGGGAUCGCCCAAUAATT	805	UUAUUGGGCGAUCCCAAUUTT

Note: Target sequence is identical to the identified sense sequence but the nucleotide "U "is converted to "T" and excluding the overhang 2 nucleotides "TT".

WHAT IS CLAIMED IS:

1. A siRNA comprising a sense strand and an antisense strand forming a duplex structure with the sense strand, wherein at least one of the two strands comprises a nucleotide sequence having at least 85% nucleotide sequence complementarity or homology to a portion of a nucleotide molecule of SEQ ID NO: 1, and wherein the siRNA is capable of inhibiting/down-regulating superoxide dismutase 1 (*SOD1*) transcript in a cell.

2. The siRNA of claim 1, wherein said at least one of the two strands has 0, 1, 2, or 3 mismatches to the portion of a nucleotide sequence of SEQ ID NO: 1; and/or
wherein said portion of the nucleotide sequence comprises a sequence selected from any one of SEQ ID NOs: 2-269; and/or
wherein said sense strand has at least 85% nucleotide sequence homology to a sequence selected from any one of SEQ ID NOs: 270-537; and/or
wherein said antisense strand has at least 85% nucleotide sequence homology to a sequence selected form SEQ ID NOs: 538-805; and/or
wherein the siRNA is capable of inhibiting/down-regulating superoxide dismutase 1 (*SOD1*) transcript in a cell by at least 10% as compared to the baseline of *SOD1* mRNA level; and/or
wherein the sense strand comprises at least 10 contiguous nucleotides; and/or
wherein the antisense strand comprises at least 10 contiguous nucleotides; and/or
wherein the sense strand and the antisense strand form a duplex structure which comprises 0, 1, 2, or 3 mismatches.

3. The siRNA of claim 1, wherein the siRNA comprises a sense strand comprising SEQ ID NO: n, and an antisense strand comprising SEQ ID NO: n+268, n is any integer between 270 to 537.

4. The siRNA of claim 1, wherein at least one nucleotide of the siRNA is chemically modified nucleotide located in the sense strand, the antisense strand, or both strands; and/or

wherein the chemically modified nucleotide is a nucleotide modified at the 5' end, 3' end, both ends, or in internal part of the strand(s); and/or

wherein at least 50% nucleotides in the sense strand, the antisense strand, and/or both strands of the siRNA are chemically modified.

5. The siRNA of claim 4, wherein the chemically modified nucleotide is one or more selected from a 2' sugar modification; base modification; a phosphorothioate (PS) backbone modification; an addition of a 5'-phosophate moiety or a 5-methyl cytosine moiety at the 5' end of the nucleotide sequence.

6. The siRNA of claim 5, wherein the 2' sugar modification is one or more selected from: 2'-fluoro-2'-deoxynucleoside (2'-F) modification, 2'-O-methyl (2'-O-Me) modification, and 2'-O-(2-methoxyethyl) (2'-O-MOE) modification; and/or

wherein the addition of a 5'-phosophate moiety is one or more selected from an additional of (E)-vinylphosphonate moiety at the 5' end of the nucleotide sequence.

7. The siRNA of claim 4, wherein the sense strand has at least 85% nucleotide sequence

homology to a sequence selected form SEQ ID NOs: 808-827, 867 with or without the addition of (E)-vinylphosphonate moiety at the 5' end; and/or

wherein the antisense strand has at least 85% nucleotide sequence homology to a sequence selected form SEQ ID NOs: 828-849, 868 with or without the addition of (E)-vinylphosphonate moiety at the 5' end.

8. The siRNA of claim 4, wherein the siRNA comprises a sense strand comprising SEQ ID NO: m, and an antisense strand comprising SEQ ID NO: m+20, m is any integer between 808 to 827; or wherein the siRNA comprises a sense strand comprising SEQ ID NO: 814, and an antisense strand comprising SEQ ID NO: 848; a sense strand comprising SEQ ID NO: 822, and an antisense strand comprising SEQ ID NO: 849; or a sense strand comprising SEQ ID NO: 814, and an antisense strand comprising SEQ ID NO: 866.

9. An oligonucleotide agent, comprising:

(a) a small interfering RNA (siRNA) according to any one of claims 1-8; and

(b) a non-targeting single-stranded oligonucleotide (accessary oligonucleotide, ACO),

wherein the ACO is about 6-22 nucleotides in length, wherein the siRNA and the ACO are covalently linked, without or with one or more linking components, to form the oligonucleotide agent.

10. The oligonucleotide agent according to claim 9, wherein the ACO is composed of one or more of RNA, DNA, BNA, LNA, GNA and PNA.

11. The oligonucleotide agent according to claim 9, wherein the ACO is about 6-18 nucleotides in length.

12. The oligonucleotide agent according to claim 11, wherein the ACO is about 8-16 nucleotides in length.

13. The oligonucleotide agent according to claim 9, wherein the siRNA comprises a sense strand having a length ranging from about 16-25 nucleotides; and/or

wherein the siRNA comprises an antisense strand having a length ranging from about 19-25 nucleotides.

14. The oligonucleotide agent according to claim 9, wherein the oligonucleotide agent capable of inhibiting/down-regulating superoxide dismutase 1 (*SOD1*) transcript in a cell by at least 50%.

15. The oligonucleotide agent according to claim 9, wherein the ACO comprises a 5' end and a 3' end, and wherein the 5' end or the 3' end of the ACO is conjugated to a linking component.

16. The oligonucleotide agent according to claim 9, wherein the sense strand and/or the antisense strand of the siRNA are covalently linked to the ACO by one or more linking components.

17. The oligonucleotide agent according to claim 9, wherein the linking component is one or

more selected from ethylene glycol chain, an alkyl chain, an alkenyl chain, an alkynyl chain, a peptide, RNA, DNA, carbohydrates, thiol linkage, a phosphodiester, a phosphorothioate, a phosphoramidate, an amide, a carbamate, a tetrazole linkage, and a benzimidazole linkage.

18. The oligonucleotide agent according to claim 17, wherein the linking component is one or more selected from:

a) Spacer phosphoramidite 18 (Phosphoramidous acid, N,N-bis(1-methylethyl)-, 19,19-bis(4-methoxyphenyl)-19-phenyl-3,6,9,12,15,18-hexaoxanonadec-1-yl 2-cyanoethyl ester);

b) Spacer-9 (3-[2-[2-[2-[bis(4-methoxyphenyl)-phenylmethoxy]ethoxy]ethoxy]ethoxy-[di(propan-2-yl)amino]phosphanyl]oxypropanenitrile);

c) Spacer phosphoramidite C3 (6-(4,4'-Dimethoxytrityl)hexyl-1-[(2-cyanoethyl)-(N,N-diisopropyl)]- phosphoramidite); and

d) Spacer-C6 Phosphoramidite (6-(4,4'-Dimethoxytrityl)hexyl-1-[(2-cyanoethyl)-(N,N-diisopropyl)]- phosphoramidite)

e) Divalent linker (DIO) 16-((bis(4-methoxyphenyl)(phenyl)methoxy)methyl)-1,1-bis(4-methoxyphenyl)-18-oxo-1-phenyl-2,5,8,11,14,17-hexaoxahenicosan-CPG.

19. The oligonucleotide agent according to claim 9, wherein the ACO is covalently linked to the 3' end, or the 5' end, or both the 3' and 5' ends of the sense strand of the siRNA; and/or

wherein the ACO is covalently linked to the 3' end, or the 5' end, or both the 3' and 5' ends of the antisense strand of the siRNA; and/or

wherein the ACO is covalently linked to one or more of internal nucleotides of the siRNA.

20. The oligonucleotide agent according to claim 9, wherein one, two or more ACOs are covalently linked to the siRNA.

21. The oligonucleotide agent according to any one of claims 9-20, wherein at least one nucleotide of the ACO is chemically modified.

22. The oligonucleotide agent according to any one of claims 9-20, wherein at least about 50% nucleotides of the ACO are chemically modified nucleotides.

23. The oligonucleotide agent according to claim 21, wherein the chemical modification of the at least about one chemically modified nucleotide is a 2' sugar modification selected from one or more of: 2′-fluoro-2′-deoxynucleoside (2′-F) modification, 2′-O-methyl (2′-O-Me), modification, and 2′-O-(2-methoxyethyl) (2′-O-MOE) modification.

24. The oligonucleotide agent according to claim 21, wherein the chemical modification of at least one chemically modified nucleotide is a phosphorothioate (PS) backbone modification.

25. The oligonucleotide agent according to claim 24, wherein the ACO comprises 6~17 phosphorothioate (PS) backbone modifications.

26. The oligonucleotide agent according to claim 13, wherein the antisense strand comprises an addition of an (E)-vinylphosphonate moiety at the 5' end of the nucleotide sequence.

27. The oligonucleotide agent according to claim 21, wherein the chemical modification of the at least about one chemically modified nucleotide is an addition of a 5-methyl cytosine moiety at the 5' end of the nucleotide sequence.

28. The oligonucleotide agent according to claim 10, wherein the ACO and/or the siRNA is conjugated to one or more conjugation groups.

29. The oligonucleotide agent according to claim 38, wherein the sense strand and/or the antisense strand of the siRNA is conjugated to one or more conjugation groups.

30. The oligonucleotide agent according to claim 38, wherein the one or more conjugation groups is selected from: a lipid, a fatty acid, a fluorophore, a ligand, a saccharide, a peptide, and an antibody.

31. The oligonucleotide agent according to claim 38, wherein the one or more conjugation groups is selected from: a cell-penetrating peptide, polyethylene glycol, an alkaloid, a tryptamine, a benzimidazole, a quinolone, an amino acid, a cholesterol, glucose and N-acetylgalactosamine.

32. The oligonucleotide agent according to any one of claims 10-38, wherein the sense strand and the antisense strand of the siRNA have nucleotide sequences that is independently at least about 85% homology to the nucleotide sequence selected from:

a) RD-12926 (SEQ ID NO: 867 and SEQ ID NO: 868),

b) siSOD1-063M1 (SEQ ID NO: 808 and SEQ ID NO: 828),

c) siSOD1-063M2 (SEQ ID NO: 809 and SEQ ID NO: 829),

d) siSOD1-063M3 (SEQ ID NO: 810 and SEQ ID NO: 830),

e) siSOD1-063M4 (SEQ ID NO: 811 and SEQ ID NO: 831),

f) siSOD1-047M1 (SEQ ID NO: 812 and SEQ ID NO: 832),

g) siSOD1-047M2 (SEQ ID NO: 813 and SEQ ID NO: 833),

h) siSOD1-047M3 (SEQ ID NO: 814 and SEQ ID NO: 834),

i) siSOD1-047M4 (SEQ ID NO: 815 and SEQ ID NO: 835),

j) siSOD1-104M1 (SEQ ID NO: 816 and SEQ ID NO: 836),

k) siSOD1-104M2 (SEQ ID NO: 817 and SEQ ID NO: 837),

l) siSOD1-104M3 (SEQ ID NO: 818 and SEQ ID NO: 838),

m) siSOD1-104M4 (SEQ ID NO: 819 and SEQ ID NO: 839),

n) siSOD1-005M1 (SEQ ID NO: 820 and SEQ ID NO: 840),

o) siSOD1-005M2 (SEQ ID NO: 821 and SEQ ID NO: 841),

p) siSOD1-005M3 (SEQ ID NO: 822 and SEQ ID NO: 842),

q) siSOD1-005M4 (SEQ ID NO: 823 and SEQ ID NO: 843),

r) siSOD1-258M1 (SEQ ID NO: 824 and SEQ ID NO: 844),

s) siSOD1-258M2 (SEQ ID NO: 825 and SEQ ID NO: 845),

t) siSOD1-258M3 (SEQ ID NO: 826 and SEQ ID NO: 846),

u) siSOD1-258M4 (SEQ ID NO: 827 and SEQ ID NO: 847),

v) siSOD1-270M3 (SEQ ID NO: 814 and SEQ ID NO: 866)

w) siSOD1-047M3^{VP} (SEQ ID NO: 814 and SEQ ID NO: 848),

x) siSOD1-005M3 ^{VP} (SEQ ID NO: 822 and SEQ ID NO: 847),

y) siSOD1-047M3-AC1 (SEQ ID NO: 850 and SEQ ID NO: 834),

z) siSOD1-005M3-AC1 (SEQ ID NO: 851 and SEQ ID NO: 842),

aa) siCON1-AC1^{VP} (SEQ ID NO: 852 and SEQ ID NO: 858),

bb) siCON2-AC1^{VP} (SEQ ID NO: 853 and SEQ ID NO: 859),

cc) siCON3-AC1^{VP} (SEQ ID NO: 854 and SEQ ID NO: 860),

dd) siSOD1-063M3-AC1^{VP} (SEQ ID NO: 855 and SEQ ID NO: 861),

ee) siSOD1-047M3-AC1^{VP} (SEQ ID NO: 850 and SEQ ID NO: 848),

ff) siSOD1-104M3-AC1^{VP} (SEQ ID NO: 856 and SEQ ID NO: 862),

gg) siSOD1-005M3-AC1^{VP} (SEQ ID NO: 852 and SEQ ID NO: 847),

hh) siSOD1-258M3-AC1^{VP} (SEQ ID NO: 857 and SEQ ID NO: 863), and

ii) siSOD1-270M3-AC1^{vp} (SEQ ID NO: 850 and SEQ ID NO: 866).

33. The oligonucleotide agent according to claim 10, wherein the ACO of the oligonucleotide agent improves the stability, bioavailability, biodistribution, and/or cellular uptake of the siRNA as compared to an oligonucleotide agent without the ACO.

34. The oligonucleotide agent according to claim 10, wherein the ACO of the oligonucleotide agent increases the biodistribution of siRNA within one or two or more target tissues as compared to an oligonucleotide agent without the ACO.

35. The oligonucleotide agent according to claim 44, wherein the target tissue is selected from: prefrontal cortex, cerebrum, cerebellum, spinal cord, muscle, lung, eye, liver and kidney.

36. A vector, comprising the siRNA according to any one of claims 1-9 and/or the oligonucleotide agent according to any one of claims 10-35.

37. A cell, comprising the siRNA according to any one of claims 1-9, the oligonucleotide agent according to any one of claims 10-35 and/or the vector of claim 36.

38. The cell according to claim 37, wherein the cell is a mammalian cell.

39. The cell according to claim 37, wherein the cell is a human cell.

40. The cell according to claim 37, wherein the cell is a host cell *in vitro, in vivo or ex vivo*.

41. A pharmaceutical composition, comprising the siRNA according to any one of claims 1-9, the oligonucleotide agent according to any one of claims 10-35, the vector of claim 36, and/or the cell according to any one of claims 37-40; and a pharmaceutically acceptable carrier.

42. The pharmaceutical composition according to claim 41, wherein the pharmaceutically acceptable carrier is one or more selected from an aqueous carrier, liposome or LNP, polymer, micelle, colloid, metal nanoparticle, non-metallic nanoparticle, bioconjugates, and polypeptide.

43. The pharmaceutical composition according to claim 41, wherein the pharmaceutical composition decreases the transcript level of the *SOD1* gene or SOD1 protein.

44. A kit, comprising the siRNA according to any one of claims 1-9, the oligonucleotide agent according to any one of claims 10-35, the vector of claim 36, the cell according to any one of claims 37-40, and/or the pharmaceutical composition according to any one of claims 41-43.

45. A method of decreasing the transcript level of a *SOD1* gene or SOD1 protein, comprising administering to a subject a pharmaceutical composition according to any one of claims 41-43.

46. A method for treating or delaying the onset or progression of a neurodegenerative disease or symptom associated with *SOD1* gene mutation, abnormal *SOD1* gene expression or abnormal SOD1 protein accumulation in a subject in need thereof, the method comprising: administering to the subject a pharmaceutical composition according to any one of claims 41-43.

47. The method according to claim 46, wherein the neurodegenerative disease or condition is selected from Amyotrophic lateral sclerosis (ALS), Alzheimer's disease (AD), Parkinson's disease (PD), and Down's syndrome (DS).

48. The method according to claim 46, wherein the pharmaceutical composition is administered to the subject intrathecally or intracerebroventricularly.

49. The method according to claim 57, wherein the ACO of the oligonucleotide agent improves the stability, bioavailability, biodistribution, and/or cellular uptake of the siRNA as compared to an oligonucleotide agent without the ACO.

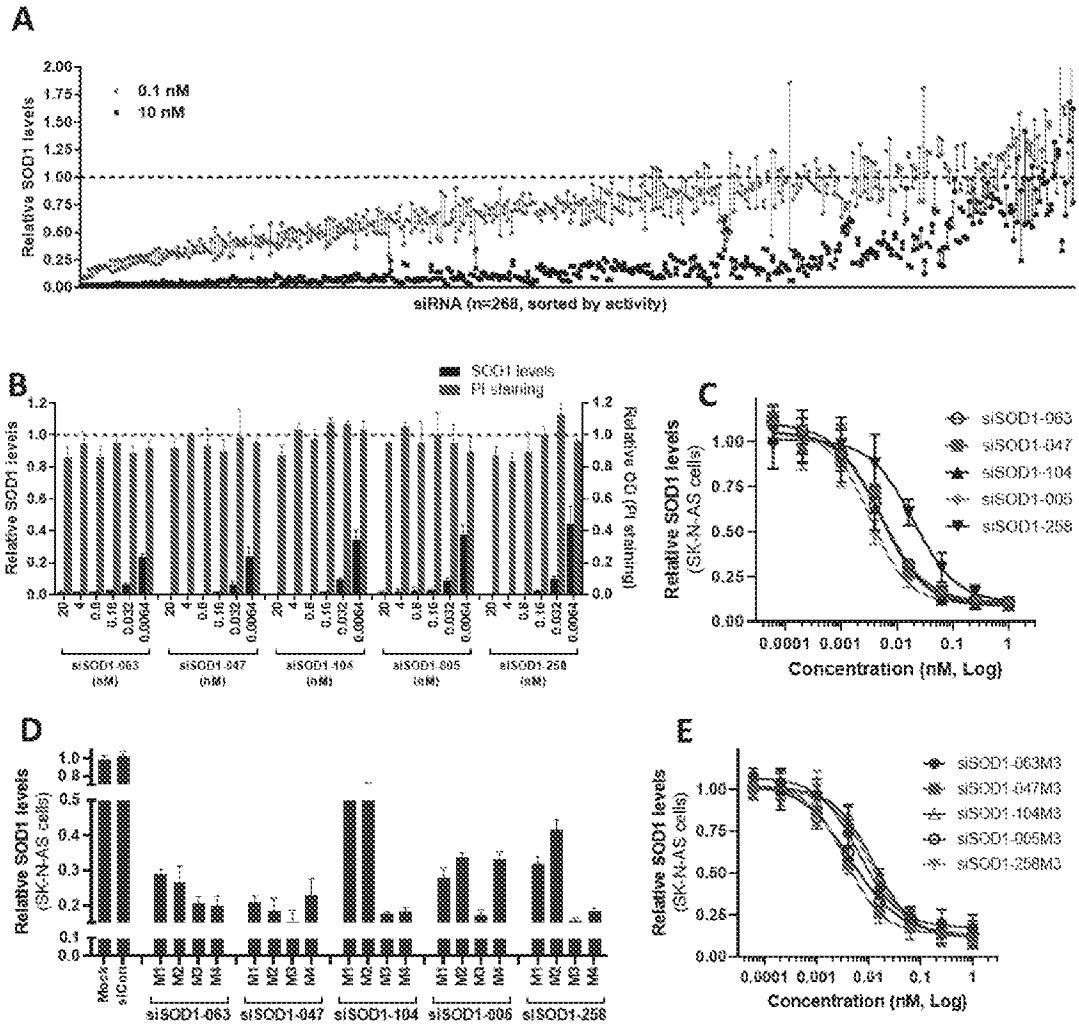
50. A use of the siRNA according to any one of claims 1-9, the oligonucleotide agent according to any one of claims 10-35, the vector of claim 36, the cell according to any one of claims 37-40, and/or the pharmaceutical composition according to any one of claims 41-43 in manufacturing a medicament for treating or delaying the onset or progression of a *SOD1*-associated neurodegenerative disease or symptom.

51. The use according to claim 64, wherein the SOD1-associated neurodegenerative disease or

symptom is selected from Amyotrophic lateral sclerosis (ALS), Alzheimer's disease (AD), Parkinson's disease (PD), and Down's syndrome (DS).

52. The siRNA according to any one of claims 1-9, the oligonucleotide agent according to any one of claims 10-35, the vector of claim 36, the cell according to any one of claims 37-40, and/or the pharmaceutical composition according to any one of claims 41-43 for use in treating or delaying the onset or progression of a SOD1-associated neurodegenerative disease or symptom.

53. The siRNA, oligonucleotide agent, vector, cell and/or pharmaceutical composition for use according to claim 52, wherein the neuro-disease or condition is selected from Amyotrophic lateral sclerosis (ALS), Alzheimer's disease (AD), Parkinson's disease (PD), and Down's syndrome (DS).


A
0.1 nM
10 nM
Relative SOD1 levels
siRNA (n=288, sorted by activity)
B
SOD1 levels
PI staining
Relative SOD1 levels
Relative OD (PI staining)
siSOD1-063
siSOD1-047
siSOD1-104
siSOD1-005
siSOD1-258
C
Relative SOD1 levels (SK-N-AS cells)
Concentration (nM, Log)
D
Relative SOD1 levels (SK-N-AS cells)
Mock
siCon
M1
M2
M3
M4
E
siSOD1-063M3
siSOD1-047M3
siSOD1-104M3
siSOD1-005M3
siSOD1-258M3
Relative SOD1 levels (SK-N-AS cells)
Concentration (nM, Log)

FIGs.1A-1E

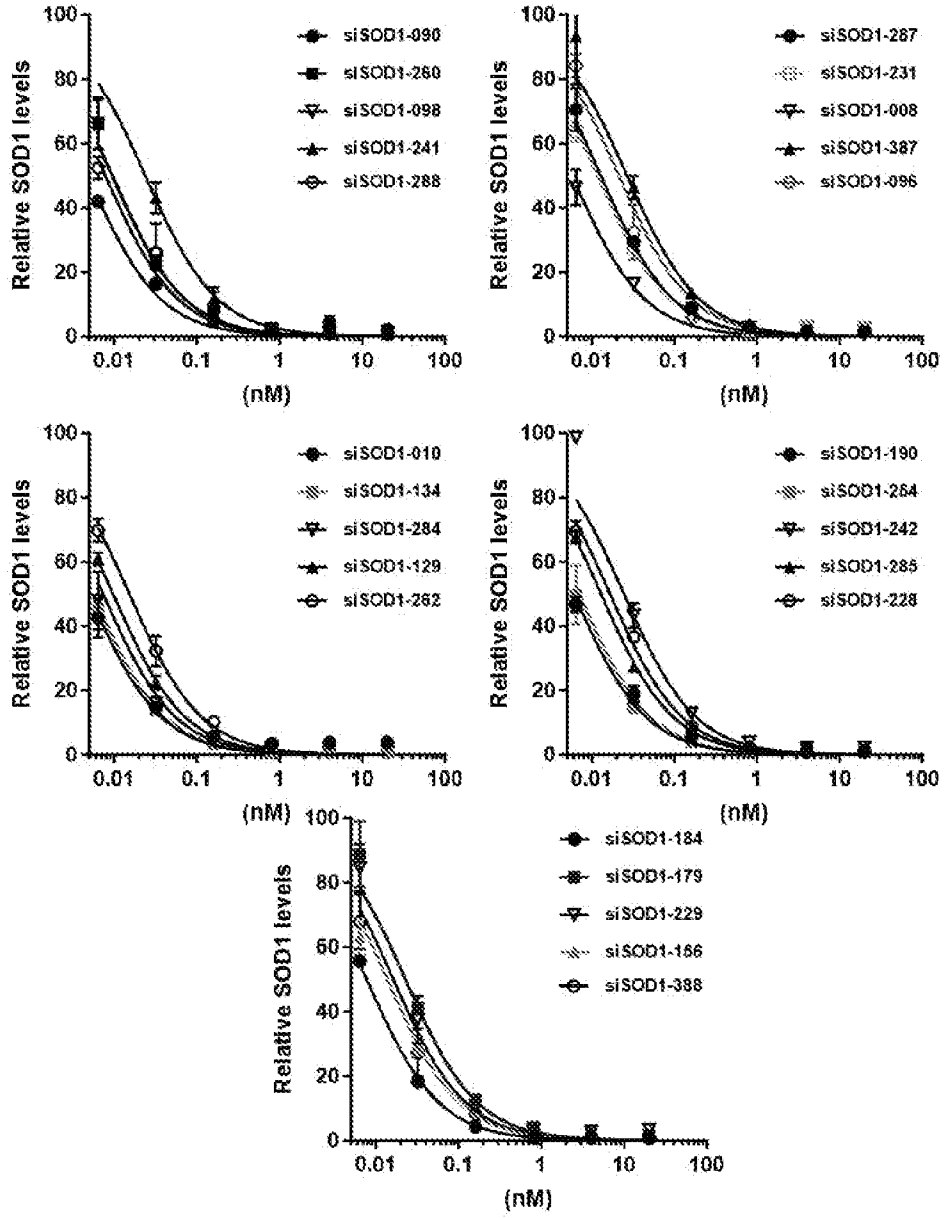

Relative SOD1 levels
(nM)
siSOD1-090
siSOD1-260
siSOD1-098
siSOD1-241
siSOD1-288
siSOD1-287
siSOD1-231
siSOD1-008
siSOD1-387
siSOD1-096
siSOD1-010
siSOD1-134
siSOD1-284
siSOD1-129
siSOD1-282
siSOD1-190
siSOD1-264
siSOD1-242
siSOD1-285
siSOD1-228
siSOD1-184
siSOD1-179
siSOD1-229
siSOD1-166
siSOD1-388

FIG.2A

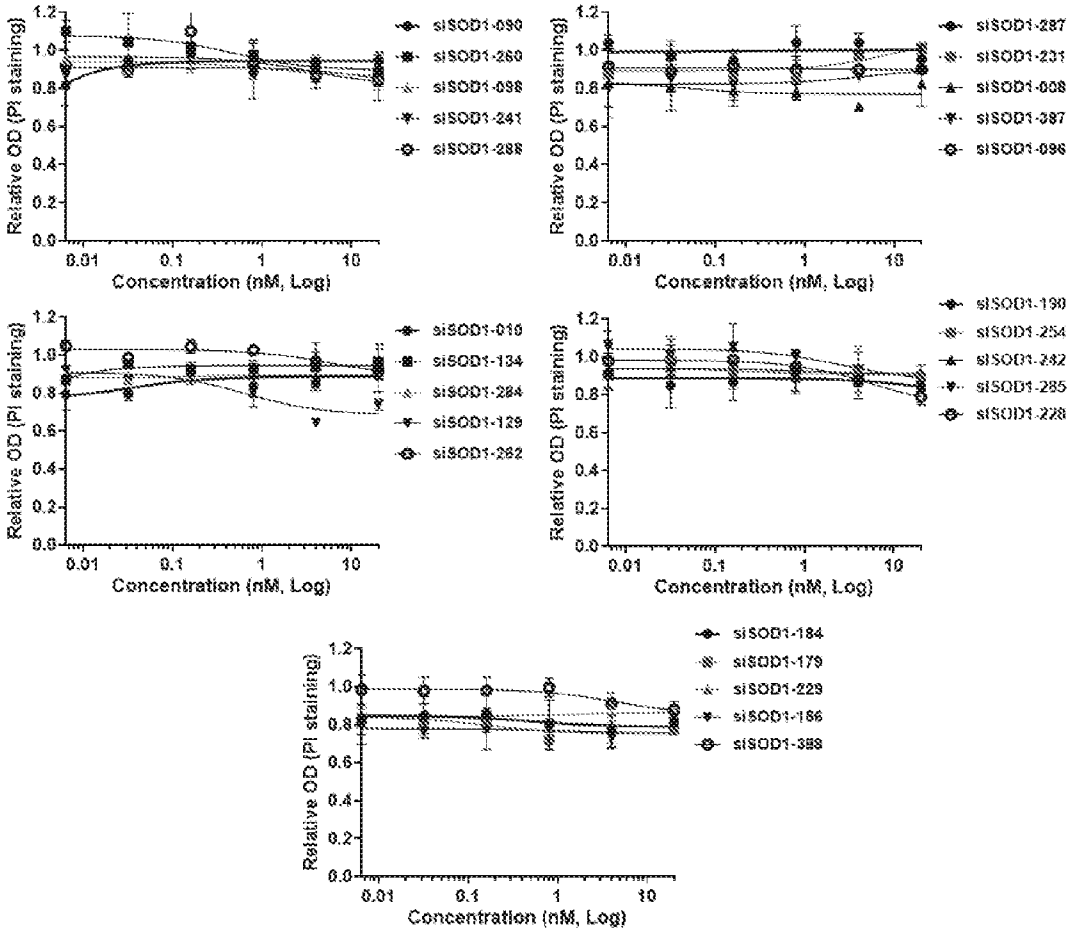

Relative OD (PI staining)
Concentration (nM, Log)
siSOD1-090
siSOD1-260
siSOD1-098
siSOD1-241
siSOD1-288
siSOD1-287
siSOD1-231
siSOD1-008
siSOD1-097
siSOD1-096
siSOD1-016
siSOD1-134
siSOD1-284
siSOD1-129
siSOD1-282
siSOD1-190
siSOD1-254
siSOD1-282
siSOD1-285
siSOD1-228
siSOD1-184
siSOD1-179
siSOD1-229
siSOD1-186
siSOD1-388

FIG.2B

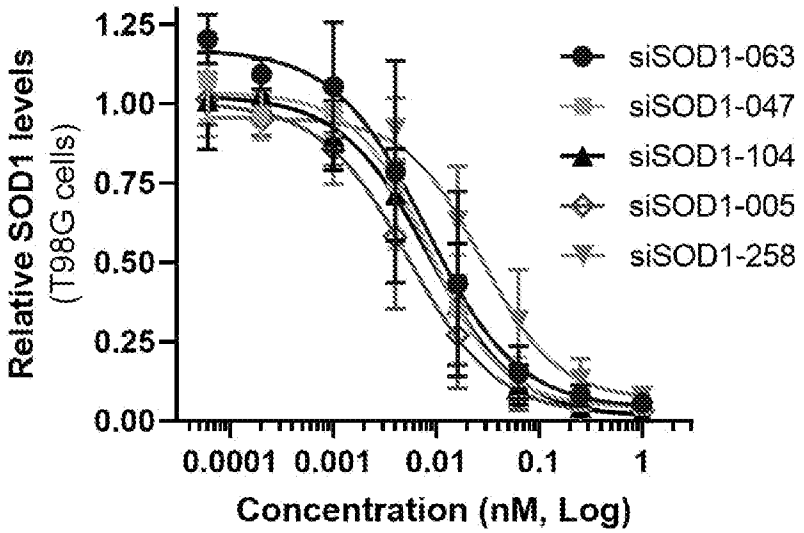

Relative SOD1 levels
(T98G cells)
Concentration (nM, Log)
siSOD1-063
siSOD1-047
siSOD1-104
siSOD1-005
siSOD1-258

FIG.3

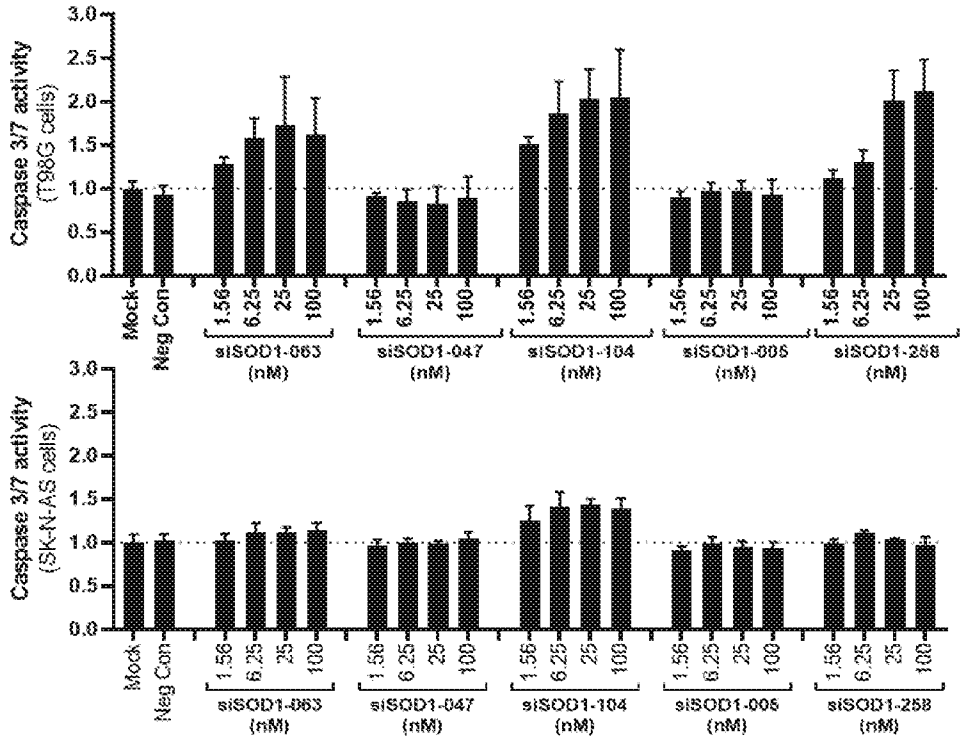

Caspase 3/7 activity (T98G cells)
Caspase 3/7 activity (SK-N-AS cells)
Mock
Neg Con
1.56
6.25
25
100
siSOD1-063 (nM)
siSOD1-047 (nM)
siSOD1-104 (nM)
siSOD1-005 (nM)
siSOD1-258 (nM)

FIG.4A

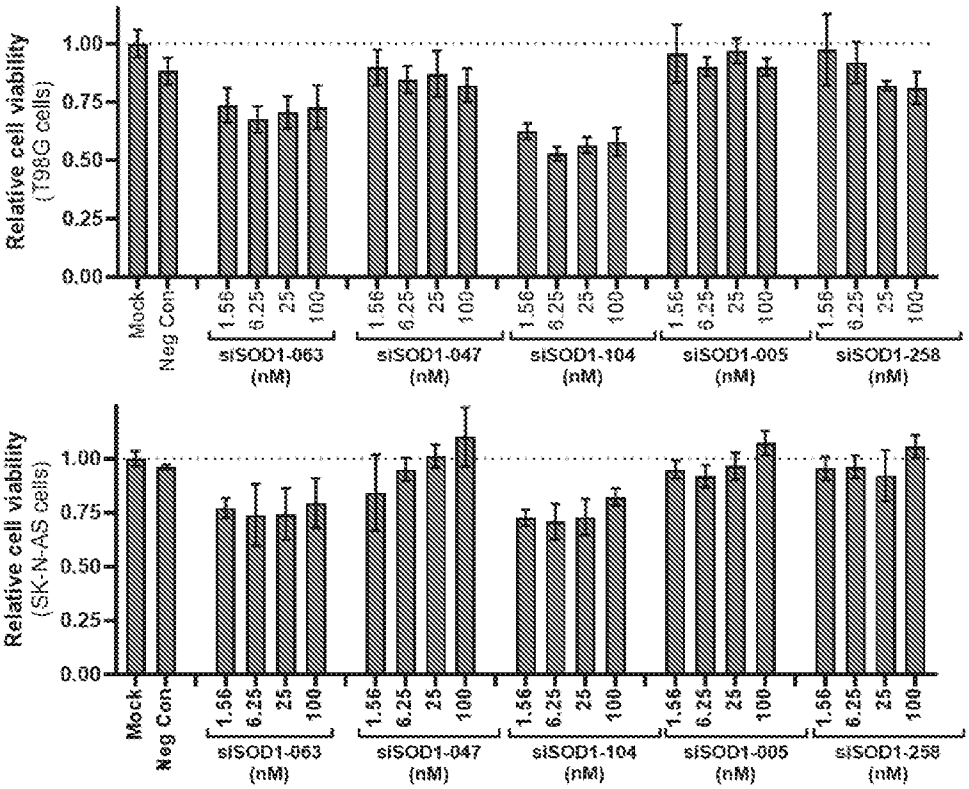

Relative cell viability (T98G cells)
Relative cell viability (SK-N-AS cells)
Mock
Neg Con
1.56
6.25
25
100
siSOD1-063 (nM)
siSOD1-047 (nM)
siSOD1-104 (nM)
siSOD1-005 (nM)
siSOD1-258 (nM)

FIG.4B

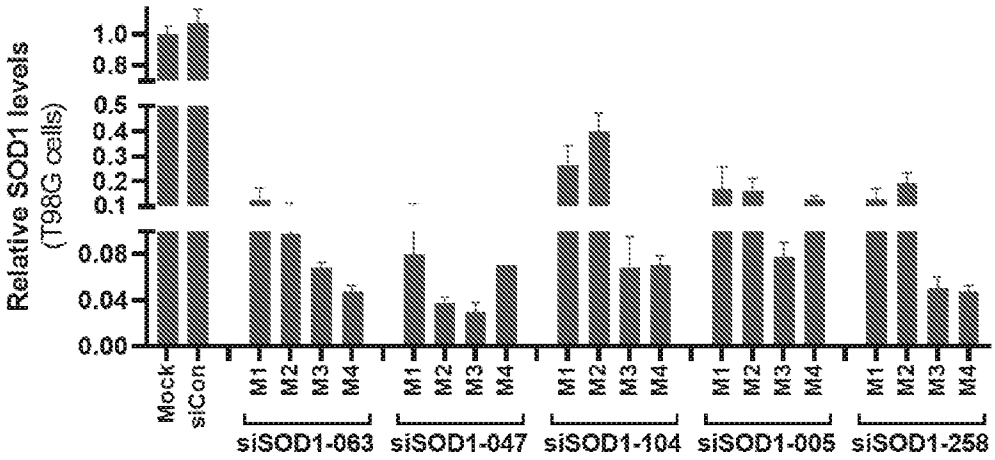

Relative SOD1 levels
(T98G cells)
1.0
0.8
0.5
0.4
0.3
0.2
0.1
0.08
0.04
0.00
Mock
siCon
M1
M2
M3
M4
siSOD1-063
siSOD1-047
siSOD1-104
siSOD1-005
siSOD1-258

FIG.5

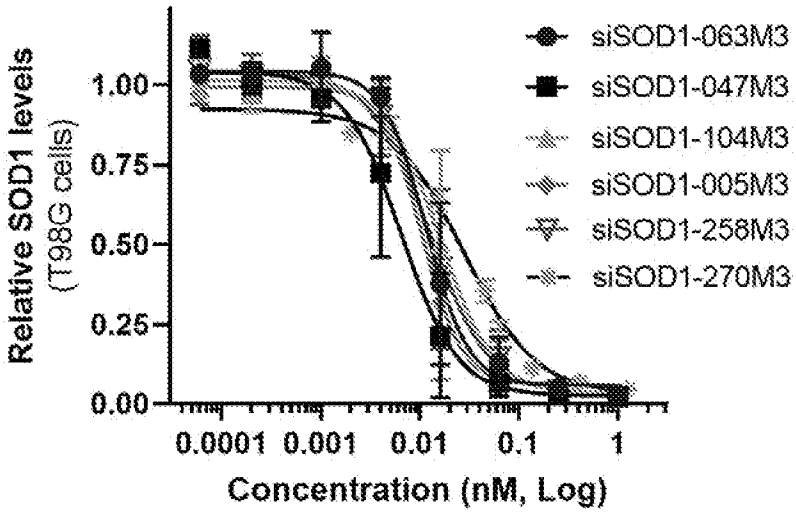
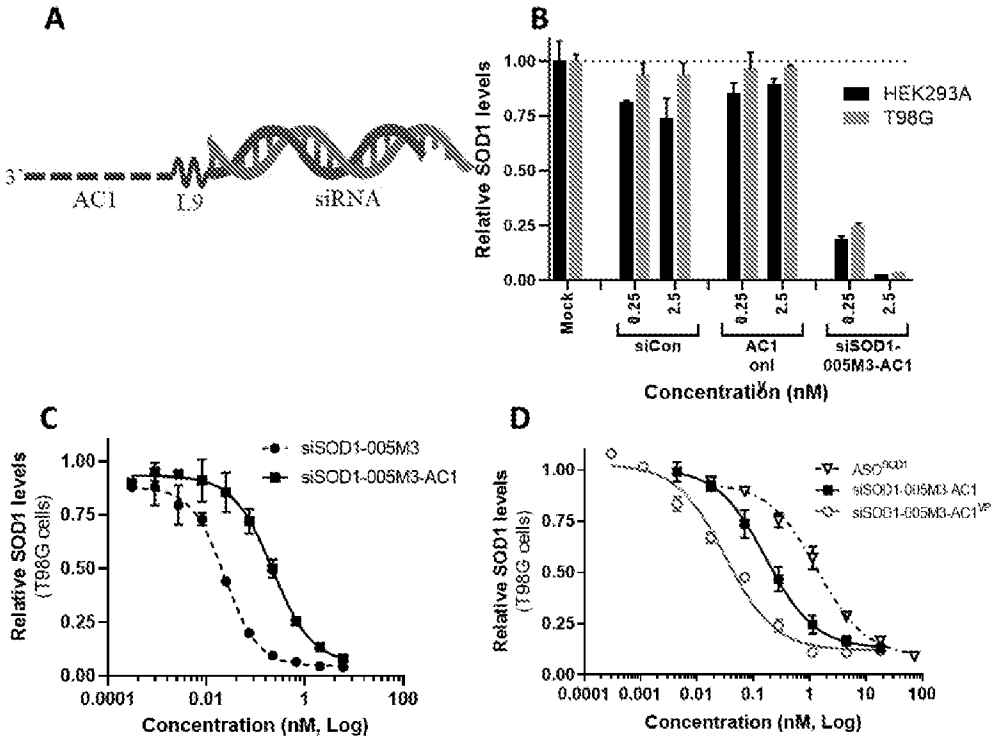
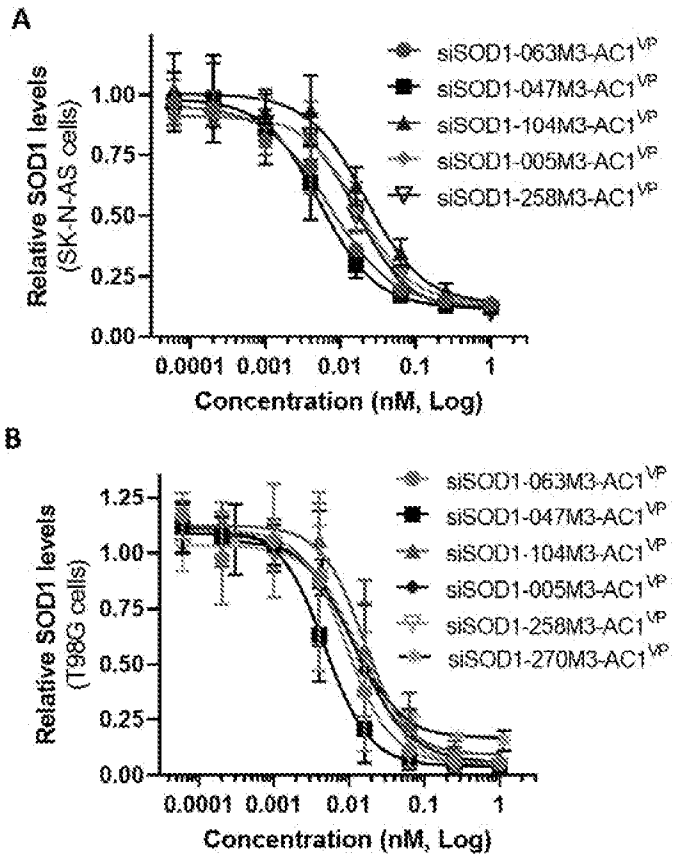

Relative SOD1 levels
(T98G cells)
1.00
0.75
0.50
0.25
0.00
0.0001
0.001
0.01
0.1
1
Concentration (nM, Log)
siSOD1-063M3
siSOD1-047M3
siSOD1-104M3
siSOD1-005M3
siSOD1-258M3
siSOD1-270M3

FIG.6



FIGs.7A-7D



FIGs.8A-8B

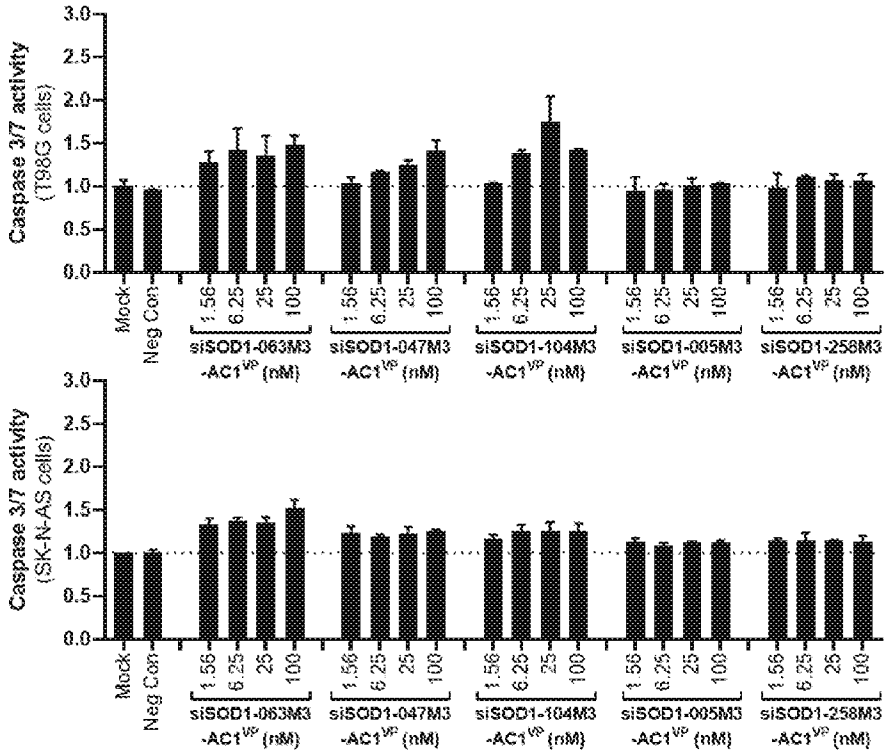

Caspase 3/7 activity
(T98G cells)
Caspase 3/7 activity
(SK-N-AS cells)
Mock
Neg Con
1.56
6.25
25
100
siSOD1-063M3
-AC1VP (nM)
siSOD1-047M3
-AC1VP (nM)
siSOD1-104M3
-AC1VP (nM)
siSOD1-005M3
-AC1VP (nM)
siSOD1-258M3
-AC1VP (nM)

FIG.9A

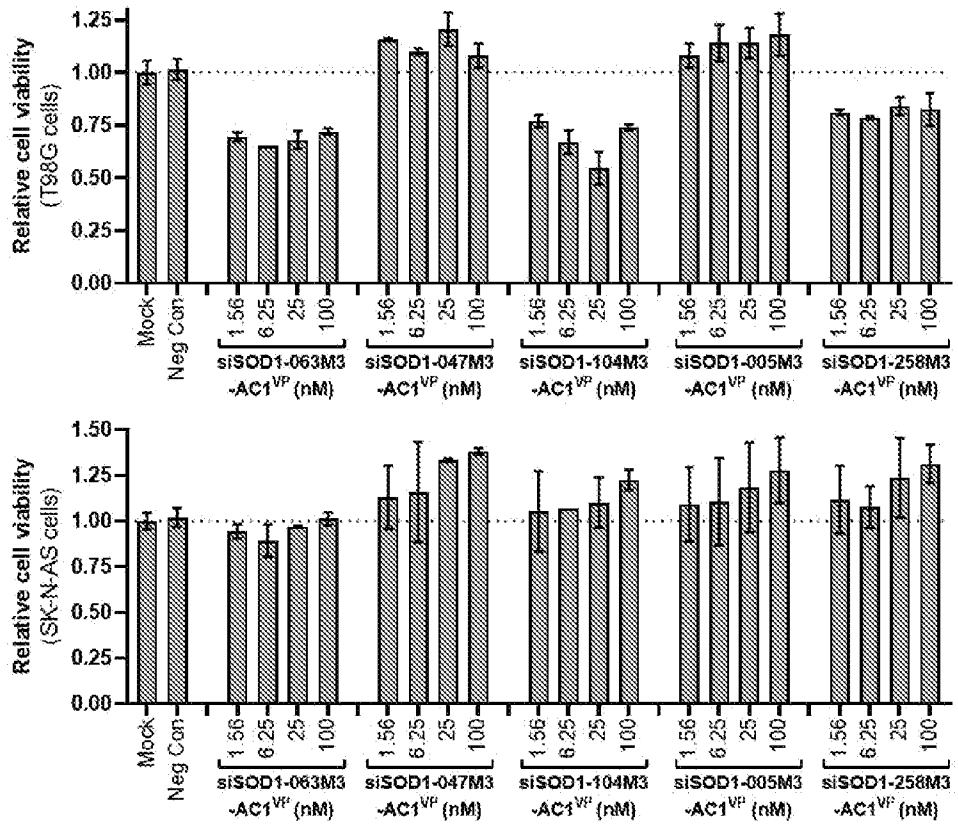

Relative cell viability
(T98G cells)
Relative cell viability
(SK-N-AS cells)
Mock
Neg Con
1.56
6.25
25
100
siSOD1-063M3
-AC1VP (nM)
siSOD1-047M3
-AC1VP (nM)
siSOD1-104M3
-AC1VP (nM)
siSOD1-005M3
-AC1VP (nM)
siSOD1-258M3
-AC1VP (nM)

FIG.9B

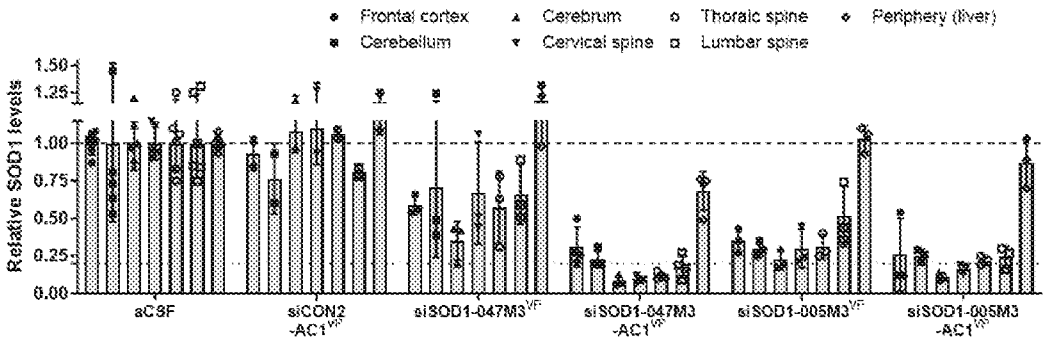
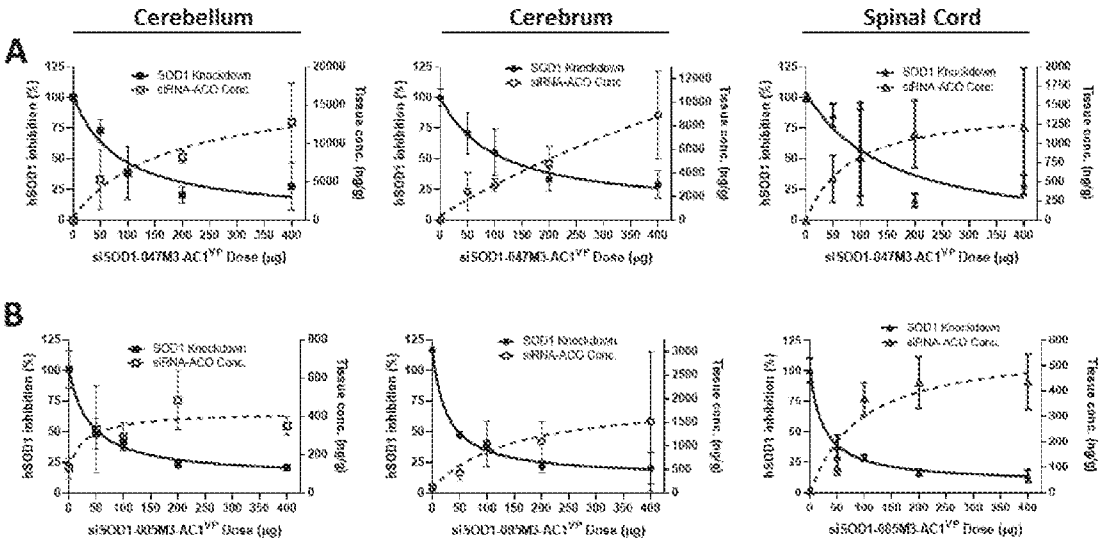
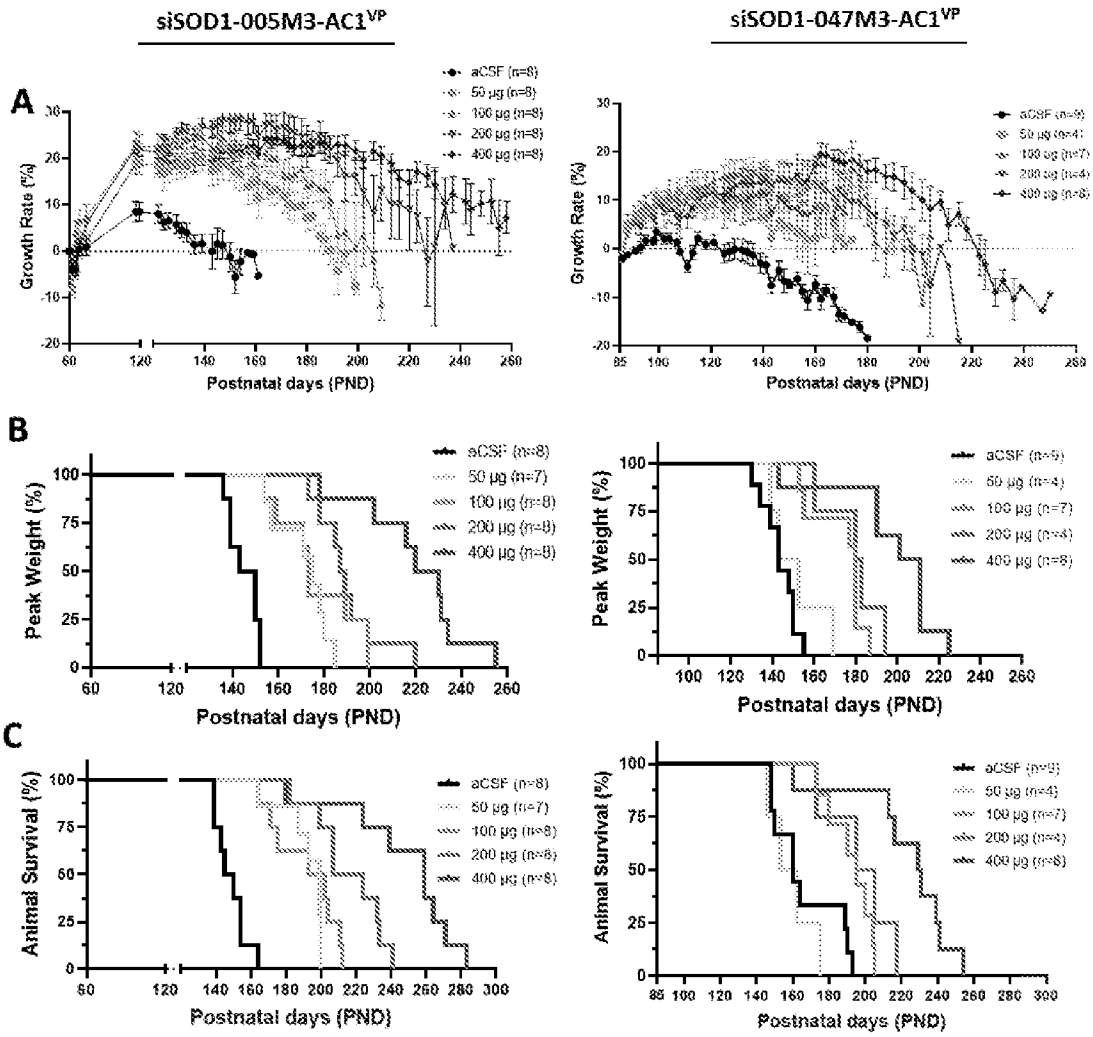


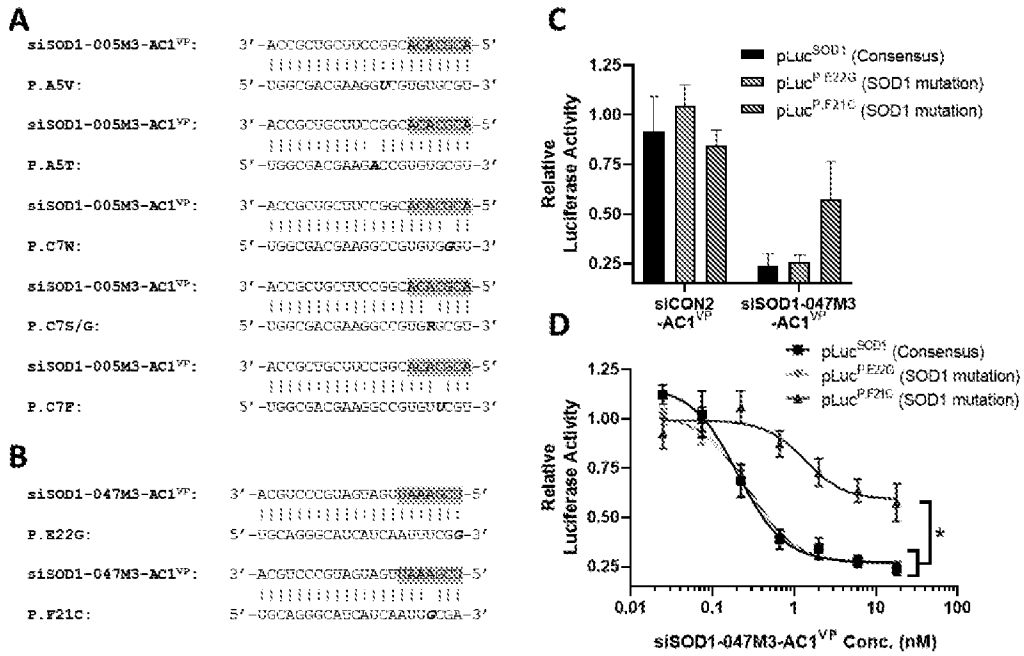
FIG.10



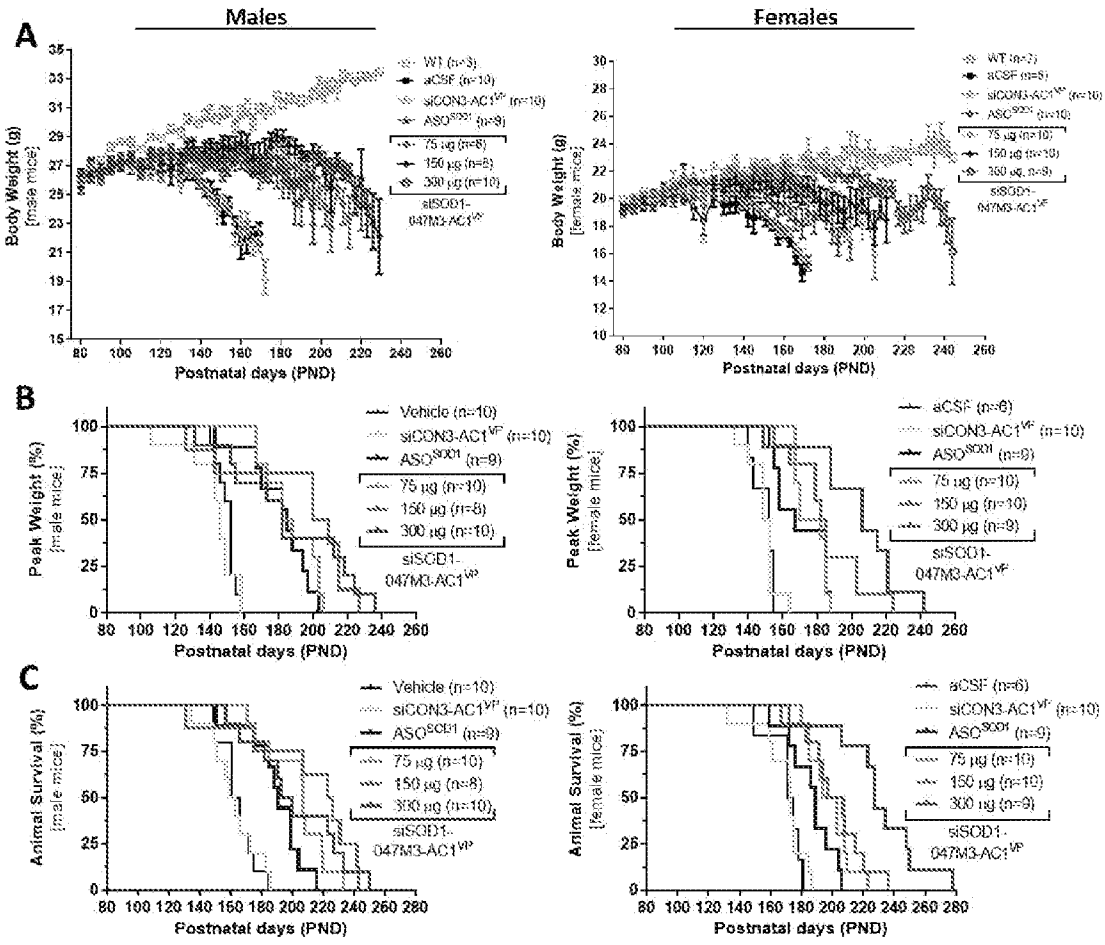
FIGs.11A-11B


siSOD1-005M3-AC1VP
siSOD1-047M3-AC1VP
A
B
C
Growth Rate (%)
Peak Weight (%)
Animal Survival (%)
Postnatal days (PND)
aCSF (n=8)
50 μg (n=8)
100 μg (n=8)
200 μg (n=8)
400 μg (n=8)
aCSF (n=8)
50 μg (n=7)
100 μg (n=8)
200 μg (n=8)
400 μg (n=8)
aCSF (n=9)
50 μg (n=4)
100 μg (n=7)
200 μg (n=4)
400 μg (n=8)

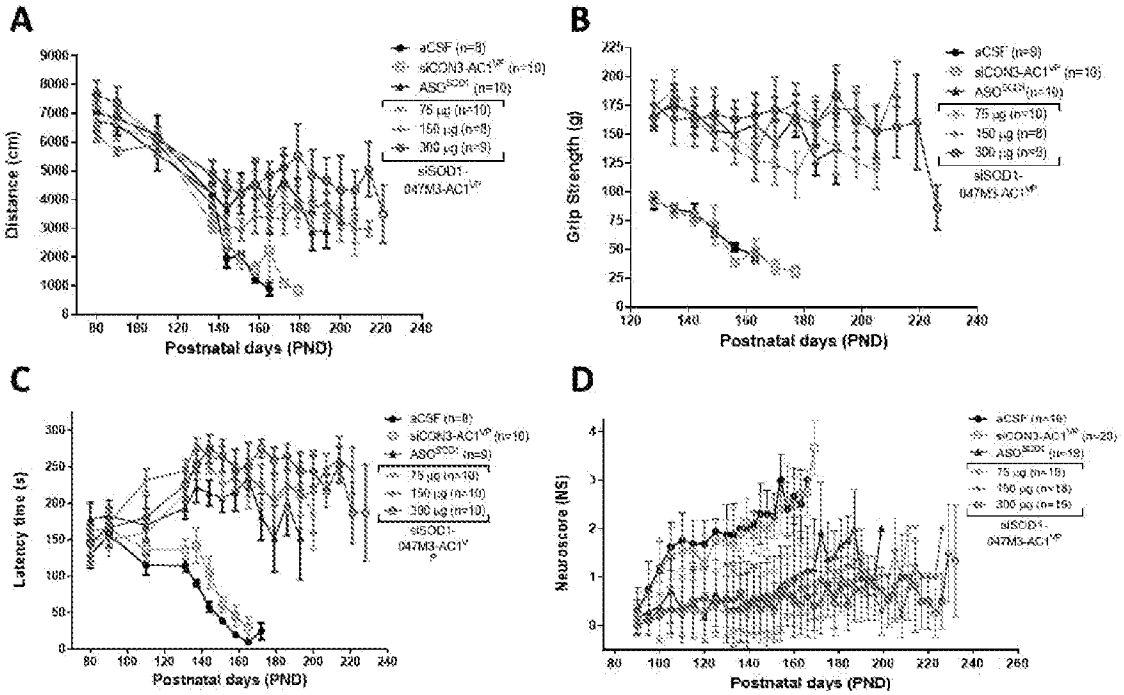
FIGs.12A-12C



FIGs.13A-13D



FIGs.14A-14C



FIGs.15A-15D

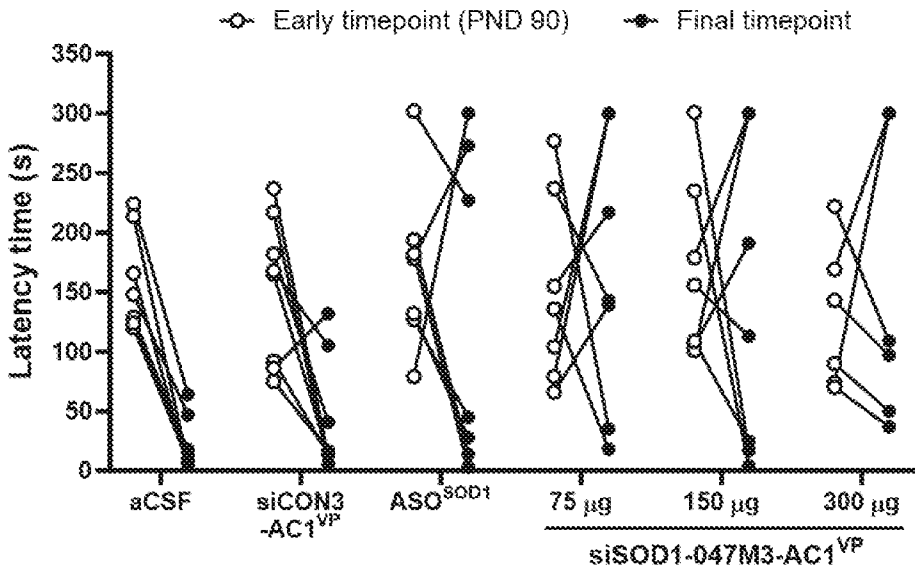


FIG.16

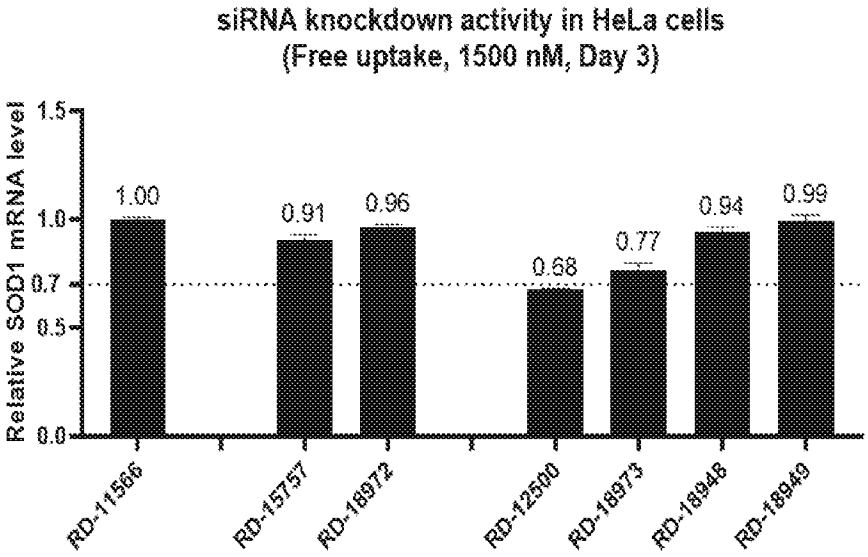


FIG.17

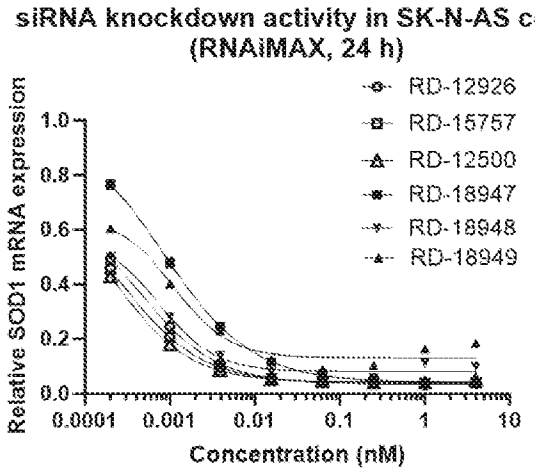


FIG.18A

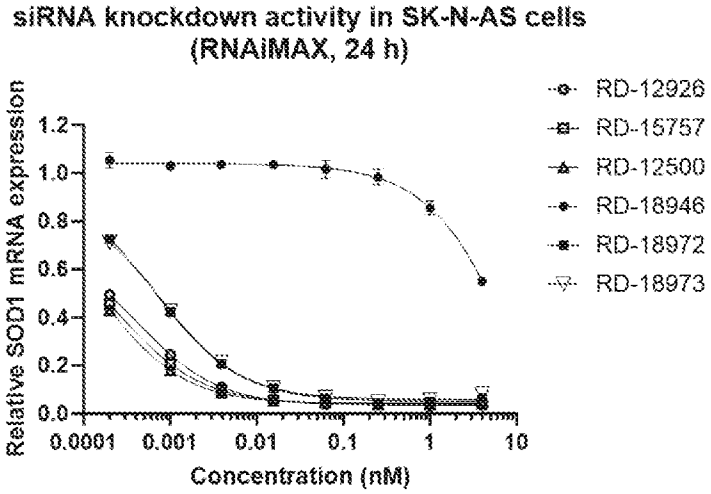


FIG.18B

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CN2024/078297

A. CLASSIFICATION OF SUBJECT MATTER

C12N 15/113(2010.01)i; A61K48/00(2006.01)i

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC:C12N; A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

CJFD,CNTXT,ENTXT,ENTXTC,WFB,WFCCP,WFCD,WFCJ,WFCOA,WFCSTA,WFFCP,WFFD,WFFJ,WFFOA,WFS, WFSTR,STN,PubMed,GenBank,ISI Web of Knowledge,CNKI:SOD1,ALS,AOC,siRNA, superoxide dismutase 1,amyotrophic lateral sclerosis,non-targeting single-stranded oligonucleotide, SEQ ID NOs: 1-805,modification

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 2023280190 A1 (RACTIGEN THERAPEUTICS) 12 January 2023 (2023-01-12) claims 1,2,62,70,107-111	1-53
Y	CN 103333913 A (Institute of Disease Control and Prevention of PLA) 02 October 2013 (2013-10-02) SEQ ID NO:2	1-53
Y	US 2005288243 A1 (UNIVERSITY OF MASSACHUSETTS) 29 December 2005 (2005-12-29) claims 1-98	1-53
Y	WANG,H.Y. et al. "Therapeutic Gene Silencing Delivered by a Chemically Modified Small Interfering RNA against Mutant SOD1 Slows Amyotrophic Lateral Sclerosis Progression" *THE JOURNAL OF BIOLOGICAL CHEMISTRY,* Vol. 283, No. 23, 26 March 2008 (2008-03-26), pages 15845-15852	1-53

☑ Further documents are listed in the continuation of Box C. ☑ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"D" document cited by the applicant in the international application
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
23 April 2024	**30 April 2024**

Name and mailing address of the ISA/CN	Authorized officer
CHINA NATIONAL INTELLECTUAL PROPERTY ADMINISTRATION **6, Xitucheng Rd., Jimen Bridge, Haidian District, Beijing 100088, China**	**LV,XiaoMeng** Telephone No. **(+86) 010-53962044**

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CN2024/078297

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	LIU,J.J. et al. "Effects of Amyotrophic Lateral Sclerosis Associated SOD1 Amino Acid Mutations on Aggregation" *SUZHOU UNIVERSITY JOURNAL OF MEDICAL SCIENCE,* Vol. 29, No. 3, 25 May 2009 (2009-05-25), pages 411-414	1-53
A	MCCAMPBELL,A. et al. "Antisense oligonucleotides extend survival and reverse decrement in muscle response in ALS models" *The Journal of Clinical Investigation,* Vol. 128, No. 8, 31 July 2018 (2018-07-31), pages 3558-3567	1-53

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CN2024/078297

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:
   a. ☑ forming part of the international application as filed.
   b. ☐ furnished subsequent to the international filing date for the purposes of international search (Rule 13*ter*.1(a)),
      ☐ accompanied by a statement to the effect that the sequence listing does not go beyond the disclosure in the international application as filed.

2. ☐ With regard to any nucleotide and/or amino acid sequence disclosed in the international application, this report has been established to the extent that a meaningful search could be carried out without a WIPO Standard ST.26 compliant sequence listing.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CN2024/078297

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☑ Claims Nos.: **45-49**
because they relate to subject matter not required to be searched by this Authority, namely:

Claims 45-49 direct to a method of decreasing the transcript level of a SOD1 gene or SOD1 protein, or a method of treating or delaying the onset or progression of a neurodegenerative disease or symptom, and therefore do not meet the requirement of Rule 39.1(iv). The search is based on the use of the pharmaceutical composition for manufacturing of a medicament.

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/CN2024/078297

Patent document cited in search report			Publication date (day/month/year)	Patent family member(s)			Publication date (day/month/year)
WO	2023280190	A1	12 January 2023	CA	3225998	A1	12 January 2023
				TW	202321449	A	01 June 2023
				AU	2022308123	A1	22 February 2024
CN	103333913	A	02 October 2013		None		
US	2005288243	A1	29 December 2005	US	2009042828	A1	12 February 2009
				US	8008271	B2	30 August 2011
				US	7498316	B2	03 March 2009
				WO	2005096781	A2	20 October 2005
				WO	2005096781	A3	02 August 2007