

PATENT COOPERATION TREATY

From the INTERNATIONAL SEARCHING AUTHORITY

PCT

NOTIFICATION OF TRANSMITTAL OF
THE INTERNATIONAL SEARCH REPORT AND
THE WRITTEN OPINION OF THE INTERNATIONAL
SEARCHING AUTHORITY, OR THE DECLARATION

To:

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Minneapolis, MN 55440-1022
ETATS-UNIS D'AMERIQUE

(PCT Rule 44.1)

Applicant's or agent's file reference 137510297WO1	Date of mailing (day/month/year) 1 April 2020 (01-04-2020)
International application No. PCT/US2019/065936	International filing date (day/month/year) 12 December 2019 (12-12-2019)
Applicant BIOGEN MA INC.	

1. ☒ The applicant is hereby notified that the international search report and the written opinion of the International Searching Authority have been established and are transmitted herewith.

Filing of amendments and statement under Article 19:

The applicant is entitled, if he so wishes, to amend the claims of the International Application (see Rule 46):

When? The time limit for filing such amendments is normally two months from the date of transmittal of the International Search Report.

How? Directly to the International Bureau of WIPO, 34 chemin des Colombettes
1211 Geneva 20, Switzerland, Facsimile No.: (41-22) 338.82.70

For more detailed instructions, see PCT Applicant's Guide, International Phase, paragraphs 9.004 - 9.011.

2. ☐ The applicant is hereby notified that no international search report will be established and that the declaration under Article 17(2)(a) to that effect and the written opinion of the International Searching Authority are transmitted herewith.
3. ☐ **With regard to any protest** against payment of (an) additional fee(s) under Rule 40.2, the applicant is notified that:
- ☐ the protest together with the decision thereon has been transmitted to the International Bureau together with any applicant's request to forward the texts of both the protest and the decision thereon to the designated Offices.
- ☐ no decision has been made yet on the protest; the applicant will be notified as soon as a decision is made.

4. Reminders

The applicant may **submit comments on an informal basis on the written opinion of the International Searching Authority** to the International Bureau. These comments will be made available to the public after international publication. The International Bureau will send a copy of such comments to all designated Offices unless an international preliminary examination report has been or is to be established.

Shortly after the expiration of **18 months from the priority date, the international application will be published** by the International Bureau. If the applicant wishes to avoid or postpone publication, a notice of withdrawal of the international application, or of the priority claim, must reach the International Bureau before the completion of the technical preparations for international publication (Rules 90**bis**.1 and 90**bis**.3).

Within **19 months** from the priority date, but only in respect of some designated Offices, a demand for international preliminary examination must be filed if the applicant wishes to postpone the entry into the national phase **until 30 months** from the priority date (in some Offices even later); otherwise, the applicant must, **within 20 months** from the priority date, perform the prescribed acts for **entry into the national phase** before those designated Offices. In respect of other designated Offices, the time limit of **30 months** (or later) will apply even if no demand is filed within 19 months. For details about the applicable time limits, Office by Office, see www.wipo.int/pct/en/texts/time_limits.html and the *PCT Applicant's Guide*, National Chapters.

Within **22 months from the priority date, the applicant may request that a supplementary international search be carried out** by a different International Searching Authority that offers this service (Rule 45**bis**.1). The procedure for requesting supplementary international search is described in the *PCT Applicant's Guide*, International Phase, paragraphs 8.006-8.032.

Name and mailing address of the International Searching Authority  European Patent Office, P.B. 5818 Patentlaan 2 NL-2280 HV Rijswijk Tel. (+31-70) 340-2040 Fax: (+31-70) 340-3016	Authorized officer EMERY, Carole Tel: +31 (0)70 340-2848
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PATENT COOPERATION TREATY

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference 137510297WO1	FOR FURTHER ACTION see Form PCT/ISA/220 as well as, where applicable, item 5 below.	
International application No. PCT/US2019/065936	International filing date (<i>day/month/year</i>) 12 December 2019 (12-12-2019)	(Earliest) Priority Date (<i>day/month/year</i>) 14 December 2018 (14-12-2018)
Applicant BIOGEN MA INC.		

This international search report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This international search report consists of a total of 5 sheets.

☒ It is also accompanied by a copy of each prior art document cited in this report.

1. Basis of the report

a. With regard to the **language**, the international search was carried out on the basis of:

- ☒ the international application in the language in which it was filed
☐ a translation of the international application into _____, which is the language of a translation furnished for the purposes of international search (Rules 12.3(a) and 23.1(b))

b. ☐ This international search report has been established taking into account the **rectification of an obvious mistake** authorized by or notified to this Authority under Rule 91 (Rule 43.6**bis**(a)).

c. ☒ With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, see Box No. I.

2. ☐ **Certain claims were found unsearchable** (See Box No. II)

3. ☐ **Unity of invention is lacking** (see Box No III)

4. With regard to the **title**,

- ☒ the text is approved as submitted by the applicant
☐ the text has been established by this Authority to read as follows:

5. With regard to the **abstract**,

- ☒ the text is approved as submitted by the applicant
☐ the text has been established, according to Rule 38.2, by this Authority as it appears in Box No. IV. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority

6. With regard to the **drawings**,

- a. the figure of the **drawings** to be published with the abstract is Figure No. 5
☒ as suggested by the applicant
☐ as selected by this Authority, because the applicant failed to suggest a figure
☐ as selected by this Authority, because this figure better characterizes the invention
 b. ☐ none of the figures is to be published with the abstract

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2019/065936

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:
 - ☒ in the form of an Annex C/ST.25 text file.
 - ☐ on paper or in the form of an image file.
 - b. ☐ furnished together with the international application under PCT Rule 13~~ter~~.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.
 - c. ☐ furnished subsequent to the international filing date for the purposes of international search only:
 - ☐ in the form of an Annex C/ST.25 text file (Rule 13~~ter~~.1(a)).
 - ☐ on paper or in the form of an image file (Rule 13~~ter~~.1(b) and Administrative Instructions, Section 713).
2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.
3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No

PCT/US2019/065936

A. CLASSIFICATION OF SUBJECT MATTER

INV. C12N15/113 A61P25/14
ADD.

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C12N

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

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

EPO-Internal, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2015/153800 A2 (ISIS PHARMACEUTICALS INC [US]) 8 October 2015 (2015-10-08) page 60 - page 61 page 65 - page 66; claim 65; examples 15-19 the whole document -----	1-41
X	ALEX MCCAMPBELL ET AL: "Antisense oligonucleotides extend survival and reverse decrement in muscle response in ALS models", JOURNAL OF CLINICAL INVESTIGATION, vol. 128, no. 8, 16 July 2018 (2018-07-16), pages 3558-3567, XP055679194, GB ISSN: 0021-9738, DOI: 10.1172/JCI99081 figure 1; table 1 the whole document ----- -/-	1-41

☒ 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

"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

24 March 2020

Date of mailing of the international search report

01/04/2020

Name and mailing address of the ISA/

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040,
Fax: (+31-70) 340-3016

Authorized officer

Spindler, Mark-Peter

INTERNATIONAL SEARCH REPORT

International application No

PCT/US2019/065936

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 2018/204786 A1 (VOYAGER THERAPEUTICS INC [US]) 8 November 2018 (2018-11-08) the whole document	1-41
A	----- SCHOCH KATHLEEN M ET AL: "Antisense Oligonucleotides: Translation from Mouse Models to Human Neurodegenerative Diseases", NEURON, vol. 94, no. 6, 21 June 2017 (2017-06-21), pages 1056-1070, XP085093439, ISSN: 0896-6273, DOI: 10.1016/J.NEURON.2017.04.010 the whole document -----	1-41

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/US2019/065936

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2015153800 A2	08-10-2015	AU 2015240761 A1	22-09-2016
		AU 2019268063 A1	12-12-2019
		CA 2942394 A1	08-10-2015
		CL 2016002509 A1	09-06-2017
		CL 2018001897 A1	16-11-2018
		CL 2019002535 A1	14-02-2020
		CN 106459972 A	22-02-2017
		EP 3126499 A2	08-02-2017
		JP 6622214 B2	18-12-2019
		JP 2017513469 A	01-06-2017
		JP 2019201664 A	28-11-2019
		KR 20170012207 A	02-02-2017
		SG 10201906520R A	27-09-2019
		SG 11201608109T A	28-10-2016
		US 2017037410 A1	09-02-2017
		US 2020040342 A1	06-02-2020
		WO 2015153800 A2	08-10-2015

WO 2018204786 A1	08-11-2018	AU 2018261790 A1	28-11-2019
		CA 3061652 A1	08-11-2018
		EP 3618839 A1	11-03-2020
		SG 11201909870S A	28-11-2019
		WO 2018204786 A1	08-11-2018

Information on Search Strategy - Pilot phase (see OJ 2015, A86)

The type of information contained in this sheet may change during the pilot for improving the usefulness of this new service.

Application Number

PCT/US2019/065936

TITLE: COMPOSITIONS AND METHODS FOR TREATING AND PREVENTING AMYOTROPHIC LATERAL SCLEROSIS

APPLICANT: BIOGEN MA INC.

IPC CLASSIFICATION: C12N15/113, A61P25/14

EXAMINER: Spindler, Mark-Peter

CONSULTED DATABASES: DOSYS, INET, WPI, NPL

CLASSIFICATION SYMBOLS DEFINING EXTENT OF THE SEARCH:

IPC:

CPC: C12N15/1137, 1- C12N2310/321, C12N2310/3525, C12N2310/11, C12N2310/315, C12N2310/3341, C12N2320/35

FI/F-TERMS:

KEYWORDS OR OTHER ELEMENTS FEATURING THE INVENTION:

SOD1, superoxide dismutase 1

ALS, amyotrophic lateral sclerosis

antisense, gapmer, ISIS666853, BIIB067

dosage regimen

PATENT COOPERATION TREATY

From the
INTERNATIONAL SEARCHING AUTHORITY

PCT

To:

see form PCT/ISA/220

WRITTEN OPINION OF THE INTERNATIONAL SEARCHING AUTHORITY (PCT Rule 43bis.1)

Date of mailing

(day/month/year) see form PCT/ISA/210 (second sheet)

Applicant's or agent's file reference
see form PCT/ISA/220

FOR FURTHER ACTION

See paragraph 2 below

International application No.
PCT/US2019/065936

International filing date (day/month/year)
12.12.2019

Priority date (day/month/year)
14.12.2018

International Patent Classification (IPC) or both national classification and IPC
INV. C12N15/113 A61P25/14

Applicant
BIOGEN MA INC.

1. This opinion contains indications relating to the following items:

- ☒ Box No. I Basis of the opinion
- ☐ Box No. II Priority
- ☐ Box No. III Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- ☐ Box No. IV Lack of unity of invention
- ☒ Box No. V Reasoned statement under Rule 43bis.1(a)(i) with regard to novelty, inventive step and industrial applicability; citations and explanations supporting such statement
- ☐ Box No. VI Certain documents cited
- ☐ Box No. VII Certain defects in the international application
- ☒ Box No. VIII Certain observations on the international application

2. FURTHER ACTION

If a demand for international preliminary examination is made, this opinion will usually be considered to be a written opinion of the International Preliminary Examining Authority ("IPEA") except that this does not apply where the applicant chooses an Authority other than this one to be the IPEA and the chosen IPEA has notified the International Bureau under Rule 66.1bis(b) that written opinions of this International Searching Authority will not be so considered.

If this opinion is, as provided above, considered to be a written opinion of the IPEA, the applicant is invited to submit to the IPEA a written reply together, where appropriate, with amendments, before the expiration of 3 months from the date of mailing of Form PCT/ISA/220 or before the expiration of 22 months from the priority date, whichever expires later.

For further options, see Form PCT/ISA/220.

Name and mailing address of the ISA:



European Patent Office
P.B. 5818 Patentlaan 2
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Tel. +31 70 340 - 2040
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Date of completion of
this opinion

see form
PCT/ISA/210

Authorized Officer

Spindler, Mark-Peter

Telephone No. +31 70 340-0



**WRITTEN OPINION OF THE
INTERNATIONAL SEARCHING AUTHORITY**

International application No.
PCT/US2019/065936

Box No. I Basis of the opinion

1. With regard to the **language**, this opinion has been established on the basis of:
 - ☒ the international application in the language in which it was filed.
 - ☐ a translation of the international application into , which is the language of a translation furnished for the purposes of international search (Rules 12.3(a) and 23.1 (b)).
2. ☐ This opinion has been established taking into account the **rectification of an obvious mistake** authorized by or notified to this Authority under Rule 91 (Rule 43bis.1(a))
3. ☒ With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, this opinion has been established on the basis of a sequence listing:
 - a. ☒ forming part of the international application as filed:
 - ☒ in the form of an Annex C/ST.25 text file.
 - ☐ on paper or in the form of an image file.
 - b. ☐ furnished together with the international application under PCT Rule 13ter.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.
 - c. ☐ furnished subsequent to the international filing date for the purposes of international search only:
 - ☐ in the form of an Annex C/ST.25 text file (Rule 13ter.1(a)).
 - ☐ on paper or in the form of an image file (Rule 13ter.1(b) and Administrative Instructions, Section 713).
4. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.
5. Additional comments:

**WRITTEN OPINION OF THE
INTERNATIONAL SEARCHING AUTHORITY**

International application No.
PCT/US2019/065936

Box No. V Reasoned statement under Rule 43bis.1(a)(i) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes: Claims	<u>1-41</u>
	No: Claims	
Inventive step (IS)	Yes: Claims	
	No: Claims	<u>1-41</u>
Industrial applicability (IA)	Yes: Claims	<u>1-41</u>
	No: Claims	

2. Citations and explanations

see separate sheet

Box No. VIII Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

see separate sheet

Re Item V

Reasoned statement with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

The following documents appeared to be relevant; the numbering will be adhered to in the rest of the procedure:

- D1 WO 2015/153800 A2 (ISIS PHARMACEUTICALS INC [US]) 8 October 2015 (2015-10-08)
- D2 ALEX MCCAMPBELL ET AL: "Antisense oligonucleotides extend survival and reverse decrement in muscle response in ALS models", JOURNAL OF CLINICAL INVESTIGATION, vol. 128, no. 8, 16 July 2018 (2018-07-16), pages 3558-3567, XP055679194

1 Inventive Step

The present application does not meet the criteria of Article 33(3) PCT, because the subject-matter of claims 1-41 does not involve an inventive step.

- 1.1 The scope of claim 20 is considered broader than the scope of the other independent claims. The following analysis is hence based on claim 20.
- 1.2 Document D1 is regarded as being the closest prior art to the subject-matter of claim 20, and discloses:
- a syringe comprising the antisense oligonucleotide ISIS666853 which is adapted for intrathecal administration of said oligonucleotide at a dose of 4, 12 or 35 mg (ex. 19)
- 1.3 The subject-matter of claim 20 therefore differs from this known syringe in that the dose is about 20 mg, about 40 mg, about 60 mg or about 100 mg.
- 1.4 No technical effect resulting from this difference can be stated.
- 1.5 The problem to be solved by the present invention may therefore be regarded as the provision of a further dosage form of antisense oligonucleotide ISIS666853.

- 1.6 The solution proposed in claim 20 of the present application cannot be considered as involving an inventive step (Article 33(3) PCT) for the following reasons:

The mere provision of a further dosage form of a known oligonucleotide does not confer an inventive step. Current claim 20 is not limited to a specific medical purpose let alone to a specific species. Any effect achieved with a fixed dose in one species (e.g. in humans) cannot be extrapolated to other species (e.g. in mice). Besides, it is also not plausible that a specific dosage regimen which provides an effect for one disease (e.g. ALS) will have the same (or at least similar) effect for a different unrelated disease (e.g. cancer).

- 1.7 Likewise, the subject-matter of the independent claims 1-8 & 21-28 is also considered not inventive.

Documents D1 and D2 both disclose the antisense oligonucleotide ISIS666853 as effective inhibitor of SOD1 *in vitro* and *in vivo* for use in treating ALS. Neither of the documents discloses treatment of human patients. However, the determination of an effective dosage regimen falls within the routine of the skilled person. For a new medical treatment based on a dosage regimen, a new teaching that will lead to a new clinical situation is required in order to involve an inventive step. In particular, the application fails to provide any unexpected technical effect arising from the lower doses (20 mg, 40 mg or 60 mg). With regard to the highest dose of 100 mg it is noted that no therapeutic effect is shown for a single dose treatment, i.e. wherein the oligonucleotide is administered only once.

It is additionally noted that claims 5-8 & 25-28 are not limited to a clear medical implementation as no therapeutic or preventive purpose is mentioned in said claims.

- 1.8 Dependent claims 9-19 & 29-41 do not contain any features which, in combination with the features of any claim to which they refer, meet the requirements of the PCT in respect of inventive step.

Claims 9, 10, 29 & 30 relate to specific SOD1 mutations. Said mutations were already known in the prior art. Said mutations do on their own not render said claims inventive.

The features of claims 11-19 & 31-41 fall within the routine of the skilled person when determining an effective dosage regimen.

- 1.9 In contrast, depending on the wording an inventive step might be acknowledged for a therapeutic treatment of ALS patients with fast progressing SOD1 mutations using 100 mg over a longer period (illustrated by fig. 5 and 7). Currently, none of the claims is limited to such a treatment.

2 Industrial Applicability and Patentability

The subject-matter of claims 1-41 is considered industrially applicable.

However, claims 1-19 & 21-41 encompass or relate to methods of treatment. The patentability can be dependent upon the formulation of the claims. The EPO, for example, does not recognise as patentable claims to the use of a compound in medical treatment, but may allow claims to a product, in particular substances or compositions for use in a first or further medical treatment.

Re Item VIII

Certain observations on the international application

The term "about" used throughout the claims is vague and unclear and leaves the reader in doubt as to the meaning of the technical features to which it refers, thereby rendering the definition of the subject-matter of the claims concerned unclear, Article 6 PCT.

motor neurons from animals treated with pri-miRNA VOYmiR127.860 packaged in AAVrh10, as compared to vehicle control lumbar motor neurons.

[00836] Shown in Table 25 and Table 26 are the levels of the vector genome in different tissues. Vector genome was determined by Droplet Digital PCR (ddPCR). Briefly, DNA was purified from LCM samples with DNA Clean and Concentrator Kit (Zymoresearch) and whole cell DNA was prepared from tissue by DNeasy Blood and Tissue Kit (Qiagen, catalog #69506) under the conditions recommended by the manufacturer. This DNA was used as template in ddPCR reactions utilizing QX200 Droplet Digital PCR System (Bio-Rad). Briefly, reactions comprised of template, 2x Supermix (BioRad, catalog#1863023), HindIII restriction endonuclease (New England Biolabs) and oligonucleotide probesets with homology specific to the vector (FAM labelled) and non-human primate host RNaseP gene (VIC labelled, [ThermoFisher Scientific, # 4403328]) were set up. Reactions were incorporated into droplets with Bio-Rad Automatic Droplet Generator with conditions and reagents recommended by the manufacturer. Droplets underwent 40 cycles of thermocycling to endpoint. After PCR was complete, samples were read on QX200 Droplet Reader (Bio-Rad). Data were analyzed by QuantaSoft software to yield copies/reaction of each target. Vector genome distribution was reported as copies of vector genome per diploid cell (VG/DC) by normalizing the copies of vector to half the copies of host. Included in the tables are the averaged group data with and without the neutralizing antibody-positive animals. The presence of neutralizing antibodies did not diminish the VG/DC levels in the CNS tissues sampled, whereas the VG/DC levels in peripheral tissues were lower than in animals that were negative for neutralizing antibodies. In the laser captured motor neurons (approximately 75 motor neurons per pool), significant vector genomes averaging 13.64 VG/DC were measured. In spinal cord white matter sections (depleted of ventral horn gray matter and pia), average (all 4 animals) levels of 35.4 vg/dc were observed. In the liver, the vg levels averaged 120.36 +/- 121.01 VG/DC (all 4 animals) whereas the sero-negative animals averaged 224.75 +/- 4.23 VG/DC. Vector genome levels in heart, lung, kidney, and ovary were markedly lower than those observed in liver or spinal cord white matter, with the levels in ovary and kidney at or approaching the limit of detection for the assay.

Table 25. Biodistribution by Vector Genome Determination in Nonhuman Primate Peripheral Tissues

Construct	Number of animals	Average Vector genomes/cell (VG/DC)				
		Liver	Heart	Lung	Ovary	Kidney
Vehicle	4	1.95+/-0.01	1.97+/-0.01	1.99+/-0.00	1.98+/-0.02	1.97+/-0.01
VOYmiR127.860	4	120.36+/-121.01	3.30+/-1.90	2.69+/-0.82	2.03+/-0.07	2.19+/-0.19

	2 (without neutralizing antibody-positive animals)	224.75+/-4.23	4.60+/-2.02	3.29+/-0.74	2.09+/-0.02	2.24+/-0.25
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Table 26. Biodistribution by Vector Genome Determination in Nonhuman Primate Spinal Cord Samples

Construct	Number of animals	Average Vector genomes/cell (VG/DC)		
		Pia Mater	White Matter	Laser Captured Motor Neurons
Vehicle	4	1.96+/-0.01	1.97+/-0.07	1.58+/-0.63
	4	201.84+/-107.93	35.40+/-8.59	13.64+/-11.67
VOYmiR127.860	2 (without neutralizing antibody-positive animals)	141.82+/-85.84	31.86+/-4.14	6.59

[00837] Precision and efficiency of pri-miRNA processing was evaluated by deep sequencing of ventral horn punches from the lumbar spinal cord, using the same methods as described above. These results showed that treatment with VOYmiR127.860 packaged in AAVrh10 resulted in a high guide to passenger ratio (>30.5), a high percentage of miRNAs with precise processing at the 5' end (90.7 +/- 4.5%), and a low level of guide and passenger strands relative to the total endogenous pool of miRNAs (0.00096 +/- 0.00054 %).

[00838] Histology was conducted on animals euthanized on Day 29. No animals in any treatment group had gross lesions at necropsy. Microscopic histopathology was conducted on spinal cord and liver, and no histopathological findings related to VOYmiR127.860 packaged in AAVrh10 were detected.

Example 8. SOD1 Knock-Down Mammalian Studies

Pharmacology and Efficacy

[00839] To study the efficacy and pharmacology of the VOYmiR127.860, studies will be conducted in SOD1-G93A mouse models of ALS.

[00840] Mice will be administered by IT administration of at least 2 dosage levels (low and high vg). Alternatively, IV dosing will be used as a surrogate route of administration.

[00841] For the pharmacology study, three groups of 10 mice/group, approximately 40 to 50 days of age and balanced for gender, will receive vehicle or VOYmiR127.860 packaged in AAVrh10 at either high or low dose levels. All animals will be euthanized after four weeks and spinal cord tissue samples will be evaluated for SOD1 suppression by RT-qPCR. Body weights and cage-side observation will also be collected.

[00842] After the pharmacology study, an efficacy study of VOYmiR127.860 packaged in AAVrh10 will be conducted in SOD1-G93A mice. Two groups of approximately 32 mice/group,

approximately 40 to 50 days of age and balanced for gender will receive vehicle or VOYmiR127-860 packaged in AAVrh10 at a high or low dose level. Endpoints will include body weight, neurological monitoring, and survival. Spinal cord tissue samples will also be evaluated for SOD1 suppression by RT-qPCR.

Pharmacology, Toxicity, and Biodistribution Study in NHPs

[00843] In order to evaluate the safety and distribution of VOYmiR127.860 packaged in AAVrh10, a study in NHP with a one-time IT infusion, will be conducted. Three time points - 4, 13 and 26 weeks - will be evaluated. The 4-week survival period after a one-time dose is intended to provide sufficient time for SOD1 suppression to be attained and to provide information on the short-term effects of VOYmiR127.860 packaged in AAVrh10. The 13-week and 26-week time points is intended to provide information on the long-term effects of VOYmiR127.860 packaged in AAVrh10.

[00844] Prior to enrollment, animals will be screened for the presence of neutralizing antibodies to the AAVrh10 capsid, as well as for abnormal serum chemistry profiles. Three dose levels will be tested. A control of vehicle only will also be tested.

[00845] Vector genomes will be quantified in all of the dose groups at 4 weeks. Tissues that have no detectable vector genomes at four weeks will not be evaluated at the 13-week or 26-week time points. Tissues that have detectable vector genomes at four weeks will be evaluated at the 13-week time point. All tissues and organs will be archived for possible vg quantification.

[00846] Primary outcome measures will include a PCR analysis of vector genomes within different CNS regions and major peripheral organs, including heart and lungs, a histopathological assessment of target and non-target tissues that have significant vector genome levels, and clinical pathology including serum chemistry, hematology, and coagulation. Safety pharmacology (central nervous, cardiovascular, and respiratory systems) and local tolerance will also be evaluated.

[00847] While the present invention has been described at some length and with some particularity with respect to the several described embodiments, it is not intended that it should be limited to any such particulars or embodiments or any particular embodiment, but it is to be construed with references to the appended claims so as to provide the broadest possible interpretation of such claims in view of the prior art and, therefore, to effectively encompass the intended scope of the invention.

[00848] All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification,

including definitions, will control. In addition, section headings, the materials, methods, and examples are illustrative only and not intended to be limiting.

CLAIMS

We claim:

1. An adeno-associated viral (AAV) vector comprising a first nucleic acid sequence comprising two inverted terminal repeats (ITRs), a promoter, an intron and a second nucleic acid sequence, wherein said second nucleic acid sequence when expressed inhibits or suppresses expression of SOD1 in a cell, wherein said second nucleic acid sequence comprises a sense strand sequence and an antisense strand sequence, wherein the sense strand sequence comprises at least 15 contiguous nucleotides differing by no more than 3 nucleotides from the nucleotide sequence of sequences listed in Table 3 and the antisense strand sequence comprises at least 15 contiguous nucleotides differing by no more than 3 nucleotides from the nucleotide sequence of sequences listed in Table 2 and wherein said sense strand sequence and antisense strand sequence share a region of complementarity of at least four nucleotides in length.
2. The AAV vector of claim 1, wherein the promoter is selected from the group consisting of CMV, CBA and H1.
3. The AAV vector of claim 2, wherein the intron is beta-globin.
4. The AAV vector of claim 3, wherein the promoter is CMV.
5. The AAV vector of claim 3, wherein the promoter is CBA.
6. The AAV vector of claim 3, wherein the promoter is H1.
7. The AAV vector of claim 1, wherein the AAV vector comprises a capsid serotype selected from the group consisting AAV1, AAV2, AAV2G9, AAV3, AAV3a, AAV3b, AAV3-3, AAV4, AAV4-4, AAV5, AAV6, AAV6.1, AAV6.2, AAV6.1.2, AAV7, AAV7.2, AAV8, AAV9, AAV9.11, AAV9.13, AAV9.16, AAV9.24, AAV9.45, AAV9.47, AAV9.61, AAV9.68, AAV9.84, AAV9.9, AAV10, AAV11, AAV12, AAV16.3, AAV24.1, AAV27.3, AAV42.12, AAV42-1b, AAV42-2, AAV42-3a, AAV42-3b, AAV42-4, AAV42-5a, AAV42-5b, AAV42-6b, AAV42-8, AAV42-10, AAV42-11, AAV42-12, AAV42-13, AAV42-15,

AAV42-aa, AAV43-1, AAV43-12, AAV43-20, AAV43-21, AAV43-23, AAV43-25, AAV43-5, AAV44.1, AAV44.2, AAV44.5, AAV223.1, AAV223.2, AAV223.4, AAV223.5, AAV223.6, AAV223.7, AAV1-7/rh.48, AAV1-8/rh.49, AAV2-15/rh.62, AAV2-3/rh.61, AAV2-4/rh.50, AAV2-5/rh.51, AAV3.1/hu.6, AAV3.1/hu.9, AAV3-9/rh.52, AAV3-11/rh.53, AAV4-8/rh.64, AAV4-9/rh.54, AAV4-19/rh.55, AAV5-3/rh.57, AAV5-22/rh.58, AAV7.3/hu.7, AAV16.8/hu.10, AAV16.12/hu.11, AAV29.3/bb.1, AAV29.5/bb.2, AAV106.1/hu.37, AAV114.3/hu.40, AAV127.2/hu.41, AAV127.5/hu.42, AAV128.3/hu.44, AAV130.4/hu.48, AAV145.1/hu.53, AAV145.5/hu.54, AAV145.6/hu.55, AAV161.10/hu.60, AAV161.6/hu.61, AAV33.12/hu.17, AAV33.4/hu.15, AAV33.8/hu.16, AAV52/hu.19, AAV52.1/hu.20, AAV58.2/hu.25, AAVA3.3, AAVA3.4, AAVA3.5, AAVA3.7, AAVC1, AAVC2, AAVC5, AAV-DJ, AAV-DJ8, AAVF3, AAVF5, AAVH2, AAVrh.72, AAVhu.8, AAVrh.68, AAVrh.70, AAVpi.1, AAVpi.3, AAVpi.2, AAVrh.60, AAVrh.44, AAVrh.65, AAVrh.55, AAVrh.47, AAVrh.69, AAVrh.45, AAVrh.59, AAVhu.12, AAVH6, AAVLK03, AAVH-1/hu.1, AAVH-5/hu.3, AAVLG-10/rh.40, AAVLG-4/rh.38, AAVLG-9/hu.39, AAVN721-8/rh.43, AAVCh.5, AAVCh.5R1, AAVcy.2, AAVcy.3, AAVcy.4, AAVcy.5, AAVCy.5R1, AAVCy.5R2, AAVCy.5R3, AAVCy.5R4, AAVcy.6, AAVhu.1, AAVhu.2, AAVhu.3, AAVhu.4, AAVhu.5, AAVhu.6, AAVhu.7, AAVhu.9, AAVhu.10, AAVhu.11, AAVhu.13, AAVhu.15, AAVhu.16, AAVhu.17, AAVhu.18, AAVhu.20, AAVhu.21, AAVhu.22, AAVhu.23.2, AAVhu.24, AAVhu.25, AAVhu.27, AAVhu.28, AAVhu.29, AAVhu.29R, AAVhu.31, AAVhu.32, AAVhu.34, AAVhu.35, AAVhu.37, AAVhu.39, AAVhu.40, AAVhu.41, AAVhu.42, AAVhu.43, AAVhu.44, AAVhu.44R1, AAVhu.44R2, AAVhu.44R3, AAVhu.45, AAVhu.46, AAVhu.47, AAVhu.48, AAVhu.48R1, AAVhu.48R2, AAVhu.48R3, AAVhu.49, AAVhu.51, AAVhu.52, AAVhu.54, AAVhu.55, AAVhu.56, AAVhu.57, AAVhu.58, AAVhu.60, AAVhu.61, AAVhu.63, AAVhu.64, AAVhu.66, AAVhu.67, AAVhu.14/9, AAVhu.t 19, AAVrh.2, AAVrh.2R, AAVrh.8, AAVrh.8R, AAVrh.10, AAVrh.12, AAVrh.13, AAVrh.13R, AAVrh.14, AAVrh.17, AAVrh.18, AAVrh.19, AAVrh.20, AAVrh.21, AAVrh.22, AAVrh.23, AAVrh.24, AAVrh.25, AAVrh.31, AAVrh.32, AAVrh.33, AAVrh.34, AAVrh.35, AAVrh.36, AAVrh.37, AAVrh.37R2, AAVrh.38, AAVrh.39, AAVrh.40, AAVrh.46, AAVrh.48, AAVrh.48.1, AAVrh.48.1.2, AAVrh.48.2, AAVrh.49, AAVrh.51, AAVrh.52, AAVrh.53, AAVrh.54, AAVrh.56, AAVrh.57, AAVrh.58, AAVrh.61, AAVrh.64, AAVrh.64R1, AAVrh.64R2, AAVrh.67, AAVrh.73, AAVrh.74, AAVrh8R, AAVrh8R A586R mutant, AAVrh8R R533A mutant, AAV, BAAV, caprine AAV, bovine AAV, AAVhE1.1,

AAVhEr1.5, AAVhEr1.14, AAVhEr1.8, AAVhEr1.16, AAVhEr1.18, AAVhEr1.35, AAVhEr1.7, AAVhEr1.36, AAVhEr2.29, AAVhEr2.4, AAVhEr2.16, AAVhEr2.30, AAVhEr2.31, AAVhEr2.36, AAVhEr1.23, AAVhEr3.1, AAV2.5T, AAV-PAEC, AAV-LK01, AAV-LK02, AAV-LK03, AAV-LK04, AAV-LK05, AAV-LK06, AAV-LK07, AAV-LK08, AAV-LK09, AAV-LK10, AAV-LK11, AAV-LK12, AAV-LK13, AAV-LK14, AAV-LK15, AAV-LK16, AAV-LK17, AAV-LK18, AAV-LK19, AAV-PAEC2, AAV-PAEC4, AAV-PAEC6, AAV-PAEC7, AAV-PAEC8, AAV-PAEC11, AAV-PAEC12, AAV-2-pre-miRNA-101, AAV-8h, AAV-8b, AAV-h, AAV-b, AAV SM 10-2, AAV Shuffle 100-1, AAV Shuffle 100-3, AAV Shuffle 100-7, AAV Shuffle 10-2, AAV Shuffle 10-6, AAV Shuffle 10-8, AAV Shuffle 100-2, AAV SM 10-1, AAV SM 10-8, AAV SM 100-3, AAV SM 100-10, BNP61 AAV, BNP62 AAV, BNP63 AAV, AAVrh.50, AAVrh.43, AAVrh.62, AAVrh.48, AAVhu.19, AAVhu.11, AAVhu.53, AAV4-8/rh.64, AAVLG-9/hu.39, AAV54.5/hu.23, AAV54.2/hu.22, AAV54.7/hu.24, AAV54.1/hu.21, AAV54.4R/hu.27, AAV46.2/hu.28, AAV46.6/hu.29, AAV128.1/hu.43, true type AAV (ttAAV), UPENN AAV 10, Japanese AAV 10 serotypes, AAV CBr-7.1, AAV CBr-7.10, AAV CBr-7.2, AAV CBr-7.3, AAV CBr-7.4, AAV CBr-7.5, AAV CBr-7.7, AAV CBr-7.8, AAV CBr-B7.3, AAV CBr-B7.4, AAV CBr-E1, AAV CBr-E2, AAV CBr-E3, AAV CBr-E4, AAV CBr-E5, AAV CBr-e5, AAV CBr-E6, AAV CBr-E7, AAV CBr-E8, AAV CHt-1, AAV CHt-2, AAV CHt-3, AAV CHt-6.1, AAV CHt-6.10, AAV CHt-6.5, AAV CHt-6.6, AAV CHt-6.7, AAV CHt-6.8, AAV CHt-P1, AAV CHt-P2, AAV CHt-P5, AAV CHt-P6, AAV CHt-P8, AAV CHt-P9, AAV CKd-1, AAV CKd-10, AAV CKd-2, AAV CKd-3, AAV CKd-4, AAV CKd-6, AAV CKd-7, AAV CKd-8, AAV CKd-B1, AAV CKd-B2, AAV CKd-B3, AAV CKd-B4, AAV CKd-B5, AAV CKd-B6, AAV CKd-B7, AAV CKd-B8, AAV CKd-H1, AAV CKd-H2, AAV CKd-H3, AAV CKd-H4, AAV CKd-H5, AAV CKd-H6, AAV CKd-N3, AAV CKd-N4, AAV CKd-N9, AAV CLg-F1, AAV CLg-F2, AAV CLg-F3, AAV CLg-F4, AAV CLg-F5, AAV CLg-F6, AAV CLg-F7, AAV CLg-F8, AAV CLv-1, AAV CLv1-1, AAV CLv1-10, AAV CLv1-2, AAV CLv-12, AAV CLv1-3, AAV CLv-13, AAV CLv1-4, AAV CLv1-7, AAV CLv1-8, AAV CLv1-9, AAV CLv-2, AAV CLv-3, AAV CLv-4, AAV CLv-6, AAV CLv-8, AAV CLv-D1, AAV CLv-D2, AAV CLv-D3, AAV CLv-D4, AAV CLv-D5, AAV CLv-D6, AAV CLv-D7, AAV CLv-D8, AAV CLv-E1, AAV CLv-K1, AAV CLv-K3, AAV CLv-K6, AAV CLv-L4, AAV CLv-L5, AAV CLv-L6, AAV CLv-M1, AAV CLv-M11, AAV CLv-M2, AAV CLv-M5, AAV CLv-M6, AAV CLv-M7, AAV CLv-M8, AAV CLv-M9, AAV CLv-R1, AAV CLv-R2, AAV CLv-R3, AAV CLv-R4, AAV CLv-R5, AAV CLv-

R6, AAV CLv-R7, AAV CLv-R8, AAV CLv-R9, AAV CSp-1, AAV CSp-10, AAV CSp-11, AAV CSp-2, AAV CSp-3, AAV CSp-4, AAV CSp-6, AAV CSp-7, AAV CSp-8, AAV CSp-8.10, AAV CSp-8.2, AAV CSp-8.4, AAV CSp-8.5, AAV CSp-8.6, AAV CSp-8.7, AAV CSp-8.8, AAV CSp-8.9, AAV CSp-9, AAV.hu.48R3, AAV.VR-355, AAV3B, AAV4, AAV5, AAVF1/HSC1, AAVF11/HSC11, AAVF12/HSC12, AAVF13/HSC13, AAVF14/HSC14, AAVF15/HSC15, AAVF16/HSC16, AAVF17/HSC17, AAVF2/HSC2, AAVF3/HSC3, AAVF4/HSC4, AAVF5/HSC5, AAVF6/HSC6, AAVF7/HSC7, AAVF8/HSC8, AAVF9/HSC9, AAV-PHP.B, AAV-PHP.A, G2B-26, G2B-13, TH1.1-32, TH1.1-35, AAVPHP.B2, AAVPHP.B3, AAVPHP.N/PHP.B-DGT, AAVPHP.B-EST, AAVPHP.B-GGT, AAVPHP.B-ATP, AAVPHP.B-ATT-T, AAVPHP.B-DGT-T, AAVPHP.B-GGT-T, AAVPHP.B-SGS, AAVPHP.B-AQP, AAVPHP.B-QQP, AAVPHP.B-SNP(3), AAVPHP.B-SNP, AAVPHP.B-QGT, AAVPHP.B-NQT, AAVPHP.B-EGS, AAVPHP.B-SGN, AAVPHP.B-EGT, AAVPHP.B-DST, AAVPHP.B-DST, AAVPHP.B-STP, AAVPHP.B-PQP, AAVPHP.B-SQP, AAVPHP.B-QLP, AAVPHP.B-TMP, AAVPHP.B-TTP, AAVPHP.S/G2A12, AAVG2A15/G2A3, AAVG2B4, AAVG2B5 and variants thereof.

8. A method for inhibiting the expression of SOD1 gene in a cell comprising administering to the cell a composition comprising an AAV vector of any one of claims 1-7.

9. The method of claim 8, wherein the cell is a mammalian cell.

10. The method of claim 9, wherein the mammalian cell is a motor neuron.

11. The method of claim 9, wherein the mammalian cell is an astrocyte.

12. A method for treating and/or ameliorating amyotrophic lateral sclerosis (ALS) in a subject in need of treatment, the method comprising administering to the subject a therapeutically effective amount of a composition comprising an AAV vector of any one of claims 1-7.

13. The method of claim 12, wherein the expression of SOD1 is inhibited or suppressed.

14. The method of claim 13, wherein the SOD1 is wild type SOD1, mutated SOD1 with at least one mutation or both wild type SOD1 and mutated SOD1 with at least one mutation.
15. The method of claim 12, wherein the expression of SOD1 is inhibited or suppressed by about 20% to about 100%.
16. The method of claim 12, wherein the ALS is familial ALS with an identified SOD1 gene mutation.
17. The method of claim 12, wherein the ALS is sporadic ALS.
18. A method for inhibiting the expression of SOD1 gene in a cell wherein SOD1 gene embraces a mutation that causes a gain of function effect inside the cell, comprising administering the cell a composition comprising an AAV vector of any one of claims 1-7.
19. The method of claim 18, wherein the cell is a mammalian cell.
20. The method of claim 19, wherein the mammalian cell is a motor neuron.
21. The method of claim 19, wherein the mammalian cell is an astrocyte.

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FIG. 1

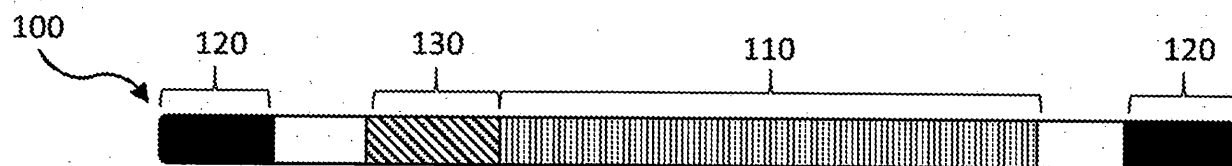


FIG. 2

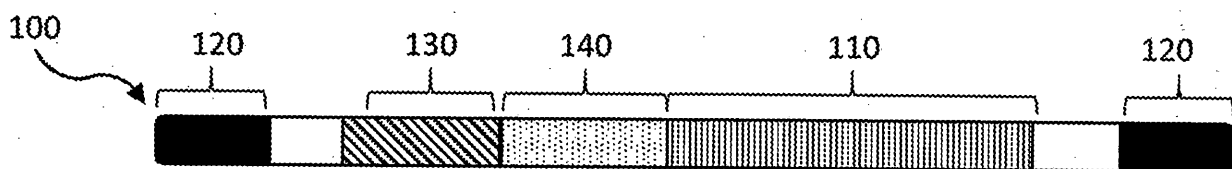
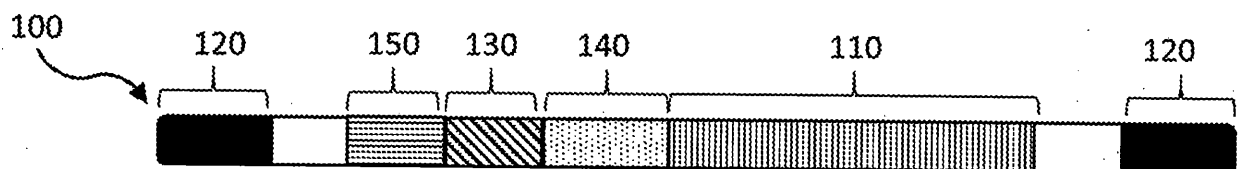


FIG. 3



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FIG. 4

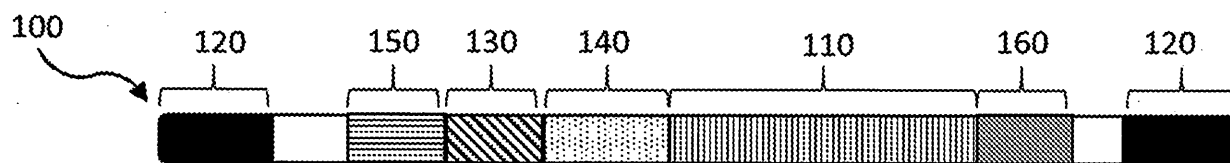


FIG. 5

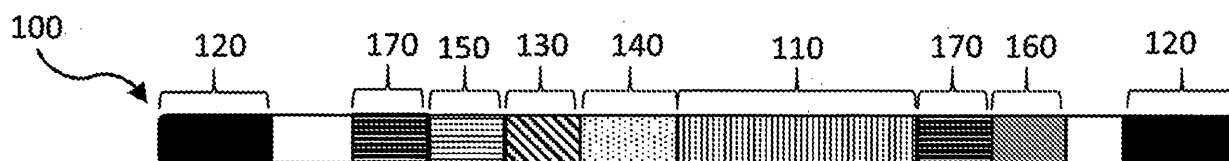
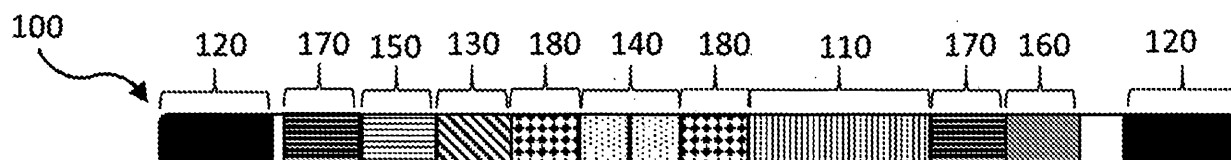


FIG. 6



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FIG. 7

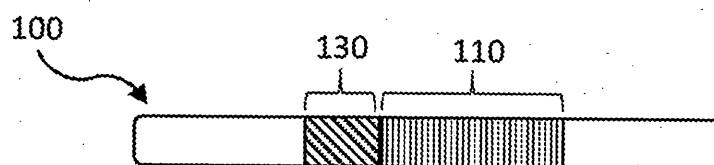


FIG. 8

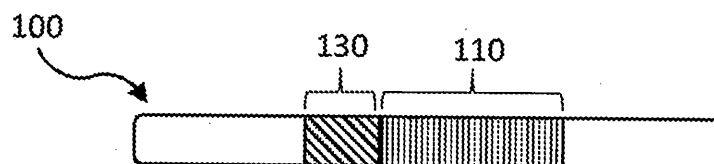
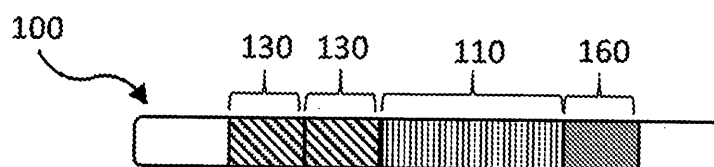


FIG. 9



INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2018/031089

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - A61K 31/713; A61K 48/00; A61P 25/28; C12N 15/11; C12N 15/113; C12N 15/86 (2018.01)

CPC - A61K 48/00; C12N 15/111; C12N 15/113; C12N 15/1137; C12N 15/86; C12N 2310/14; C12N 2750/14143; C12Y 115/01001 (2018.08)

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)

See Search History document

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

USPC - 424/93.2; 435/6; 435/375; 435/455; 435/320.1; 514/44A; 536/23.1; 536/24.5 (keyword delimited)

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

See Search History document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X - Y	WO 2016/077687 A1 (VOYAGER THERAPEUTICS, INC.) 19 May 2016 (19.05.2016) entire document	1-5, 7-21 6
Y	US 2013/0171726 A1 (BENITEC, INC) 04 July 2013 (04.07.2013) entire document	6
A	US 2017/0037410 A1 (IONIS PHARMACEUTICALS, INC.) 09 February 2017 (09.02.2017) entire document	1-21
A	US 2008/0113375 A1 (KHVOROVA et al) 15 May 2008 (15.05.2008) entire document	1-21
A	US 2014/0349390 A1 (VERITAS BIO, LLC) 27 November 2014 (27.11.2014) entire document	1-21
A	WO 2016/077689 A1 (VOYAGER THERAPEUTICS, INC.) 19 May 2016 (19.05.2016) entire document	1-21



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

"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

29 August 2018

Date of mailing of the international search report

13 SEP 2018

Name and mailing address of the ISA/US

Mail Stop PCT, Attn: ISA/US, Commissioner for Patents
P.O. Box 1450, Alexandria, VA 22313-1450

Facsimile No. 571-273-8300

Authorized officer

Blaine R. Copenheaver

PCT Helpdesk: 571-272-4300
PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (January 2015)

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2018/031089

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

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

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:
See extra sheet(s).

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-21 to the extent that they read on a sense nucleic acid sequence of SEQ ID NO:1085 and an antisense nucleic acid sequence of SEQ ID NO:916.

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet (2)) (January 2015)

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2018/031089

Continued from Box No. III Observations where unity of invention is lacking

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees need to be paid.

Group I+: claims 1-21 are drawn to sense nucleic acid sequences and antisense nucleic acid sequences for inhibiting expression of SOD1, and compositions and methods comprising the same.

The first invention of Group I+ is restricted to a sense nucleic acid sequence and an antisense nucleic acid sequence, and compositions and methods comprising the same, wherein the sense nucleic acid sequence is selected to be SEQ ID NO: 1085; and the antisense nucleic acid sequence is selected to be SEQ ID NO:916. It is believed that claims 1-21 read on this first named invention and thus these claims will be searched without fee to the extent that they read on a sense nucleic acid sequence of SEQ ID NO:1085 and an antisense nucleic acid sequence of SEQ ID NO:916.

Applicant is invited to elect additional sense nucleic acid sequences and antisense nucleic acid sequences, each with specified SEQ ID NO, to be searched in a specific combination by paying an additional fee for each set of election. An exemplary election would be a sense nucleic acid sequence and an antisense nucleic acid sequence, and compositions and methods comprising the same, wherein the sense nucleic acid sequence is selected to be SEQ ID NO: 1086; and the antisense nucleic acid sequence is selected to be SEQ ID NO:917. Additional sense nucleic acid sequences and antisense nucleic acid sequences will be searched upon the payment of additional fees. Applicants must specify the claims that read on any additional elected inventions. Applicants must further indicate, if applicable, the claims which read on the first named invention if different than what was indicated above for this group. Failure to clearly identify how any paid additional invention fees are to be applied to the "+" group(s) will result in only the first claimed invention to be searched/examined.

The inventions listed in Groups I+ do not relate to a single general inventive concept under PCT Rule 13.1, because under PCT Rule 13.2 they lack the same or corresponding special technical features for the following reasons:

The Groups I+ formulas do not share a significant structural element responsible for inhibiting expression of a SOD1 gene, requiring the selection of alternatives for the sense nucleic acid sequence and the antisense nucleic acid sequence, where "the sense strand sequence comprises at least 15 contiguous nucleotides differing by no more than 3 nucleotides from the nucleotide sequence of sequences listed in Table 3 and the antisense strand sequence comprises at least 15 contiguous nucleotides differing by no more than 3 nucleotides from the nucleotide sequence of sequences listed in Table 2".

Additionally, even if Groups I+ were considered to share the technical features of an adeno-associated viral (AAV) vector comprising a first nucleic acid sequence comprising two inverted terminal repeats (ITRs), a promoter, an intron and a second nucleic acid sequence, wherein said second nucleic acid sequence when expressed inhibits or suppresses expression of SOD1 in a cell, wherein said second nucleic acid sequence comprises a sense strand sequence and an antisense strand sequence, wherein the sense strand sequence comprises at least 15 contiguous nucleotides and the antisense strand sequence comprises at least 15 contiguous nucleotides and wherein said sense strand sequence and antisense strand sequence share a region of complementarity of at least four nucleotides in length; these shared technical features do not represent a contribution over the prior art.

Specifically, WO 2016/077689 A1 to Voyager Therapeutics, Inc. discloses an adeno-associated viral (AAV) vector (such modulatory polynucleotides may be encoded by or contained within plasmids or vectors or recombinant adeno-associated viruses (AAV) and may comprise artificial microRNAs, Para. [0009]) comprising a first nucleic acid sequence comprising two inverted terminal repeats (ITRs) (may be located between the 5' end of the flip ITR and the 3' end of the flop ITR in an expression vector, Para. [0051]), a promoter (modulatory polynucleotide may be located downstream of a promoter such as, but not limited to, CMV, U6, CBA or a CBA, Para. [0048]), an intron (promoter with a SV40 intron in an expression vector, Para. [0041]) and a second nucleic acid sequence, wherein said second nucleic acid sequence when expressed inhibits or suppresses expression of SOD1 in a cell (the modulatory polynucleotides of the invention may target any gene known in the art. As a non-limiting example, the gene may be SOD1, Para. [00139]; the first payload region sequence may be a guide strand of a siRNA construct and the second payload region sequence may be a passenger strand of an siRNA construct. The passenger and guide sequences may be substantially complementary to each other, Para. [0075]), wherein said second nucleic acid sequence comprises a sense strand sequence and an antisense strand sequence, wherein the sense strand sequence comprises at least 15 contiguous nucleotides (The 19mers, along with the 5' most position of the sense strand are shown in Table 4 along with the antisense strand which is the reverse complement of the sense strand, Para. [00331]) and the antisense strand sequence comprises at least 15 contiguous nucleotides (3' arm of the stem loop comprises a guide strand. This strand is also known as the antisense strand in that it reflects homology to a target. The guide strand may be between 15-30 nucleotides in length, 21-25 nucleotides or 22 nucleotides in length, Para. [0058]) and wherein said sense strand sequence and antisense strand sequence share a region of complementarity of at least four nucleotides in length (The passenger and guide strands may be completely complementary across a substantial portion of their length, Para. [0061]).

The inventions listed in Groups I+ therefore lack unity under Rule 13 because they do not share a same or corresponding special technical features.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2018/031089

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:

☒ in the form of an Annex C/ST.25 text file.

☐ on paper or in the form of an image file.

b. ☐ furnished together with the international application under PCT Rule 13*ter*. I(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.

c. ☐ furnished subsequent to the international filing date for the purposes of international search only:

☐ in the form of an Annex C/ST.25 text file (Rule 13*ter*. I(a)).

☐ on paper or in the form of an image file (Rule 13*ter*. I(b) and Administrative Instructions, Section 713).

2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

SEQ ID NOs: 916-925 and 1085-1094 were searched.

Possible steps after receipt of the international search report (ISR) and written opinion of the International Searching Authority (WO/ISA)

General information

For all international applications, the competent International Searching Authority (ISA) will establish an international search report (ISR) accompanied by a written opinion of the International Searching Authority (WO/ISA). The WO/ISA may be responded to by

- filing informal comments with the **International Bureau of WIPO (IB)** (where no demand for international preliminary examination (**demand**) is filed)
- filing amendments under Art. 19 PCT (this can be done whether or not a **demand** is filed)
- filing amendments under Art. 34 PCT and/or formal observations in response to objections raised in the **WO/ISA** (where a **demand** is actually filed)

This document explains these possibilities.

Filing informal comments

After receipt of the **ISR and WO/ISA**, the applicant may file informal comments on the **WO/ISA**, **directly with the IB** (see International Search and Preliminary Examination Guidelines 2.15). These will be communicated to the designated/elected Offices, together with the International Preliminary Report on Patentability (**IPRP**) at 30 months from the priority date.

Amending claims under Art. 19 PCT

The applicant may file **amended claims** under Art. 19 PCT, **directly with the IB** by the later of the following dates:

- 2 months from the date of mailing of the **ISR** and the **WO/ISA**
- 16 months from the priority date

However, any such amendment received by the **IB** after the expiration of the applicable time limit shall be **considered to have been received on time** by the **IB**, if it reaches it **before** the technical preparations for international publication have been completed (the 15th day prior to the date of publication, see PCT Applicant's Guide, International Phase, 9.013).

For further information, please see Rule 46 PCT as well as form PCT/ISA/220.

Please also note that, when filing amended claims under Art. 19 PCT, such amendments shall be **accompanied by a letter** identifying the amendments made and also the basis for the amendments in the application as originally filed (Rule 46.5(b) PCT). Where a **demand** is filed, failure to comply with this requirement may result in the amendments being ignored in the International Preliminary Examination Report (**IPER**), see Rule 70.2(c-bis) PCT.

Filing a demand for international preliminary examination

In principle, the **WO/ISA** will be considered to be the written opinion of the International Preliminary Examining Authority (**IPEA**). Where the **WO/ISA** issued by the **EPO** as **ISA** gives a positive opinion on the international application and the invention to which it relates, filing a **demand** with the **EPO** as **IPEA** would normally be unnecessary, since a positive **IPRP** would anyway be established by the **IB** based on the **WO/ISA** (see also further below).

If the applicant wishes to file a **demand** (for example, to allow him to argue his case in international preliminary examination with regard to objections raised in a negative **WO/ISA** before the **IPEA** issues an **IPER**), this must be done before expiration of **3 months after the date of mailing of the ISR and WO/ISA** or **22 months after priority date**, whichever expires later (Rule 54*bis* PCT). Amendments under Art. 34 PCT can be filed with the **IPEA**, normally at the same time as filing the demand (Rule 66.1(b) PCT) or within the time limit set for reply to any written opinion issued during international preliminary examination by the **IPEA**.

If a **demand** is filed at the **EPO** as **IPEA** and no comments/amendments have been received by the time the **EPO** starts drawing up the **IPER** (Rule 66.4*bis* PCT), the **WO/ISA** will be transformed by the **IPEA** into an **IPER** (also called the **IPRP (Chapter II)** which would merely reflect the content of the **WO/ISA** (OJ 10/2011, 532). The **demand** can still be withdrawn (Art. 37 PCT).

Please also note that, when filing amendments under Art. 34 PCT, such amendments shall be accompanied by a letter which identifies the amendments made and also the basis for the amendments in the application as originally filed (Rule 66.8(a) PCT). Failure to comply with this requirement may result in the amendments being ignored in the **IPER (IPRP (Chapter II))**, see Rule 70.2(c-*bis*) PCT.

Filing a request for supplementary international search

The applicant may, with the **IB**, file a request for **supplementary international search** under Rule 45*bis*.1 PCT. The present **ISR** and **WO/ISA** may also be taken into account in the execution of that supplementary international search, provided that these are available to the Authority charged with this task before it starts the supplementary search (Rule 45*bis*.5 PCT).

This kind of request **cannot be filed specifying the ISA** who did the international search.

More information on this topic can be found in the **PCT Applicant's Guide**, Chapter 8 (<http://www.wipo.int/pct/en/guide/ip08.html>).

End of the international phase

Where no **demand** is filed, at the end of the international phase, the **IB** will transform the **WO/ISA** into the **IPRP (PCT Chapter I)** (Rule 44*bis* PCT), which will then be transmitted together with possible informal comments to the designated Offices. Where a demand is filed, the **WO/ISA** is not transformed into an **IPRP (Chapter I)** by the **IB**, but rather the **IPEA** will establish an **IPER**, (the **IPER** is the same as the **IPRP (PCT Chapter II)**, see Rule 70.15 PCT).

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Antisense oligonucleotides extend survival and reverse decrement in muscle response in ALS models

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Research Article

Neuroscience

Therapeutics

Mutations in superoxide dismutase 1 (SOD1) are responsible for 20% of familial ALS. Given the gain of toxic function in this dominantly inherited disease, lowering SOD1 mRNA and protein is predicted to provide therapeutic benefit. An early generation antisense oligonucleotide (ASO) targeting SOD1 was identified and tested in a phase I human clinical trial, based on modest protection in animal models of SOD1 ALS. Although the clinical trial provided encouraging safety data, the drug was not advanced because there was progress in designing other, more potent ASOs for CNS application. We have developed next-generation SOD1 ASOs that more potently reduce SOD1 mRNA and protein and extend survival by more than 50 days in SOD1^{G93A} rats and by almost 40 days in SOD1^{G93A} mice. We demonstrated that the initial loss of compound muscle action potential in SOD1^{G93A} mice is reversed after a single dose of SOD1 ASO. Furthermore, increases in serum phospho-neurofilament heavy chain levels, a promising biomarker for ALS, are stopped by SOD1 ASO therapy. These results define a highly potent, new SOD1 ASO ready for human clinical trial and suggest that at least some components of muscle response can be reversed by therapy.

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Antisense oligonucleotides extend survival and reverse decrement in muscle response in ALS models

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Mutations in superoxide dismutase 1 (SOD1) are responsible for 20% of familial ALS. Given the gain of toxic function in this dominantly inherited disease, lowering SOD1 mRNA and protein is predicted to provide therapeutic benefit. An early generation antisense oligonucleotide (ASO) targeting SOD1 was identified and tested in a phase I human clinical trial, based on modest protection in animal models of SOD1 ALS. Although the clinical trial provided encouraging safety data, the drug was not advanced because there was progress in designing other, more potent ASOs for CNS application. We have developed next-generation SOD1 ASOs that more potently reduce SOD1 mRNA and protein and extend survival by more than 50 days in SOD1^{G93A} rats and by almost 40 days in SOD1^{G93A} mice. We demonstrated that the initial loss of compound muscle action potential in SOD1^{G93A} mice is reversed after a single dose of SOD1 ASO. Furthermore, increases in serum phospho-neurofilament heavy chain levels, a promising biomarker for ALS, are stopped by SOD1 ASO therapy. These results define a highly potent, new SOD1 ASO ready for human clinical trial and suggest that at least some components of muscle response can be reversed by therapy.

Introduction

Amyotrophic lateral sclerosis (ALS) is caused by the loss and dysfunction of neurons in motor pathways and leads to severe weakness, stiffness, and ultimately death in 3–5 years. Approximately 15%–20% of ALS can be associated with a genetic cause. Among the genetically defined ALS cases, about 20% are associated with mutations in the superoxide dismutase 1 (SOD1) gene. Although the exact toxicity of SOD1 mutations remains incompletely understood, there is a consensus that there is a toxic gain of function, and some key data support this idea. First, overexpression of mutant SOD1 in mice or rats recapitulates important aspects of human ALS, including loss of neuromuscular junction innervation and motor neuron death (1, 2). Second, loss of SOD1, while resulting in eventual motor neuron dysfunction, does not result in motor neuron death (3, 4). Third, in humans, individual disease mutations are associated with varying levels of SOD1 activity, yet there is no correlation between disease severity and SOD1 activity (5–7). Given the toxic gain-of-function role of SOD1, lowering levels of SOD1 is predicted to be therapeutic.

One method of lowering SOD1 is to target the SOD1 mRNA using antisense oligonucleotides (ASOs) that bind to the SOD1 mRNA by Watson-Crick base pairing. The ASO activates RNase H1 that destroys the targeted RNA. Direct delivery of ASOs to cerebral spinal fluid (CSF) affords widespread distribution of ASOs throughout the brain and spinal cord in rodents and nonhuman primates (8–13). ASOs for CNS disorders have advanced to clinical trial for Huntington's disease (registered at ClinicalTrials.gov, NCT02519036), SOD1-associated ALS (NCT02623699), and Alzheimer's disease (NCT03186989). Most impressively, a splicing-modifying ASO has recently been approved by the FDA (14) for spinal muscular atrophy (SMA) (15).

An SOD1 ASO was used both in the first demonstration of the utility of ASOs in animal models of CNS disease (8, 16) and the first human clinical trial (9). Although there was tremendous enthusiasm for the early SOD1 ASO (ASO 333611), effects in the animal models were modest and advances in ASO technology identified other, more effective ASO designs for CNS disease, such that it became likely that a more potent ASO could be developed. We describe here the recent development of more potent ASOs with substantial prolongation of survival in SOD1 rodent models. The increased efficacy of these ASOs allowed us to test how changes in disease markers may be halted or reversed in those models, a key question for application of this therapy in human clinical trials. Suppression of SOD1 after disease onset reversed the disease course, indicating that intervention in symptomatic patients may not only halt disease but also reverse some aspects of it. The work described here provided the foundation for advancement of this compound to the ongoing human clinical trial in SOD1 ALS patients (NCT02623699).

Conflict of interest: AM, GST, BJF, LS, YL, MZ, ST, DLG, and DWS are employees of Biogen. TC, CFB, HBK, and EES are employees of Ionis Pharmaceuticals. Ionis Pharmaceuticals provides antisense oligonucleotides to TMM. Biogen provides support to TMM for clinical studies. TMM has participated in a medical advisory board for Biogen and is a consultant for Cytokinetics. TMM and Washington University have licensed technology regarding SOD1 protein kinetic measurements to C2N. MC is a consultant for Cytokinetics, Lilly, Orion, Biohaven, Wave and Biogen.

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<https://doi.org/10.1172/JCI99081>.



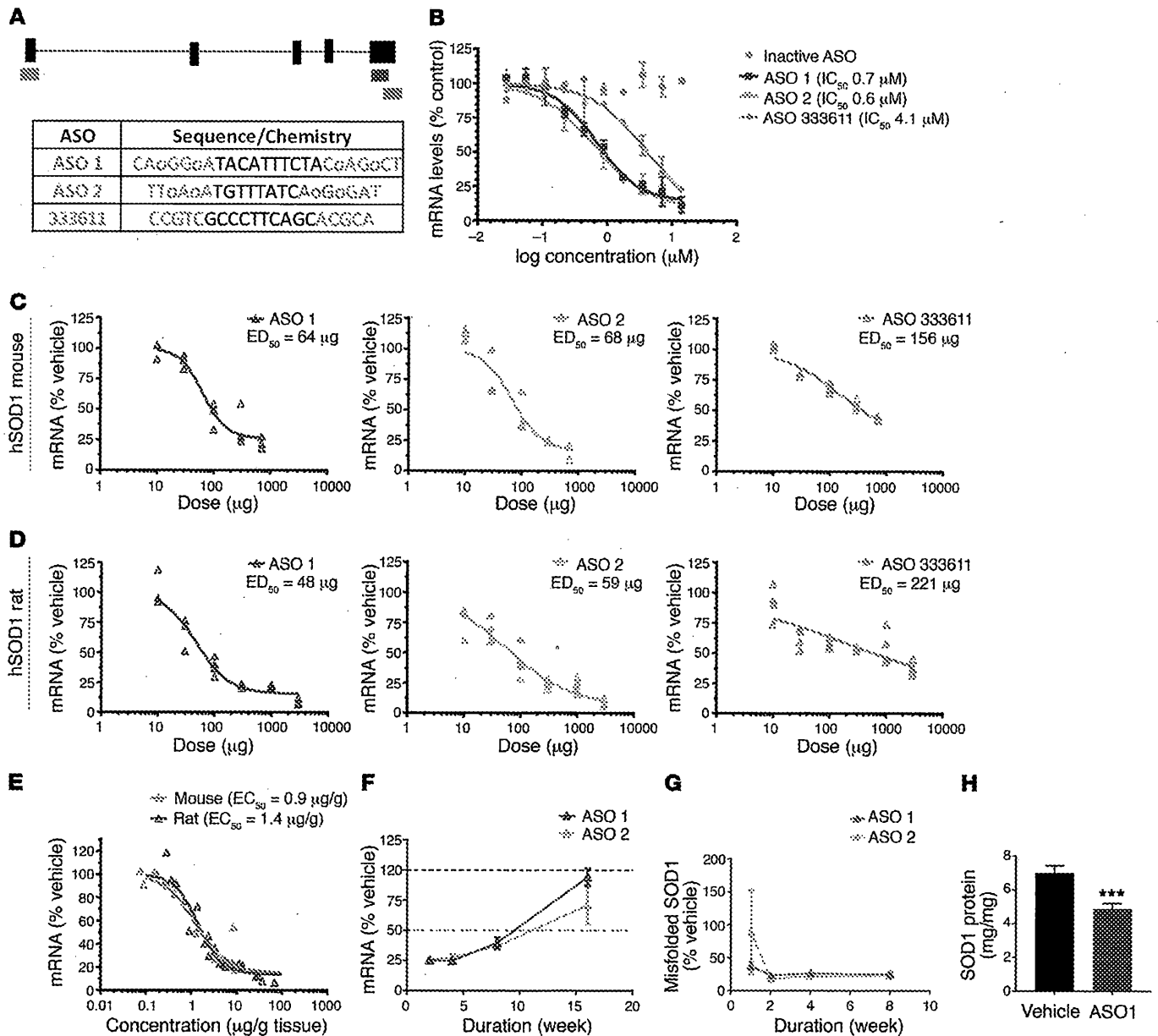


Figure 1. New SOD1 ASOs reduce mRNA and protein in vitro and in vivo, and are more potent than the previous SOD1 lead. (A) Oligonucleotides containing phosphorothioate backbone modifications (unmodified phosphodiester linkages noted with red o) and 2'-O-methoxyethylribose (MOE; orange) and (S)-2',4'-constrained 2'-O-ethyl (cEt; blue) groups in the 5' and 3' wings were targeted to the 3' UTR of SOD1 mRNA (ASO 1 and ASO 2), exon 1 of SOD1 mRNA (333611), or nothing in the human or rodent genome (inactive ASO). The sequences evaluated and location of chemical modifications are provided. (B) SHSY5Y cells were treated with SOD1 ASOs by electroporation. After 24 hours, SOD1 mRNA was measured. New SOD1 ASOs are more potent than the previous lead ASO ($n = 2$ per concentration, average $\pm$ range). (C and D) Candidate ASOs for human SOD1 were screened in mice (C) and rats (D) expressing human SOD1. ASOs were injected i.c.v. (mice) and intrathecally (rats) and mRNA was measured in lumbar spinal cord 2 weeks after dosing. Potent ASOs were identified that lowered SOD1 mRNA in both species with ED_{50} between 50 μ g and 70 μ g ($n = 3$ per dose, individual animals). (E) Tissue concentrations of ASO were measured and correlated with SOD1 mRNA lowering, demonstrating an EC_{50} of 0.9 μ g/g in mice and 1.4 μ g/g in rats ($n = 24$). (F) A single intrathecal bolus of 500 μ g ASO 1 or ASO 2 was given to SOD1^{G93A} rats. SOD1 mRNA levels were assessed in the spinal cord at 2, 4, 8, and 16 weeks after bolus. ASO 1 and ASO 2 suppressed SOD1 mRNA levels for more than 8 weeks ($n = 2-7$ per time point, average $\pm$ SEM). (G) A single intraventricular bolus of ASO 1 or ASO 2 was given to SOD1^{G93A} rats. Misfolded SOD1 protein from spinal cord was assessed at 1, 2, 4, and 8 weeks after bolus ($n = 7$ per time point, average $\pm$ SEM). (H) Six weeks after a single intraventricular bolus of ASO 1, SOD1 protein was quantified in the lumbar spinal cord of SOD1^{G93A} rats. *** $P = 0.0005$.

Results

Next-generation SOD1 ASOs more potently reduce mRNA and protein in rodent models compared with previous ASOs. Based on an in vitro screen of over 2,000 ASOs targeting the human SOD1 gene and subsequent optimization, 2 particularly potent ASOs targeting the 3' UTR were selected for further testing (Figure 1A). In a

human neuroblastoma cell line (SH-SY5Y), these new ASOs (ASO 1 and ASO 2) potently lowered SOD1 mRNA in a dose-dependent manner (Figure 1B). The new ASOs were more efficacious than the previous SOD1 ASO, 333611 (Figure 1B and Supplemental Figure 1A; supplemental material available online with this article; <https://doi.org/10.1172/JCI99081DS1>). To determine the potency of these

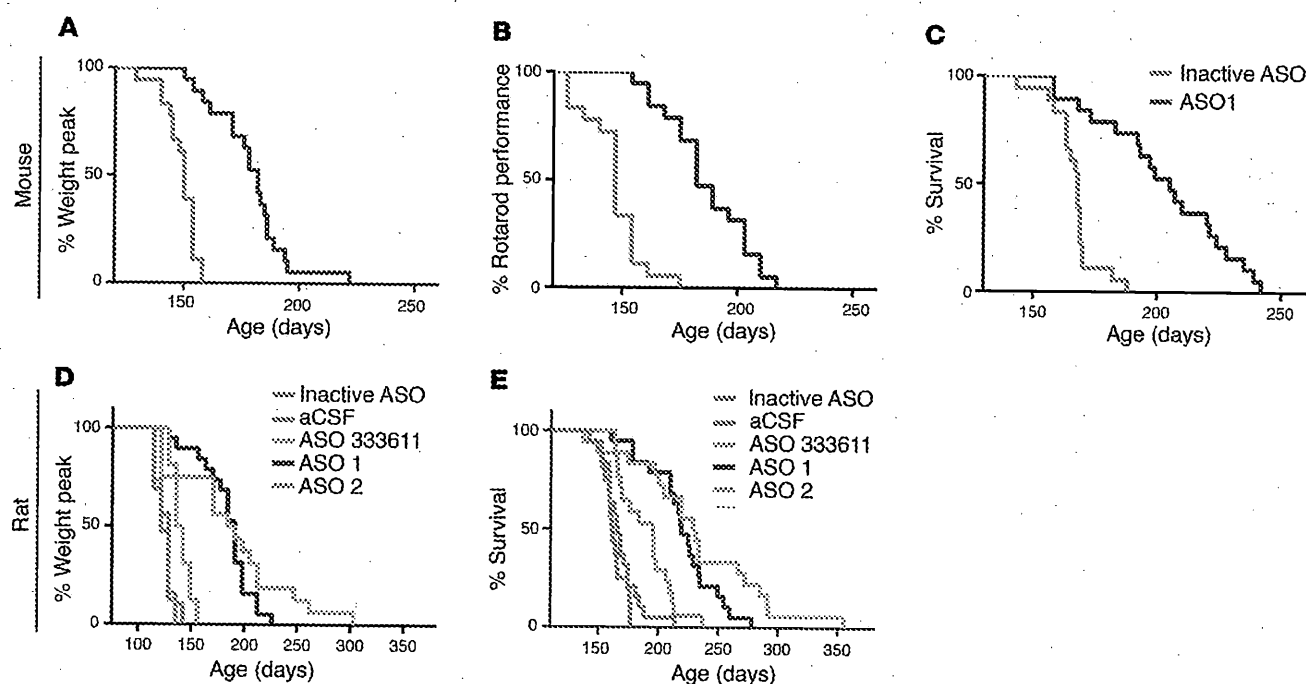


Figure 2. SOD1 ASOs prolong onset, survival, and motor performance of SOD1^{G93A} mutant animals. (A–C) Mice were dosed i.c.v. twice, at day 50 and again at day 94, each time with 300 μ g ASO 1 ($n = 20$ per treatment group, all females). (A) Onset of disease was scored as percentage of animals losing 10% of peak body weight. Median onset for mice treated with the control ASO was 140 days, whereas treatment with ASO 1 increased median onset to 183 days ($P < 0.0001$, log-rank Mantel-Cox). (B) Motor performance was tested through the rotarod test: mice were tested once a week starting at 80 days of age until they could not stay on the rod for at least 30 seconds. ASO 1 treatment significantly increased rotarod performance: in the control group, the median age at which 50% decrease in performance was reached was 147 days, whereas in the ASO 1-treated animals, the median increased significantly to 182 days ($P < 0.0001$, 2-way ANOVA). (C) The median survival of mice treated with the control ASO was 168 days, whereas the treatment with ASO 1 increased median survival to 205 days ($P < 0.0001$, log-rank Mantel-Cox). (D and E) Rats were injected intrathecally with a 1,000 μ g single bolus dose of inactive control ASO ($n = 16$), aCSF vehicle control ($n = 19$), ASO 333611 ($n = 17$), ASO 1 ($n = 19$), or ASO 2 ($n = 18$). (D) Rats treated with ASO 1 or ASO 2 maintained weight 70 days ($P < 0.0001$) and 67 days ($P < 0.001$) longer, respectively, than rats treated with aCSF. 333611 delayed onset of weight loss modestly (median 139 days, compared with aCSF median 121 days). (E) Survival was markedly prolonged in the ASO 1 and ASO 2 treatment groups by 53 days ($P < 0.0001$) and 64 days ($P < 0.0001$), respectively, compared with aCSF control treatment in which rats survived to a median age of 166 days. This represents a 32% (ASO 1) and 39% (ASO 2) extension of survival. P values were determined by log-rank (Mantel-Cox) test.

compounds in vivo, ASOs were delivered to the CSF of mice and rats expressing human SOD1^{G93A} via intraventricular (mice) or intrathecal (rats) bolus injection. CSF administration distributes ASOs throughout the brain and spinal cord (8–13). Human SOD1 mRNA was reduced in spinal cords of SOD1^{G93A} mice in a dose-dependent manner (Figure 1C) with approximately 75% maximum reduction of SOD1 following a single injection of ASO 1 or ASO 2. Again, ASO 1 and ASO 2 were substantially more active and efficacious than the previous ASO 333611. Similar dose-dependent reductions in human SOD1 mRNA were achieved in SOD1^{G93A} rats (Figure 1D), with ASO 1 and ASO 2 exhibiting greater activity than 333611 (Figure 1D). Potency of the lead ASO 1 was indistinguishable in SOD1^{G93A} mice and rats, with a lumbar cord EC₅₀ of 0.9 μ g/g and 1.4 μ g/g tissue, respectively (Figure 1E). ASO 1 and ASO 2 also exhibit prolonged duration of action, with nearly 10 weeks of sustained SOD1 mRNA lowering following a single intrathecal bolus injection in SOD1^{G93A} rats (Figure 1F). Similarly, misfolded SOD1 also exhibits sustained reduction after a single bolus injection (Figure 1G). Consistent with our previous findings, native SOD1 mRNA (Supplemental Figure 1B) and protein (Figure 1H) are also reduced in spinal cord tissue following ASO treatment.

Treatment with SOD1-lowering ASO significantly delays disease onset and extends survival in SOD1^{G93A} mice and rats. Expression of

mutant SOD1 in SOD1^{G93A} rodent models is known to cause severe atrophy of the limbs and trunk that leads to loss of motor function and eventually death (17). To investigate whether a SOD1-lowering ASO strategy could delay disease parameters, SOD1^{G93A} mice were injected intraventricularly with bolus doses of 300 μ g ASO 1 at 50 and 94 days of age. Weight and performance on rotarod were tested weekly. Mice that received ASO 1 maintained weight 26 days longer and performed better on rotarod than mice injected with a control inactive ASO (inactive ASO) at similar concentrations (Figure 2, A and B). Median survival for ASO 1-treated mice was 37 days longer compared with inactive ASO, representing a 22% extension of survival in the mouse model (Figure 2C).

SOD1^{G93A} transgenic rats were injected intrathecally with a single bolus dose of artificial CSF (aCSF) or 1,000 μ g ASO vehicle, ASO 333611, ASO 1, ASO 2, or inactive ASO. ASO 333611 is the previous SOD1 ASO as described in reference 8 and used in a phase I study in SOD1 ALS participants (9). Rats assigned to the lead ASO treatment groups performed better in all categories when compared with the vehicle treatment group. SOD1^{G93A} rats treated with ASO 1 or ASO 2 maintained weight 70 days ($P < 0.0001$) and 67 days ($P < 0.001$) longer, respectively, than rats treated with aCSF (Figure 2D). In contrast, the intrathecal bolus of ASO 333611 or inactive ASO did not significantly delay onset

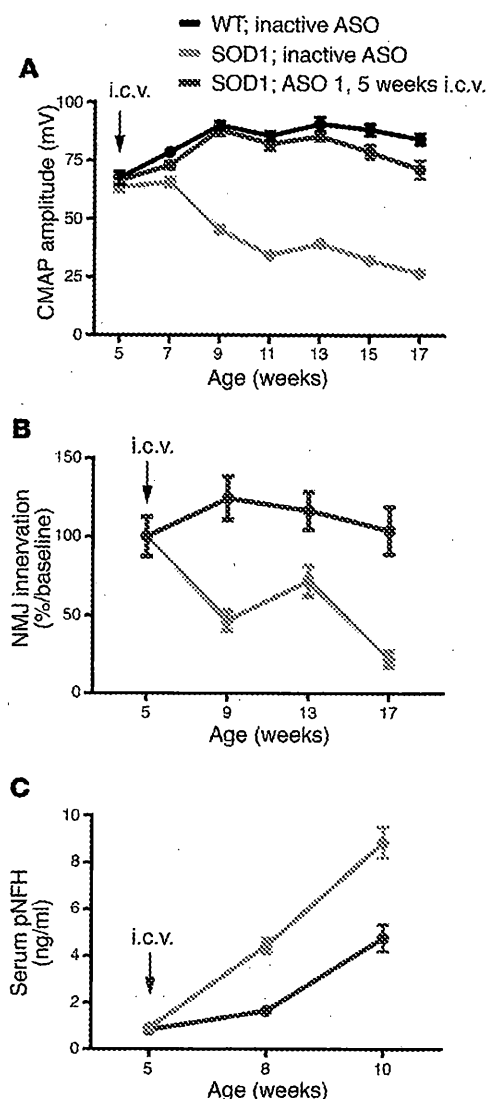


Figure 3. One single i.c.v. injection of SOD1 ASO extensively preserves neuromuscular synapses and neuronal loss in SOD1^{G93A} mutant mice. (A–C) Mice were injected once at 5 weeks of age with a single dose of 300 μ g ASO 1. Compound muscle action potential (CMAP), neuromuscular junctions (NMJ), and phospho-neurofilament heavy chain (pNFH) were measured at multiple time points ($n = 12$ per group; average $\pm$ SEM). (A) Results validated a significant effect of ASO 1 as compared with the inactive ASO (significant at weeks 7–17). CMAP values recorded from ASO 1-treated animals were not significantly different from those recorded from WT animals except at weeks 15 and 17 ($P < 0.001$ and $P < 0.0001$, respectively, 2-way ANOVA). (B) ASO 1 significantly protected SOD1 mice from NMJ loss at the tibialis anterior muscle. Although there was a significant difference in NMJ number between WT animals and SOD1 animals injected with a control ASO at all ages analyzed (data not shown), ASO 1-treated mice did not show significant differences compared with WT mice ($P < 0.0001$). (C) Blood was collected at baseline (5 weeks) before i.c.v. injection, and then again at 8 and 10 weeks of age. pNFH serum levels were significantly decreased at 8 and 10 weeks of age by ASO 1 treatment ($P < 0.001$, 2-way ANOVA).

of phospho-neurofilament in SOD1^{G93A} mice. To test whether SOD1 reduction by ASO could affect markers of disease, SOD1^{G93A} mice were injected intraventricularly once at 5 weeks of age with 100 μ g ASO 1 and evaluated for changes in compound muscle action potential (CMAP), neuromuscular junction innervation, and serum phospho-neurofilament heavy chain (pNFH) levels. In SOD1^{G93A} mice, CMAP declines over time, preceding the loss of motor neurons (18, 19). SOD1^{G93A} mice treated with ASO 1 at 5 weeks maintained CMAP over the next 12 weeks, whereas the control-treated animals' CMAP was reduced by more than half over the same time period (Figure 3A). Consistent with CMAP electrophysiological demonstration of preserved muscle function, ASO 1-treated mice maintained innervation of the tibialis anterior muscles in the hind limbs, whereas control-treated animals showed evidence of denervation of more than 75% of muscle endplates (Figure 3B). pNFH increases in CSF and serum in ALS rodent models, human patients with ALS, and other neurodegenerative diseases and it has been proposed as a potential pharmacodynamics marker (20–22). SOD1 mice treated with ASO 1 showed lower levels of pNFH compared with SOD1 mice treated with control ASO (Figure 3C).

Reversal of SOD1-mediated neuronal dysfunction may be achieved by treatment with SOD1 ASO and is measurable by CMAP and pNFH levels. Two major questions regarding markers of disease in neurodegenerative diseases are: to what extent therapeutics may be able reverse disease and whether this reversal can be measured. SOD1^{G93A} mice were treated at 9 weeks of age when CMAP had clearly begun to decline and pNFH had begun to rise (Figure 4, A and B). Treatment with ASO 3, an SOD1 ASO with dose-dependent reductions in SOD1 mRNA similar to those in ASO 1 and ASO 2 (Supplemental Figure 4), demonstrated a sustained increase in CMAP whereas control ASO-treated mice showed a continued steady decline (Figure 4A). Similarly, pNFH, which at time of injection (9 weeks) had already begun to rise in SOD1^{G93A} mice (Figure 3C), was still significantly lower 8 weeks after treatment with ASO 3 (inactive ASO-treated group, 22 ng/ml $\pm$ 1.9 ng/ml; ASO 3-treated group, 12 ng/ml $\pm$ 1.2 ng/ml; mean $\pm$ SEM, $P < 0.001$) (Figure 4B). We also found that both pNFH serum and CSF levels were responsive to SOD1 ASO treatment in ALS

when compared with the aCSF group (delayed by 17.5 days and 7 days, respectively). Survival was markedly prolonged in the ASO 1 and ASO 2 treatment groups by 53 days ($P < 0.0001$) and 64 days ($P < 0.0001$), respectively (Figure 2E), compared with aCSF control treatment in which rats survived to a median age of 166 days. This represents a 32% (ASO 1) and 39% (ASO 2) extension of survival. The effect was similar for both male and female rats (Supplemental Figure 2). This result is substantially improved compared with published results using ASO 333611 infused intraventricularly (10 days, ref. 8) or compared with ASO 333611 delivered intrathecally in this study (195-day median survival, 29-day prolongation) (Figure 2E).

To assess if timing of administration impacts disease onset or survival, SOD1^{G93A} mice were dosed with either ASO 1 or inactive control by i.c.v. bolus at a single time point, either day 80 or 110. Disease onset was delayed and survival markedly extended regardless of the time point of injection (Supplemental Figure 3). There was no significant difference in the onset or survival between the 80-day active ASO treatment and the 110-day active ASO treatment.

SOD1 ASO treatment preserves compound muscle action potential, maintains neuromuscular innervation, and results in lower levels



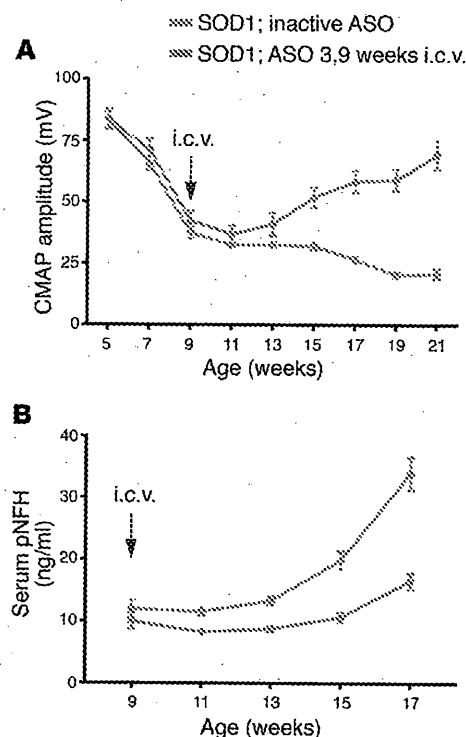


Figure 4. SOD1 ASO injected at 9 weeks of age is able to reverse CMAP amplitudes and lower serum pNFH levels in SOD1^{G93A} mutant mice. (A and B) Mice were injected once at 9 weeks of age with either a control ASO or with ASO 3 (100 μ g). (A) CMAP amplitudes at the tibialis anterior muscles were recorded at baseline (5 weeks) and then every other week thereafter ($n = 12$ per group, average $\pm$ SEM). On the week of dosing (9 weeks), CMAP was recorded prior to i.c.v. injection. Typically, SOD1 mice show a steady decline in the CMAP amplitudes recorded at the tibialis anterior; however, one single dose of ASO 3 at 9 weeks of age was able to reverse the trend and by 15 weeks the ASO 3-treated mice had CMAP amplitudes significantly higher than mice treated with a control ASO ($P < 0.001$, 2-way ANOVA). (B) Blood was collected from each animal at 9, 11, 13, 15, and 17 weeks of age and pNFH levels were quantified. pNFH serum levels of control mice showed a steady increase whereas those of ASO 3-treated mice did not. Levels at 15 weeks: inactive ASO, 19.9 ng/ml $\pm$ 5.1 ng/ml; ASO 3, 10.6 ng/ml $\pm$ 2.5 ng/ml. Levels at 17 weeks: inactive ASO, 33.96 ng/ml $\pm$ 9.3 ng/ml; ASO 3, 16.5 ng/ml $\pm$ 4.3 ng/ml. $P < 0.0001$, 2-way ANOVA, $n = 12$ per group, average $\pm$ SEM.

rats (Supplemental Figure 5). Furthermore, it was recently demonstrated that motor neuron-enriched miR-218 is temporally increased in ALS rat model CSF and its levels correlate with motor neuron loss (23). Here, we found that miR-218 CSF levels were also responsive to SOD1 ASO therapy (Supplemental Figure 5). These results suggest that SOD1-mediated neuronal dysfunction is, at least in part, reversible with a potent therapeutic.

SOD1 ASOs lower SOD1 mRNA and protein in nonhuman primates. To evaluate the distribution and pharmacokinetic properties of the new SOD1 ASO in a larger brain, ASO 1 was delivered via intrathecal injection to cynomolgus monkeys. ASO 1 was optimized for targeting human SOD1, but it is complementary to nonhuman primate (NHP) SOD1 with a 1-base mismatch, and it is active in NHP cells (Supplemental Figure 6). ASO 1 was evaluated in NHPs at doses of 4 mg, 12 mg, and 35 mg delivered intrathecally, with each monkey receiving 5 doses. One week after the final dose, SOD1 mRNA was lowered broadly in the CNS in a dose-

dependent manner (Figure 5A). Quantification of drug levels in various tissues confirmed a pharmacokinetic/pharmacodynamic relationship, with an estimated composite EC₅₀ of 20 μ g/g tissue (Figure 5B). This is consistent across all tissues assessed (Supplemental Figure 7). The decrease in potency in NHP SOD1 compared with human SOD1 in transgenic rodents (Figure 1E, EC₅₀ 0.9–1.4 μ g/g) is likely attributable, in part, to the single mismatch of ASO 1 to the NHP SOD1 transcript. SOD1 protein levels in the tissue were lowered by approximately 50% (Figure 5C). A corresponding dose-dependent decrease in CSF SOD1 levels was also observed, confirming CSF SOD1 levels reflect target reduction in the CNS in the larger NHP brain (Figure 5D). CSF SOD1 protein remains reduced approximately 100 days after the last administration (Figure 5E). These data in NHPs demonstrate effectiveness of the SOD1 ASO in a larger animal and are an important step toward understanding the use of the compound in human clinical trials.

Discussion

ASOs targeting SOD1 were previously used to demonstrate widespread distribution of ASOs in the CNS following CSF delivery, proof of principle in animal models, and important early safety in a first-in-humans clinical study (8, 9, 24). Benefiting from advancements in ASO technology, here we describe other, more potent ASOs that demonstrate a prolonged survival benefit in both mouse and rat SOD1 models, the longest to date using a relevant therapeutic. With this added ASO potency, we were able to test whether treating after the disease progressed would also provide benefit, and we found a notable reversal of the CMAP. These data establish a novel therapeutic for SOD1-mediated ALS, which is currently under clinical investigation (NCT02623699).

For many genetically linked neurodegenerative diseases, patients remain asymptomatic for more than 5 decades but when they develop signs of disease, the progression is relatively rapid over a period of about 5 years. Understandably, there is concern that once the disease starts, it may be difficult or impossible to slow or reverse the neurodegenerative process. We have attempted to address the importance of timing in 2 approaches. The first was to limit the intervention to a single time point, ranging from early in the mouse's life to well after some disease processes, such as denervation of the tibialis, have occurred. Using mouse clinical measures such as disease onset or survival, timing was irrelevant to the magnitude of benefit. Potentially of greater interest are the data in Figure 4A showing reversal of a specific, highly quantifiable endpoint in this very rapid model of ALS. CMAP is the summation of the amount of functional innervation to a given muscle. The fact that this measure increases in mice in the ASO treatment setting demonstrates a remarkable ability for the motor system to recover. Concomitant with this, there was decreased denervation at the neuromuscular junction, reinforcing the functional recovery (Figure 4B). We also demonstrated that serum and CSF pNFH levels, which are increased in rodent and human ALS, were decreased by this therapy. This defines, for the first time, serum and CSF pNFH levels as a pharmacodynamic marker for ASO therapy. Perhaps even more importantly, these neurofilament data suggest that any particularly effective therapy in humans may reverse the rise in serum/CSF pNFH. Taken together, these

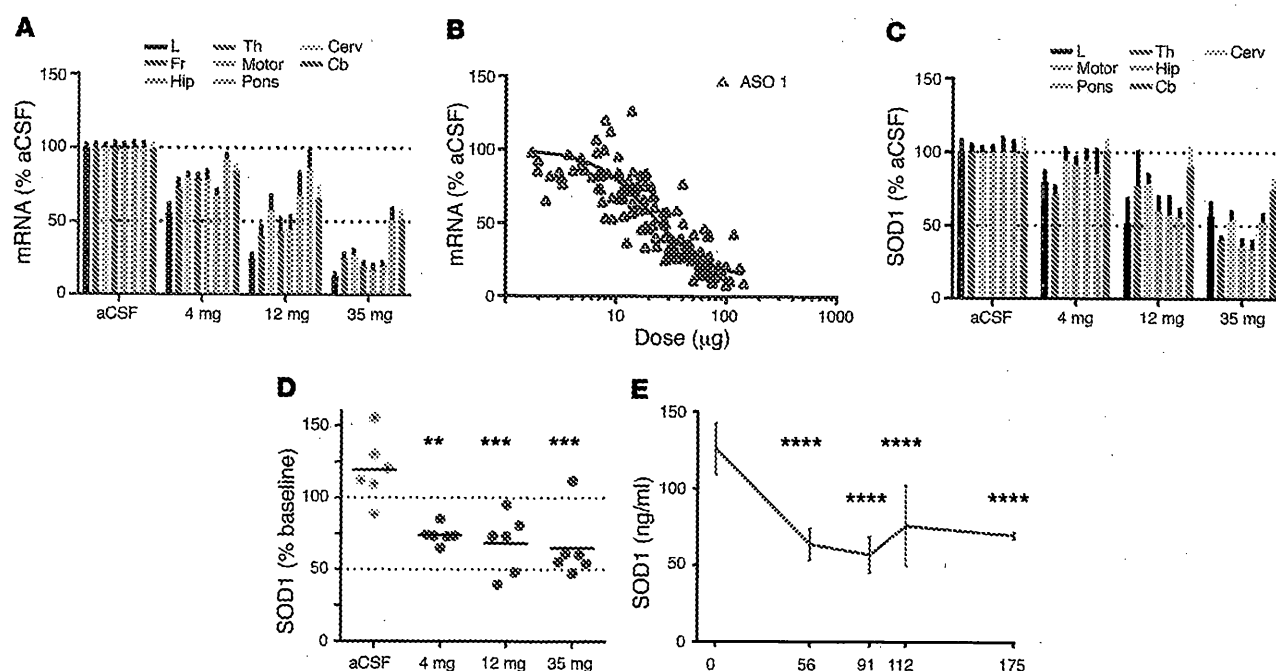


Figure 5. SOD1 ASO lowers SOD1 in nonhuman primate CNS and CSF. (A–C) The safety of the ASO was assessed in cynomolgus monkeys, which also provided an opportunity to understand the on-target activity of the ASO and the utility of SOD1 in the cerebrospinal fluid as a marker of target suppression in the tissue. Monkeys were dosed with artificial CSF or 4, 12, or 35 mg ASO 1, with each monkey receiving 5 doses. One week after the final dose, SOD1 mRNA and protein were quantified and associated with ASO concentration across brain regions. (A) A dose-dependent lowering of mRNA across CNS regions, including the lumbar spinal cord, was observed ($n = 6$, average $\pm$ SEM). L, lumbar; Th, thoracic; Cerv, cervical; Fr, frontal cortex; Motor, motor cortex; Hip, hippocampus; Pons, pons; Cb, cerebellum. (B) A pharmacokinetic/pharmacodynamic relationship could be observed, with an estimated IC_{50} of 20 μ g/g tissue ($n = 126$, each point representing one tissue). (C) A dose-dependent lowering of SOD1 protein across CNS regions, including the lumbar spinal cord, was observed ($n = 6$, average $\pm$ SEM). aCSF, artificial CSF. (D) A dose-dependent lowering of SOD1 protein in CSF as compared with the vehicle-treated group was observed ($n = 6$; line, average; 4 mg, $P = 0.0019$; 12 mg, $P = 0.0006$; 35 mg, $P = 0.0003$; 1-way ANOVA). ** $P < 0.005$; *** $P < 0.001$. (E) CSF SOD1 protein remains significantly reduced as compared with day 0, roughly 100 days following the last intrathecal ASO administration ($n = 6$, average $\pm$ SEM; **** $P = 0.0001$; 1-way ANOVA).

data show reversal of neurodegeneration and define electrophysiologic and serum pharmacodynamic markers that can be directly translated to human clinical studies. miR-218, a motor neuron-specific microRNA, also shows changes in CSF with SOD1 ASO treatment in SOD1^{G93A} rats and may be another outstanding pharmacodynamic marker for motor neuron-targeted therapeutics (23). Measuring miR-218 in human CSF will be a critical next step.

Clinical trials in a rare population such as SOD1-related ALS may be challenging given the relatively small number of patients affected by the disease, but much of the groundwork for such a trial has been established. Natural history studies of SOD1 ALS have defined survival times in this patient group. For people with A4V mutations in SOD1, which represent 50% of the SOD1-associated ALS population in the United States, survival is 1.2 years. If we anticipate a change of 50% in survival in patients, as few as 52 participants would be needed to determine if an ASO changed survival (25). An even more important first step in clinical trials is to determine if SOD1 in the CSF is lowered by the ASO. The NHP CSF data included here demonstrate that an intrathecally administered ASO can reduce CSF target protein concentrations, confirming CSF protein as a translational pharmacodynamic end point. Similar clinical measures of CSF SOD1 protein are crucial to demonstrate drug action and pharmacodynamic activity for ongoing clinical trials (24). Recent SOD1 protein kinetics (26) have determined the half-life of CSF SOD1 in humans to be 25 ± 7.4 days.

One concern for this approach is the safety associated with

loss of SOD1 expression. While safety will remain an important consideration in human clinical studies, for SOD1 ALS there are several data points that provide some reassurance. As noted above, extensive evidence supports the primary toxicity of SOD1 mutations as resulting from gaining a novel toxic property. Overexpression of mutant SOD1 in mice or rats mimics important aspects of ALS in humans, including loss of neuromuscular junction innervation and motor neuron death (1, 2). Loss of SOD1 results in motor neuron dysfunction but not motor neuron death (3, 4), and neither loss nor increase of normal SOD1 activity in mouse models of SOD1 ALS alters survival (27). In humans, individual disease mutations are associated with varying levels of SOD1 activity, and some patients with disease-causing mutations even have apparently normal SOD1 activity (7). Lastly, SOD1 ASOs have been found to be safe in humans (9). A clinical trial based on this prior work and the work presented here is already underway in SOD1-related ALS (NCT02623699) using ASO 1.

For many neurodegenerative diseases, the proteins implicated by genetic forms of the disease are also implicated in the pathways or pathology of a broad number of cases. Based on this experience, it was anticipated that SOD1 would be involved broadly in ALS. The data regarding this issue are not yet clear. Using some staining protocols, misfolded SOD1 may be found in pathological samples from non-SOD1 genetic ALS (28). However, other studies have not replicated these findings (29). The simple explanation of using different reagents and samples does not fully explain the discrep-

Table 1. Antisense oligonucleotides

ASO	Sequence/Chemistry
ASO 1	5'-TACATTCTA-3'
ASO 2	5'-TGAATGTTTATCA-3'
ASO 3	5'-TTTAATCT-3'
333611	5'-CTTGGCCCTTCAGACCA-3'
Inactive ASO (in vitro)	5'-CTGAAGGTT-3'
Inactive ASO (in vivo)	5'-CTGAAGGTT-3'

Oligonucleotides containing phosphorothioate backbone modifications (unmodified phosphodiester linkages noted with red o) and MOE (orange) and cEt (blue) groups in the 5' and 3' wings were targeted to the 3' UTR of SOD1 mRNA (ASO 1, ASO 2, and ASO 3), exon 1 of SOD1 mRNA (333611), or nothing in the rodent genome (inactive ASOs). The sequences evaluated and the location of chemical modifications are provided. Different control (inactive) ASOs were used for in vitro and in vivo studies. All cytosine residues are 5' methylcytosine.

ancies. Perhaps most intriguing are data showing that lowering SOD1 in human astrocytes or oligodendrocytes decreases the toxicity of these cells to cultured motor neurons (30, 31). Overall, while these data are suggestive that SOD1 may be involved more broadly in ALS than the 1%–2% of the ALS population with genetic SOD1 mutations, the data are not yet strong enough to push forward a SOD1-lowering therapy in all ALS. As further data emerge on SOD1 in sporadic ALS and as we learn more about safety and efficacy of ASOs in the SOD1 genetic population, SOD1 ASOs for ALS without known SOD1 mutations will likely need to be reconsidered.

ASO therapeutics for neurologic diseases are in clinical trial or are anticipated shortly for clinical trial for a number of disorders. Phase III trials for a splicing ASO for spinal muscular atrophy (SMA) were stopped early because of success and led to recent FDA approval of these drugs for clinical use (14). A splicing ASO recently received conditional approval for Duchenne muscular dystrophy (32). ASOs for Huntington's disease are currently in phase I trial in Europe. ASO lowering of tau showed impressive efficacy in mouse preclinical studies (12) and is in early clinical trials (NCT03186989). ASOs targeting C9ORF72 expansions that cause ALS/FTD showed efficacy in stem cell-derived neurons (33, 34) and mouse models (35) and are likely to be in human clinical trial in the near future. Thus, the approach outlined here for targeting mRNA in the brain and spinal cord with ASOs is increasingly being used for neurological disorders and hopefully will become part of a clinical treatment paradigm, as in SMA.

While we are enthusiastic and encouraged by the demonstration of reversal in these studies, we recognize that these results may or may not directly translate to humans. Subsequent data from human clinical studies compared with the data presented here may be the best opportunity to fully evaluate the translational potential of SOD1 models. There has been much prior criticism of SOD1 models and their failure to predict efficacy in humans, but for the vast majority of these failed clinical studies, 2 important components were often missing: first, a demonstration that the pathway targeted by the drug was involved in the participants being treated; and second, a pharmacodynamic

marker to demonstrate that the drug worked as designed in humans. The ASO SOD1 trial in humans with SOD1 ALS is directly on target and the pharmacodynamic marker, SOD1 in the CSF, has been clearly defined (26).

There are other methods to target SOD1 mRNA. Indeed, recent data in animal models using virally driven RNAi showed promising results (36, 37). While there may be some additional issues surrounding delivery of virus, these strategies are plausible. Antibodies that target SOD1 are currently being developed (38–41). In addition, small molecules targeting SOD1 have been considered. Pyrimethamine has been tested in phase I/II trial and showed an approximately 10%–15% lowering of SOD1 in CSF (42). Arimoclomol, a heat shock protein activator, was tested in rapidly progressive ALS. Patients treated with arimoclomol showed a trend toward longer survival (43). It is possible that some of these approaches could be used in conjunction with ASOs. All, including ASOs, require ongoing studies in human SOD1 ALS to determine success.

In conclusion, ASOs targeting SOD1 show extension of survival in SOD1 mice and rat models and reversal of CMAP. These ASOs are also effective in NHPs. Given the growing experience with ASOs in clinical trials, the new SOD1 ASOs are an exciting therapeutic for human SOD1 ALS.

Methods

Antisense oligonucleotides. The antisense oligonucleotides used in the present study are described in Table 1 and were manufactured and provided by Ionis Pharmaceuticals.

Intrathecal bolus injection in the rat. SOD1^{G93A} transgenic rats received a 30- μ l intrathecal bolus injection via polyethylene catheter placed between the L3 and L5 vertebrae of the lumbar spinal cord, as previously described (44). For proof-of-concept studies, 65-day-old rats were given 1000 μ g ASO 1, ASO 2, ASO 3, ASO 333611, inactive ASO, or an artificial cerebrospinal fluid (aCSF) vehicle control. All ASOs were provided by Ionis Pharmaceuticals and diluted in aCSF. All treatment groups were sexual phenotype- and litter-matched to control for drift in copy count or sexual phenotype-induced variances. In addition, researchers were blinded to the identity of the ASO at the time of treatment and throughout the duration of the experiment. For pharmacokinetic and pharmacodynamic studies, adult animals were dosed and tissue was collected 2 weeks after dosing for RNA quantification, unless otherwise noted.

Dosing of mice. Intracerebroventricular (i.c.v.) infusions were performed as previously described (45). Surgeries were conducted according to Biogen Guidance for Aseptic Rodent Surgery. Animals were anesthetized by isoflurane inhalation. The eyes of the animal were treated with ocular lubricant. The fur of the scalps and anterior backs were clipped and the animal was placed in a stereotaxic apparatus. Buprenorphine was administered subcutaneously prior to incision (1.0 mg/kg). An 11.5-cm, slightly off-center incision was made in the scalp. A 25-gauge needle attached to a Hamilton syringe was placed at bregma level and the needle was then moved to the appropriate anterior/posterior and medial/lateral coordinates (0.2 mm anterior/posterior and 1 mm to the right medial/lateral). The proper amount of injection solution was injected at rates of approximately 1 μ l/second for a total of 10 μ l. This flow rate has been shown to deliver sufficient compound with consistency and no side effect to the animal.

The incision was sutured closed using 1 horizontal mattress stitch with 50 Ethilon suture. Depending on the experiment, mice were injected either once (at day 35) or twice (at day 50 and day 94) in order to obtain sustained mRNA lowering of SOD1.

Motor assessments in the rat. Prior to surgery, rats were assessed for baseline weight and general motor capabilities. Following surgery, rats were monitored weekly for changes in these values. Weight loss was calculated from the time of surgery to identify disease onset (peak weight) (46). When an animal began to show paralysis in any portion of the body, a HydroGel pack (Clear H₂O) was placed on the bottom of the home cage to limit dehydration. Monitoring continued until the rats reached end-stage, defined as loss of the ability to right itself after being placed on its side, or inability to ambulate from a given location. The date of end-stage and subsequent euthanasia was recorded for survival analyses.

Euthanasia and tissue collection. For proof-of-concept studies, upon reaching end-stage, rats were exsanguinated by transcardial perfusion with heparinized (0.03%) PBS. Sections of the brain and spinal cord were flash frozen in liquid nitrogen for RNA and protein analysis. For pharmacodynamic and pharmacokinetic studies, following in-life completion, animals were euthanized by CO₂ asphyxiation as approved by IACUC protocol. For RNA analysis, 2-mm sections of lumbar spinal cord were collected. Adjacent to the RNA sampling, pieces of lumbar cord were also collected for ASO quantification. Each dissected region was then frozen at -80°C until analysis.

Rotarod in mice. Mice were given 1 day to become familiar with the rotarod apparatus before the test by placing them on a rod slowly rotating at 2 rpm for 5 minutes. To determine motor function deficiency, mice were put on the rod at increasing speeds from 2 rpm to 40 rpm in 180 seconds and at 40 rpm for an additional 60 seconds. Tests were given 2 times in a day. Mice were tested once a week starting at 80 days of age until they could not stay on the rod for at least 30 seconds.

Compound muscle action potential. CMAP recordings were performed at the indicated time points under isoflurane anesthesia. Disposable monopolar 28-gauge needle electrodes were used for stimulation and recording. The sciatic nerve was stimulated near the sciatic notch with constant-current monophasic square-wave pulses (0.1 millisecond duration, 2-second intervals). For CMAP recordings, the recording electrode was placed intramuscularly 1 mm deep in the tibialis anterior. Stimulation current was increased until a maximum CMAP was obtained, and recordings were performed using a current level 0.5 mA above this value (supramaximal stimulation). The amplitude represents the peak-to-peak amplitude of the biphasic CMAP waveform (averaged over 4 stimuli). For each animal, right and left leg amplitudes were averaged.

NMJ staining and counting. Tibialis anterior muscle samples were postfixed in 4% paraformaldehyde (PFA) for 15 minutes after collection and then incubated into 20% sucrose for 24 hours for cryoprotection. Samples were shipped to Clarapath Inc. for analysis. All samples were sectioned using the Clarapath Inc. tape-transfer system at 20 µm in a cryostat. Sections were collected directly onto glass slides. Slides were stained with a rabbit antivesicular acetylcholine transporter (VachT; 1:11,000); the antibody was provided by Target ALS through Covance. The secondary antibody used was Alexa Fluor 488 donkey anti-rabbit (Life Technologies). All slides were counterstained with TMR-α-bungarotoxin (Life Technologies) at a concentration of 1:750. All stained slides were imaged using a ×20 objective (0.75 NA) at a resolution of 0.45 µm/pixel. A single focal plane was collected for all samples. A multiband fluorescence filter set was used to simulta-

neously image GFP and RFP. Both automated and manual counting methods for TMR-α-bungarotoxin and VachT were performed.

pNFH quantification. pNFH serum levels were measured through the ELLA microfluidic ELISA platform (Protein Simple) according to the manufacturer's instructions. Briefly, a maximum of 100 µl blood was collected through facial vein puncture at the indicated time points. Serum samples were prepared by centrifugation of BD Vacutainer SST tubes (BD Diagnostics) and stored at -80°C until used.

Gyrolab assay for misfolded SOD1 protein. Rat spinal cord samples were homogenized in 50 mM Tris pH 7.4, 100 mM NaCl, 10% glycerol, 1% Triton X-100 and then diluted at 1:30. Misfolded SOD1 protein was measured with the following antibodies: capture antibody D3H5 (40) (working concentration: 100 µg/ml); and detection antibody B8H10 (working concentration, 50 nM; Medimabs MM-0070-P). Concentrations were measured on a Gyrolab XP Workstation based on a standard curve of denatured recombinant human SOD1 protein.

Cynomolgus monkey SOD1 protein quantification. Protein levels were measured using an ELISA method specifically qualified to quantify the amount of SOD1 in cynomolgus monkey brain tissue and CSF. Briefly, microtiter plates were coated with SD-G6 antibody diluted in 0.2 M carbonate-bicarbonate buffer and blocked with SuperBlock-PBS (Thermo Fisher Scientific) for 2 hours at ambient temperature. The SOD1 reference standard was generated at Biogen and was used to prepare standards and quality controls (QCs) to final concentrations in SuperBlock-PBS. Cynomolgus brain tissue, CSF samples, and the endogenous QC (EQC) were diluted 1:64 with SuperBlock-PBS. Plates were incubated for 1 hour with sample, washed, and then incubated with 2.5 µg/ml SOD-100 antibody diluted in SuperBlock-PBS for 1 hour, then washed and incubated for 1 hour with 1:32,000 dilution goat anti-rabbit IgG HRP-conjugated antibody. After washing, TMB substrate was added.

Study approval and experimental animals. SOD1^{G93A} rats (Taconic Biosciences, model 2148) were bred in-house on a Sprague-Dawley background. All rats were kept on a 12-hour light/dark cycle. SOD1^{G93A} mice (B6.CgTg(SOD1*G93A)Gur/J) were bred and maintained at the Jackson Laboratories breeding facility. Food and water were provided ad libitum. Breeding and experimental procedures were completed in accordance with an animal use protocol approved by the Animal Studies Committee at Washington University School of Medicine, Biogen, and Ionis Pharmaceuticals. All mouse studies were conducted using female animals only.

The NHP study was performed at Covance Laboratories GmbH. Covance Laboratories GmbH test facility is fully accredited by the AAALAC. All procedures in the study plan were in compliance with the German Animal Welfare Act and were approved by the local IACUC. Briefly, 30 healthy cynomolgus monkeys (*Macaca fascicularis*) approximately 2–5 years of age and between 2–6 kg in weight at study start were used for testing. NHPs were administered, by intrathecal bolus injection, 5 doses of ASO or aCSF on days 1, 14, 28, 56, and 84 with 1.0 ml dosing volume using a 35 mg/ml dosing solution. NHPs were euthanized on day 91. CSF collection was performed predose at day 1 (baseline) and at day 91, just before necropsy. To determine the time frame for SOD1 protein recovery, CSF was collected from a separate cohort of animals predose on day 1 (baseline), and predose on days 56, 91, 112, and 119. NHPs received neurological examinations predose on days 1, 14, 28, 56, 84, and 91 and at least once every 2 weeks between days 28 and 91.



Statistics. $P < 0.05$ was considered significant for all studies. For survival studies for mice and rats, a log-rank Mantel-Cox test was used. One-way ANOVA followed by Tukey's post hoc test was performed for Figure 5, D and E. IC_{50} values were calculated with the formula $\log(\text{inhibitor})$ vs. response - variable slope (4 parameters), using Prism6 software formula $\log(\text{agonist})$ vs. response - find EC anything, with the following constraints: bottom > 0 , top = 100, $F = 50$ for ED_{50} and EC_{50} , and $F = 80$ for EC_{50} . For all other comparisons, a 2-way ANOVA was used.

Author contributions

AM, TC, AJW, CFB, HBK, EES, DLG, MC, and TMM designed the study. TC, AJW, GST, AS, BJE, KMS, MLH, MS, LS, YL, MZ, ST, DLG, and DWS collected data. AM, TC, AW, GST, CFB, HK, ES, DLG, and TMM analyzed data. NC, JA, and BW designed and

generated the electrophysiology data in Figures 2 and 3. AM, TC, GST, CFB, HBK, EES, DLG, and TMM wrote the manuscript.

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Antisense Oligonucleotides: Translation from Mouse Models to Human Neurodegenerative Diseases

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Multiple neurodegenerative diseases are characterized by single-protein dysfunction and aggregation. Treatment strategies for these diseases have often targeted downstream pathways to ameliorate consequences of protein dysfunction; however, targeting the source of that dysfunction, the affected protein itself, seems most judicious to achieve a highly effective therapeutic outcome. Antisense oligonucleotides (ASOs) are small sequences of DNA able to target RNA transcripts, resulting in reduced or modified protein expression. ASOs are ideal candidates for the treatment of neurodegenerative diseases, given numerous advancements made to their chemical modifications and delivery methods. Successes achieved in both animal models and human clinical trials have proven ASOs both safe and effective. With proper considerations in mind regarding the human applicability of ASOs, we anticipate ongoing *in vivo* research and clinical trial development of ASOs for the treatment of neurodegenerative diseases.

A Re-emergence of Antisense Technology

Recent years have seen a re-emergence of antisense oligonucleotides (ASOs) as valuable tools to inform on disease mechanisms and as powerful therapeutics for disease intervention. Part of this recent success can be attributed to improvements made to the ASO structure and chemical modifications that have made ASOs more advantageous and efficient as potential strategies for the treatment of disease. ASOs can exert their gene-targeting effects through different mechanisms of action, making them amenable to a variety of molecular targets and disease processes. These advancements in ASO technology have been coupled with the surprising empiric finding that, despite being highly charged and large, ASOs distribute widely throughout the CNS when delivered to the cerebral spinal fluid (CSF). This characteristic has greatly enabled the application of ASOs as therapeutic strategies for CNS disorders, many of which currently have no adequate treatment.

The first ASO to enter clinical trial, targeted against p53 in acute myelogenous leukemia, drew upon 20 years of chemical advancements prior to its introduction in 1993 (Bayever et al., 1993). This milestone was closely followed by the first market approval for the antisense therapeutic, fomivirsen, in 1998 for the treatment of cytomegalovirus retinitis in patients with immunodeficiency (Marwick, 1998). Since then, numerous ASOs have progressed to human clinical trial for diseases, including cancer, diabetes, and muscular dystrophy (Bennett and Swayze, 2010), taking advantage of the various mechanisms and synthetic structures now available for the design of ASO therapies. Neurodegenerative diseases, specifically, are in imminent need of an effective therapy, and ASOs offer one possible strategy. Considering the remarkable clinical trial successes that, to date, have been achieved in diseases like spinal muscular atrophy and amyotrophic lateral sclerosis, researchers and patients are enthusiastic about ASO application to neurodegenerative diseases. Many neurodegenerative diseases collectively are characterized by the dysfunction or abnormal accumulation of toxic

proteins. Therefore, strategies aimed at reducing levels of the toxic protein or creating non-toxic modifications are attractive approaches for clinical development. ASOs and their gene-targeting effects provide a promising solution, and their successful translation from rodent models to human patients underscores their vast potential.

Harnessing Antisense Oligonucleotide Structure and Function

Structure and Chemical Modifications

ASOs are small, single-stranded sequences of DNA, 8–50 base pairs in length, composed of a phosphate backbone and sugar rings. The structure and sequence allows for specific, complementary matching with target RNA by Watson-Crick base pairing. ASO properties, including solubility, binding, potency, and stability, derive from various modifications to both the backbone and sugar rings. Backbone modifications both increase affinity to the RNA target and protect the ASO from nuclease degradation. One of the first and most commonly used modifications made to the phosphate backbone replaced the singly bonded oxygen group with a sulfur ion, creating a phosphorothioate (PS) linkage (Figure 1). This modification endows the ASO with enhanced stability and protection against nuclease degradation, increased binding to plasma proteins to maintain stable concentrations in serum, better cellular uptake, and the ability to recruit RNase H enzyme for target degradation (Bennett and Swayze, 2010). A major disadvantage to the PS backbone modification is the potential inflammatory response generated at high concentrations (Crooke, 2007) and prohibitive affinity for plasma protein binding (Geary et al., 2001), which may produce adverse effects and other limitations in a clinical setting.

Creating PS molecules and other ASOs with nuclease resistance and enhanced potency must be balanced against activation of the innate immune response. For instance, CpG oligodeoxynucleotides motifs (i.e., an unmethylated “C” followed by a “G” base separated by a phosphodiester linkage,

	Phosphorothioate (PS) DNA	Morpholino	Peptide nucleic acid (PNA)	2'-O-Methyl (2'-O-Me)	2'-O-Methoxyethyl (2'-O-MOE)	Locked nucleic acid (LNA)
STRUCTURE						
PROPERTIES/USES	Nuclease resistant High affinity protein binding for cellular uptake	Nuclease resistant Stable	Highly nuclease resistant High binding affinity Low toxicity	Enhanced target affinity Nuclease resistant Well-tolerated	Enhanced target affinity Nuclease resistant Well-tolerated	Potently binds to target Nuclease resistant Increased toxicity
	Supports RNase H activity Design used in clinical trial for SMA, ALS, and HD	Supports non-degrading mechanisms Translation inhibition Splice modification	Supports non-degrading mechanisms Translation inhibition Splice modification	Supports non-degrading and RNase H* activity "with gapmer" design Used in clinical trial with PS backbone	Supports non-degrading and RNase H* activity "with gapmer" design	Non-degrading and RNase H* activity "with gapmer" design

Figure 1. ASO Chemical Modifications, Properties, and Common Uses

Decades of synthetic chemical research have produced various ASO structural designs to enhance the pharmacokinetic properties, target affinity, and tolerability profile for ASO application. Common modifications have been made to the phosphodiester backbone to create phosphorothioate (PS) DNA, morpholino, and peptide nucleic acid (PNA) designs, which all confer excellent nuclease resistance for more potent ASO activity. Notably, PS designs exhibit stability and high-binding affinity to proteins, enabling efficient uptake into cells and support RNase-H-mediated cleavage of the target. Therefore, PS modifications are broadly employed in preclinical studies and are the primary design for human clinical trials for spinal muscular atrophy (SMA), amyotrophic lateral sclerosis (ALS), and Huntington's disease (HD). In combination, 2' ribose substitutions—2'-O-methyl (2'-O-Me), 2'-O-methoxyethyl (2'-MOE), and locked nucleic acid (LNA)—greatly enhance target binding, increase resistance to degradation by nucleases, and generally confer less toxicity than unmodified designs. Uniform 2' modifications support non-degrading mechanisms of action, including splice site modification and translational inhibition; however, introduction of a "gapmer" strategy will permit RNase-H-mediated activity. Both 2'-O-methyl and 2'-O-methoxyethyl modifications are commonly used in preclinical research and clinical trials.

common in particular base sequences) are potent immune activators (Younis et al., 2006) and, therefore, are often avoided in designing ASOs for in vivo application. An exception might be when immune stimulation is a desired property, for example with immunotherapies (Vollmer and Krieg, 2009). Yet, even with these considerations, it is likely that all ASOs are recognized as foreign DNA by toll-like receptors of the innate immune system. Much of the chemistry at the 2' position has been focused on ways to stabilize the ASO without activating an immune response. In concert with the PS backbone modification, recent ASO pharmacology typically includes 2' ribose sugar modifications (Figure 1). Of these, the 2'-O-methyl (2'-O-Me) and 2'-O-methoxyethyl (2'-MOE) are the most frequently used, enabling stronger binding affinity to target RNA and enhanced resistance to nuclease degradation (Bennett and Swayze, 2010). These modifications may help to overcome the disadvantageous immunostimulatory properties of the PS backbone. Both 2'-O-Me and 2'-MOE design are reported to have a lowered pro-inflammatory response and be well tolerated compared to unmodified motifs (Henry et al., 2000; Tiuk et al., 2009). ASOs consisting of fully modified 2' sugar moieties do not enable RNase H enzyme activity but instead support non-degrading ASO mechanisms, such as changes to alternative splicing or translational inhibition (Bennett and Swayze, 2010). Activating RNase H and thus cleaving the target mRNA requires a creative pharmacologic manipulation to produce a chimeric or "gapmer" design, which takes advantage of the tolerability of 2'-O-Me or 2'-MOE sugar rings combined with the RNase-H-mediated degradation property of the PS backbone (Figure 1). A gapmer consists of 2' modified sugar ring bases separated by a central region of unmodified nucleotides to

enable target degradation without inducing overt inflammatory responses (Geary et al., 2015).

ASO chemistry continues to evolve, and modifications, including a morpholino backbone, peptide nucleic acid (PNA) chemistry, and locked nucleic acid (LNA) modifications, are becoming more prevalent in preclinical and clinical use (Figure 1). Both morpholinos and PNA designs afford highly stable RNA complementarity and, although they do not stimulate degradation of their target, they are useful for other mechanisms of action discussed below (Bennett and Swayze, 2010). LNA sugar moieties are similar to the 2'-O-methyl structures but exhibit greater potency and can be designed to support RNase H activity. Although these modifications are advantageous for their potency and nuclease-resistant properties, problems associated with toxicity or poor penetration continue to be explored (McMahon et al., 2002; Swayze et al., 2007). As various ASOs move closer toward clinical trial, ASO design remains an important avenue of development.

Mechanisms of Action

Before ASOs can exert their gene-modulating effects, they must overcome membrane barriers and traffic to subcellular locations, typically the nucleus. These events can be viewed as limiting steps for ASO activity and underscore the importance of effective chemical designs to modulate both uptake and distribution at the cellular level. The proposed entry method of ASOs into cells involves association with high- and low-binding plasma proteins, internalization into lysosomal or endosomal compartments, and trafficking within the cell (Geary et al., 2015), although the exact details of these processes are complex and may differ across cell types. A recent review of phosphorothioate ASO cellular uptake and intracellular trafficking summarizes

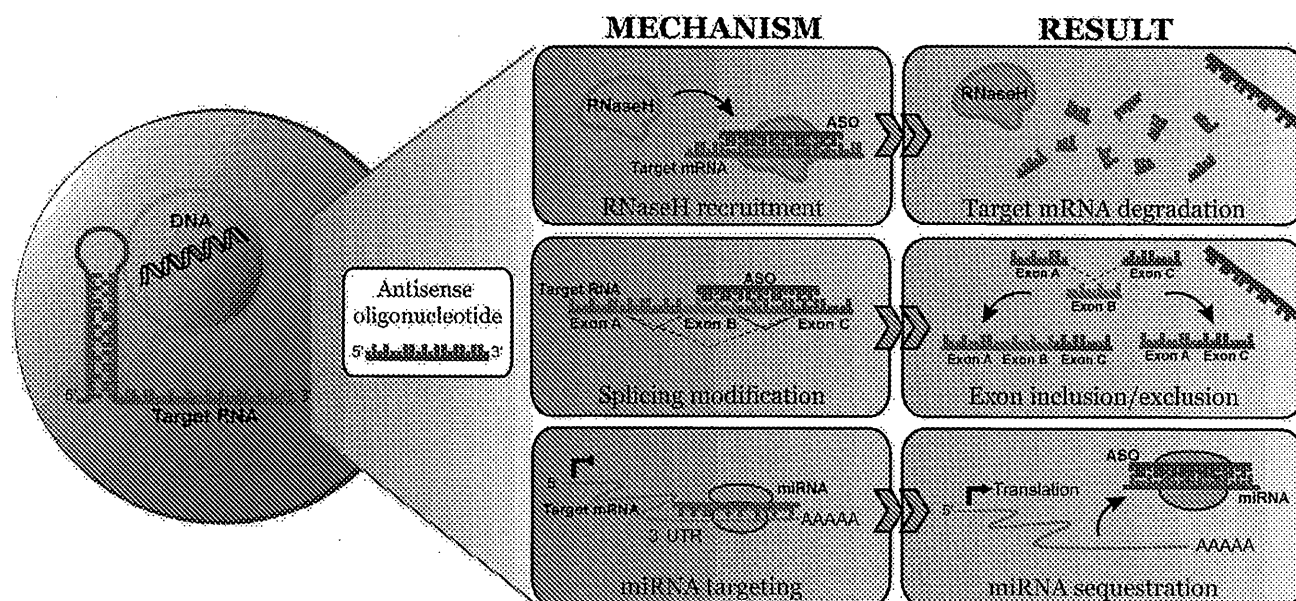


Figure 2. Common ASOs Mechanisms of Action

When administered, ASOs bind to target RNA with base pair complementarity and exert various effects based on the ASO chemical structure and design. Three mechanisms, commonly employed in preclinical models of neurodegenerative disease and human clinical trial development, are shown. These mechanisms include mRNA target degradation via recruitment of the RNase H enzyme, alternative splicing modification to include or exclude exons, and miRNA inhibition to inhibit miRNA binding to its target mRNA.

the reported receptors, proteins, and pathways involved (Crooke, 2007). In brief, proteins that interact with ASOs at the cell surface and within intracellular organelles, in part, determine their subcellular trafficking and escape into the cytosol; however, many questions regarding specific protein interactions remain unanswered. Once inside cells, ASOs are able to travel between the cytosol and nucleus via proposed binding to cellular molecules (Lorenz et al., 2000). The single-stranded ASOs bind to their target RNA through Watson-Crick base pairing, which, depending on the target and ASO design, can trigger distinct mechanisms of action by which the ASO ultimately degrades or modifies its target. These mechanisms can be divided into several common categories, discussed below: RNase-mediated degradation; mRNA modification; or microRNA inhibition (Figure 2).

RNase-mediated degradation is particularly advantageous in the context of many neurodegenerative disorders in which a distinct RNA/protein dysregulation or accumulation is recognized as the central cause of disease. It is not surprising, therefore, that RNase activation continues to be employed in preclinical ASO development and several clinical trials. Degradation of the mRNA target via this mechanism begins in the nucleus, where the DNA-RNA pairing of ASOs to their mRNA target triggers recruitment of the enzyme RNase H1 (Wu et al., 2004). Binding of the ASO to mRNA mimics the DNA-RNA binding that occurs during DNA replication, in which RNase H cleaves the RNA primer from newly synthesized DNA (Cerritelli and Crouch, 2009). For ASO therapeutics, destruction of the mRNA prohibits its translation into protein, yielding a reduction of the intended target. RNase H activation is facilitated by the PS backbone modification or gapmer ASO design. Because RNase H specifically cleaves RNA, the ASO itself avoids degradation

and, therefore, is able to bind to additional target mRNA, enabling sustained mRNA and, in turn, protein reduction. Destruction of the cognate mRNA is the most common mechanism by which ASOs are used, yet several additional mechanisms can be employed to achieve protein lowering or manipulation (Bennett and Swayze, 2010). Other possible mechanisms of ASO-mediated protein reduction include translational inhibition or altered RNA stability via RNA modification.

RNA modification is a second, common mechanism by which ASOs can function. Acting within the nucleus, these ASOs pair with the target mRNA but, given their design, will not initiate the direct degradation of the mRNA. Instead, ASOs modify the mRNA at the 5' cap or polyadenylation site to prevent mRNA translation or alter RNA stability, thereby lowering the intended mRNA and protein in a non-degrading fashion (Bennett and Swayze, 2010). In addition, one surprisingly effective use of RNA-modifying ASOs has been to change alternative splicing patterns of mRNA. ASO binding to intron/exon junctions can affect recruitment and binding of splicing factors or destabilize splicing sites (Havens and Hastings, 2016). Such a strategy might be most beneficial for disorders with a known splicing defect to either restore protein function or to exclude mutated stretches of DNA. The degree of shift in splicing patterns has been striking in animals, as will be discussed below, and has advanced to successful clinical trial use. The most notable of these is the ASO design to promote the inclusion of exon 7 in the survival motor neuron (*SMN2*) gene, now US Food and Drug Administration (FDA)-approved for the treatment of spinal muscular atrophy (FDA News Release, 2016a).

ASOs can also act through binding of microRNA or other small RNA targets to inhibit their actions. MicroRNAs are sequences of

approximately 22 base pairs in length and function in the regulation of gene expression through binding target mRNAs. ASOs act by binding these microRNA (miRNA) targets to inhibit their mRNA interaction by sequestration or degradation (Davis et al., 2006, 2009). Dysregulation of miRNAs has been identified in numerous disease states, including cancer, virology, and neurodegenerative disease, highlighting these small RNAs as potential targets for therapeutic intervention. This strategy is already being applied successfully in clinical trials for hepatitis C infection (Janssen et al., 2013). Although no miRNA-targeted ASO for neurodegenerative disorder is in clinical use, emerging knowledge on the roles of miRNA in disease continues to provide motivation for their future application to human patients.

Delivery Methods to the CNS

In order for ASOs to be effective, they must achieve delivery to affected regions or cells at appropriate concentrations. In addition, they must be stable and maintain efficacy over time for feasible treatment throughout the course of disease. Diseases of the CNS often pose problems that limit efficient and effective drug delivery. Most notably, the blood brain barrier restricts entry of certain molecules based on size, charge, or solubility (Partridge, 1998), which will not only limit the effective concentrations that reach the brain or spinal cord but also completely exclude certain drugs. ASOs lack the ability to cross the blood brain barrier, a limitation that was initially considered a reason for their exclusion as a therapeutic approach for neurodegenerative diseases. However, in the clinical setting, multiple drugs, including chemotherapies, some spasticity medications, and pain medications, are routinely delivered to the CSF that circulates throughout the CNS (Boitros and Christo, 2014). Despite the prediction that highly charged ASOs would remain localized to the site of CSF infusion, empiric data strikingly show widespread distribution throughout the brain and spinal cord in mice (DeVos et al., 2013; Kordasiewicz et al., 2012; Lagier-Tourenne et al., 2013; Passini et al., 2011), rats (Smith et al., 2006), and non-human primates (Kordasiewicz et al., 2012; Passini et al., 2011; Fligo et al., 2014; Smith et al., 2006) using intraventricular or intrathecal injection. Intrathecal ASO delivery has already been implemented in human clinical trials for amyotrophic lateral sclerosis and spinal muscular atrophy with safe and tolerable results (Chiriboga et al., 2016; Miller et al., 2013). The adequate distribution achieved with intrathecal delivery and the growing clinical experience with ASO delivery by this method will likely lead to an increasing number of clinical studies being performed with an intrathecal delivery route. Intraventricular delivery in humans remains open to further study and may have some practical advantages in patients with anatomic abnormalities, such as severe scoliosis, that make intrathecal delivery more challenging.

Intraventricular or intrathecal injections are relatively invasive compared with orally delivered medications, yet thus far have been well tolerated. Ongoing research is focused on developing novel ASO delivery methods for the CNS, including oral, subcutaneous, and intranasal routes. Success with intrathecal delivery programs, including current and potential commercialization of some ASO candidates, is likely to spark innovative approaches to make a successful therapy even easier to administer. On the other hand, with the urgent need for neurodegenerative disease

therapies, intrathecal delivery will likely continue as a standard method.

Challenges for Antisense Application

Potential Side Effects

One general concern often raised regarding ASO application for human diseases is the possibility of adverse side effects. ASO toxicities and off-target effects are known to be both sequence and chemistry dependent, and thus, each ASO molecule must be considered independently and with a complete toxicology evaluation. However, there are some general points to consider.

The first consideration is on-target toxicity as a result of lowering levels of a total protein. The effects of gene deletion in rodents provide a useful first-pass evaluation of the potential toxicities resulting from protein reduction, but these models have the potential to be over-interpreted in both directions. Whereas protein reduction from birth—as in knockout mouse experiments—may be deleterious, lowering this same protein in the adult—as is proposed in many ASO programs—may be very well tolerated. Conversely, protein reduction from birth may be assumed safe, but this conclusion may be misguided by the known caveat for potential compensation of gene deletion in mice. ASO administration with substantial lowering of mRNA and protein in diseased and age-matched rodents and in non-human primates may provide the best reassurance of safety for an ASO approach. Yet, ASO studies in non-human primates are typically in a relatively small number of animals and for limited periods of time. Thus, the broad array of potential problems associated with lowering protein levels remains unknown and is still challenging to predict in the adult human. For many neurodegenerative diseases, this small theoretical risk is dwarfed by the ongoing, known hazard of the disease-causing protein, yet ASOs will continue to be evaluated for their on-target toxicity in preclinical and clinical testing.

Second, concerns for “off-target” toxicity focus on binding to other mRNAs and immune activation. For human clinical candidates, each ASO is screened in silico against the human genome and “hits” with one, two, or three mismatches are empirically tested for ASO-mediated changes of these mRNAs (Kamola et al., 2015; Monia et al., 1992). Thus, ASOs with potential overlap can be routinely screened against. In vivo, the ASO is recognized as foreign DNA by toll-like receptors and likely causes “off-target” activation of the immune system (Agrawal and Kandimalla, 2004). This response has been largely mitigated by screening for chemistries and sequences that are well-tolerated, such as avoiding potent immune-activating CpG motifs, as mentioned above. Extensive research and development has dampened some of the concerns for adverse side effects, and preclinical and clinical ASO application appears promising. However, on- and off-target issues, some of which may not yet be recognized, remain an important consideration.

Alternative Approaches

In areas of research for which ASO application may be currently limited or absent, alternative approaches for mRNA and protein modulation are available, including RNAi and antibody-mediated therapeutics, as well as traditional small molecules. RNAi uses small double-stranded RNA molecules that are processed within the cell and assembled into an RNA-induced silencing complex

(RISC) for targeting cellular mRNA. Similar to the mechanisms by which ASOs act, RNAi enables gene expression changes, such as mRNA degradation, alternative splicing manipulation, miRNA inhibition, and transcriptional silencing (Ail  et al., 2008; Carthew and Sontheimer, 2009). In vivo delivery of the RNA sequences requires viral packaging, and off-target effects remain a concern, though continued research and safety data are likely to allay some of this apprehension in the future.

A second alternative to ASOs involves antibodies that are targeted against a protein of interest. Once bound to their protein target, antibodies stimulate the host's immune system to sequester, neutralize, or damage that protein. Intracellular delivery of antibodies may be difficult and, therefore, ineffective against CNS diseases that stem from intracellular mechanisms. However, diseases with extracellular targets may benefit from an antibody-mediated strategy. Cumulative evidence points to extracellular proteins, including tau (Clavaguera et al., 2009; de Calignon et al., 2012; Holmes and Diamond, 2014), amyloid beta (Eisele et al., 2010; Meyer-Luehmann et al., 2006), and alpha-synuclein (Luk et al., 2012; Mougencot et al., 2012), as the drivers of pathological propagation within the brain. In support of this and the therapeutic application of antibodies, anti-A β immunization abolished A β deposition in Alzheimer's disease (AD) model mice (Meyer-Luehmann et al., 2006) and anti-tau antibody treatment cleared tau pathology in mutant tau mice (Yanamandra et al., 2013). Antibodies targeted against A β , solanezumab, and aducanumab are currently in clinical trial and reporting efficacy (Sevigny et al., 2016; Siemers et al., 2016), suggesting antibody-mediated therapeutics are on par with ASOs in their clinical application.

Application to Neurodegenerative Diseases

The application of ASOs for the treatment of neurodegenerative diseases has shown great success in mouse models of disease, allowing ASOs to be steadily applied to human disease through clinical trial developments (Figure 3). ASOs are currently being applied to several neurodegenerative diseases but have been most pivotal in the treatment of spinal muscular atrophy, completing phase III status and achieving FDA drug approval within the past year. Not too far behind are ASO therapeutics for amyotrophic lateral sclerosis and Huntington's disease, in which clinical trials are ongoing. These neurodegenerative diseases or their genetic subpopulations may be benefiting most readily from an ASO intervention strategy, given the well-characterized, single-gene disruption that can be targeted. For this reason and others, ASO application in Alzheimer's disease and related dementias continues to be challenging. Translational success of ASOs may also be limited in different diseases, given the predictive challenges posed by available mouse models and by the potential downsides of long-term protein lowering. However, it is clear that ASOs and their applications continue to develop as researchers try to harness their mechanisms of action toward treating neurodegenerative diseases.

Amyotrophic Lateral Sclerosis

Amyotrophic lateral sclerosis (ALS), also known as Lou Gehrig's disease, is characterized by progressive loss of motor neurons and muscle wasting. Patients are typically diagnosed at age 40–70 and succumb to the disease in only 2–5 years from the

time of diagnosis. An overwhelming 90% of ALS cases have no known cause, termed sporadic or singleton ALS (sALS), whereas 10% of cases comprise familial ALS (fALS) and arise from gene mutations (Pasinelli and Brown, 2006). Of the fALS cases, 15%–20% are caused by mutations within the Cu/Zn superoxide dismutase 1 gene (*SOD1*). More recently, fALS cases have been linked to the abnormal hexanucleotide repeat expansion within the chromosome 9 open reading frame 72 (*C9orf72*) gene, accounting for about 40% of familial cases (Majourie et al., 2012). Current treatment options for ALS patients are ineffective at halting disease progression. As major factors leading to fALS, both *C9orf72* and *SOD1* have been at the forefront of therapeutic targets for ALS and sparked preclinical research and clinical translation of ASO therapies to human patients. In addition, other targets for ASOs, including miR-155, have also shown promise.

***SOD1*.** Since its identification in 1993 as a contributing factor to fALS (Fossen et al., 1993), *SOD1* has become an important target for disease intervention. Mutations in *SOD1* have been identified throughout its transcript, although the effect of the mutation on *SOD1* enzymatic activity does not correlate with disease characteristics. Mouse data demonstrate that expression of mutant *SOD1* causes motor neuron disease whereas genetic deletion of *SOD1* does not (Philips and Rothstein, 2016). Taken together, these data support a toxic gain of function associated with the aggregation of misfolded *SOD1*. The first study using an ASO to target *SOD1* by RNase-H-mediated degradation identified striking widespread ASO distribution throughout the brain and spinal cord of rat and non-human primate, affording extensive *SOD1* mRNA and protein knockdown (Smith et al., 2006). This pivotal study confirmed ASOs as an effective technique to target CNS proteins in vivo. Importantly, ASOs were able to reduce the mutated (G93A) form of *SOD1*, resulting in extended survival (Smith et al., 2006). Given its promising preclinical results, ASO 333611 entered phase I testing in patients with *SOD1*-related fALS on January 2010 and was completed in January 2012 (NCT01041222; <https://clinicaltrials.gov>). This first-in-man study for intrathecal administration of the ASO demonstrated an excellent safety profile and established important pharmacokinetic measurements (Miller et al., 2013). This study did not show a reduction in *SOD1* protein; however, low concentrations of ASO were used as a conservative, initial test. *SOD1*-directed ASOs continue to progress through clinical trial testing for patient use. In late 2015, the second generation of *SOD1*-targeting ASO compound, BIIB067 (IONIS-*SOD1*Rx), entered phase I/II trial (NCT02623699; <https://clinicaltrials.gov>), with a single-dose cohort to be followed by a multiple-dose cohort in *SOD1* fALS patients. Escalating doses in this study are anticipated to garner more effective *SOD1* reduction compared to its initial phase I dose. Importantly, *SOD1* measured in the CSF will be an important pharmacodynamics marker for an *SOD1*-targeted ASO to determine efficacy in this ongoing study and to help direct design of future clinical trials (Crisp et al., 2015; Winer et al., 2013).

***C9orf72*.** The emergence of *C9orf72* expansion as the most frequent cause to date of fALS has directed efforts toward identifying its toxic role in the pathogenesis of ALS as well as frontotemporal dementia (FTD) (DeJesus-Hernandez et al., 2011). Not

ANTISENSE OLIGONUCLEOTIDE
PRECLINICAL RESEARCH

HUMAN CLINICAL TRIAL
PHASE: I II III

Spinal muscular atrophy: *SMN2*

Model: Type III SMA mouse	Model: <i>Smn</i> ^{-/-} , <i>hSMN2</i> ^{+/+} , <i>SMNΔ7</i> ^{+/+} mouse	Model: Non-human primate	Drug: IONIS-SMN _{rx} (hulishersen)
Key results ¹⁴ : Reduced tail/ear necrosis	Key results ¹⁴ : Motor neuron survival	Profile ¹⁴ : Good tissue distribution	Clinical Trial: NCT01494701 (Phase I) ¹⁵
Model: Δ7-SMA mouse	Improved NMJ structure		NCT01839856 (Phase II)
Key results ¹⁵ : Attenuated motor deficits	Improved motor function		NCT02193074 (Phase III)
	Extended survival		NCT02292537 (Phase III)
			FDA approval: Spinraza TM

Amphotrophic lateral sclerosis: *SOD1*

Model: SOD1-G93A rat	Model: Non-human primate	Drug: BIB067 (IONIS-SOD1 _{rx})
Key results ¹⁶ : Reduced mutated SOD1	Profile ¹⁶ : Good tissue distribution	Clinical Trial: NCT01041222 (Phase I) ¹⁷
Extended survival		NCT02623699 (Phase III)

Huntington's disease: *huntingtin*

Model: YAC128 mouse	Model: BACHD mouse	Model: Non-human primate	Drug: IONIS-HTT _{rx}
Key results ¹⁸ : Improved motor behavior	Key results ¹⁸ : Improved motor behavior	Profile ¹⁸ : Good tissue distribution	Clinical Trial: Phase I/II
Model: R6/2 mouse	Reduced polyQ aggregates		(Canada/Europe)
Key results ¹⁹ : Extended survival			

Amphotrophic lateral sclerosis: *C9orf72*

Model: Patient iPSC-differentiated neurons	Model: C9-BACexp mouse cortical neurons
Key results ²⁰⁻²² : Reduced RNA foci	Key results ²³ : Reduced RNA foci and dipeptide proteins
Corrected gene expression	Model: C9 ^{ΔG} BAC mouse
	Key results ²⁴ : Reduced RNA foci and dipeptide proteins
	Improved cognitive behavior

Tauopathies: *MAPT* and *tau*

Model: Mutant tau (E10+14) mouse	Model: Mutant tau (P301) mouse	Model: Non-human primate
Key results ²⁵ : Lowered total tau	Key results ²⁶ : Reversed tau pathology	Profile ²⁷ : Good tissue distribution
Model: Human-tau (hTau, Tau.N279K) mice	Prevented neuronal loss	
Key results ²⁸ : Reduced 4R tau mRNA/protein	Extended survival	

Alzheimer's disease: *APP*

Model: SAMP8 mouse	Model: Tg2576 mouse
Key results ²⁹⁻³¹ : Reduced APP protein	Key results ³² : Reduced APP protein
Improved learning/memory	Improved learning and memory
Decreased Aβ deposition	

Figure 3. Progression of Select ASO Candidates for the Treatment of Neurodegenerative Diseases

Successful translation of ASOs from preclinical rodent models to human clinical trials is evident, particularly for the treatment of neurodegenerative diseases. Spinal muscular atrophy targeting the survival motor neuron 2 (*SMN2*) gene and familial ALS targeting the superoxide dismutase 1 (*SOD1*) gene. The *C9orf72* gene is an additional target in ALS, with key pathological improvements identified in patient iPSC-derived neurons and novel mouse models. ASOs targeting the huntingtin gene demonstrate notable efficacy in Huntington's disease models and are in human clinical trial in Canada and Europe. ASOs for application in Alzheimer's disease and tauopathies, most notably those targeting amyloid precursor protein (*APP*) and tau (*MAPT*), respectively, show promise in preclinical development. 1, Hua et al. (2010); 2, Williams et al. (2009); 3, Porensky et al. (2012); 4, Passini et al. (2011); 5, Chiriboga et al. (2016); 6, Smith et al. (2006); 7, Miller et al. (2013); 8, Stanek et al. (2013); 9, Kordasiewicz et al. (2012); 10, Donnelly et al. (2013); 11, Lagier-Tourenne et al. (2013); 12, Sareen et al. (2013); 13, O'Rourke et al. (2016); 14, Jiang et al. (2016); 15, Sud et al. (2014); 16, Schoch et al. (2016); 17, DeVos et al. (2017); 18, Kumar et al. (2000); 19, Erickson et al. (2012); 20, Farr et al. (2014).

only is the normal physiological function of *C9orf72* unknown, but the exact mechanism by which the abnormal *C9orf72* hexanucleotide (GGGGCC) expansion mediates disease pathogenesis remains under debate. Loss of normal *C9orf72* function, gain of RNA toxicity, and gain of protein toxicity have all been proposed mechanisms of disease (Ling et al., 2013). Initially, one of the major hurdles to *C9orf72* research was the lack of a rodent model that recapitulates both the molecular and behavioral characteristics of the disease, making it difficult to test potential therapeutics, including ASO strategies, in an in vivo setting. Researchers initially used human induced pluripotent stem cell (iPSC)-derived neurons and fibroblasts from *C9orf72*-positive ALS patients to test ASO efficacy with promising results (Donnelly et al., 2013; Lagier-Tourenne et al., 2013; Sareen et al., 2013). More recently, mouse models expressing the expanded

C9orf72 gene have been developed, enabling in vivo study of ASOs (Jiang et al., 2016; Liu et al., 2016; O'Rourke et al., 2016; Peters et al., 2016).

Early studies validated ASOs that bind within the repeat expansion or within surrounding N-terminal regions of the *C9orf72* mRNA transcript that act by either recruiting RNase H to degrade the transcript or by blocking the interaction between the repeat expansion and RNA-binding proteins (Donnelly et al., 2013). Although no reduction in RNA levels was observed with ASOs designed to hinder RNA-binding protein interaction, they effectively reduced toxic RNA foci (Donnelly et al., 2013; Lagier-Tourenne et al., 2013; Sareen et al., 2013), restored normal gene expression markers (Donnelly et al., 2013; Lagier-Tourenne et al., 2013; Sareen et al., 2013), and protected against glutamate toxicity (Donnelly et al., 2013). This important finding

supports a gain of function by a toxic RNA mechanism. The recent development of animal models that express the abnormally expanded *C9orf72* gene aims to bridge the gap between ASO application in iPSC-derived cells and disease models (Jiang et al., 2016; Liu et al., 2016; O'Rourke et al., 2016; Peters et al., 2015). In one of these models, ASOs were shown to suppress several pathological features of *C9orf72* ALS, including RNA foci and dipeptide proteins in primary cortical neurons cultured from mice expressing the *C9orf72* expansion (O'Rourke et al., 2016). ASOs targeting the expanded *C9orf72* gene in a novel mouse model similarly reduced the expression of RNA foci and dipeptide proteins and also attenuated cognitive impairments (Jiang et al., 2016).

Together, these studies provide support for RNA or protein toxicity as a primary disease mechanism and successful mitigation of pathology with ASO use. Because RNA foci and dipeptide products are generated from both sense and antisense directions of the *C9orf72* transcript, it remains unclear whether reducing the antisense transcript will also be important for therapeutics. Whereas deletion of *C9orf72* from birth in mice does show abnormalities (Jiang et al., 2016; O'Rourke et al., 2016), raising some concerns for an ASO-mediated lowering strategy, the short-term administration of ASO to the endogenous mouse homolog was well tolerated (Jiang et al., 2016; Lagier-Tourenne et al., 2013). These promising *in vivo* data suggest that ASOs for *C9orf72* ALS and ALS/FTD are likely to be in initial clinical trial in the near future.

miR-155 and Other Targets. Whereas targeting *SOD1* and *C9orf72* have been at the forefront of ALS research, other targets also show promise in modulating disease pathogenesis. Intraventricular delivery of ASOs against miR-155, a target miRNA found to be upregulated in ALS tissues, resulted in de-repression of miR-155 targets throughout the brain and spinal cord and remarkably led to a significant extension in survival of *SOD1*^{G93A} mutant mice (Butovsky et al., 2015; Koval et al., 2013). It is clear that miR-155 plays a pathological role in ALS models and likely in humans, and an ASO strategy can be effective in achieving an improved outcome. Other preclinical studies have identified acetylcholine (Gotkine et al., 2013), phospholipase A2 α (Solomonov et al., 2016), p75 (Tumer et al., 2003), and prostate apoptosis response 4 (Pedersen et al., 2000) as potential targets for ASOs to mitigate ALS, and putative targets like ataxin-2 have been proposed for future research (van den Heuvel et al., 2014).

Concluding Remarks. Positive strides made in the preclinical and clinical development of an ASO strategy have greatly increased enthusiasm for ASOs as an applicable therapy for ALS. ASOs have not only enabled increased understanding of disease processes but also have shown successful translation to human patients in the case of *SOD1*. Notably, this clinical effort implemented an intrathecal administration of ASOs to CNS in adults, a strategy that is likely to be employed in subsequent ASO trials targeting *SOD1* in ALS and in trials aimed at other neurodegenerative disorders.

Spinal Muscular Atrophy

Spinal muscular atrophy (SMA) results from the deletion of the survival motor neuron (*SMN1*) gene, a loss that is predicted to be lethal in all other species except human. Humans express a second form of the *SMN* gene—*SMN2*—which is able to partially

restore *SMN* protein expression and function. The number of *SMN2* gene copies expressed determines the degree to which *SMN* function is restored, which in turn affects the severity of the disease. *SMN2* cannot fully compensate for the loss of *SMN1* in disease due to a C/T nucleotide variation within exon 7 that causes weakening of the 3' splice site and exon 7 skipping. The resulting protein product is unstable and degraded. Therefore, strategies designed to improve the ability of the *SMN2* gene to produce full-length, functional *SMN* protein are paramount for disease intervention. ASOs have been advantageous in designing treatment strategies targeted against splicing regulatory elements of *SMN2* to promote exon 7 inclusion, with remarkably successful results in mouse models and human clinical trials, and has led to the first FDA-approved treatment of SMA.

SMN2 Exon 7 trans-Splicing Targeting ISS-N1. Initial efforts to pinpoint targetable sequences within *SMN2* to regulate exon 7 splicing identified an inhibitory splicing sequence, known as intronic splicing silencer N1 (ISS-N1), located in intron 7 of the *SMN2* gene. When targeted against ISS-N1, 2'-O-Me-modified ASOs potently stimulated exon 7 inclusion and increased *SMN* protein levels in patient fibroblasts (Singh et al., 2006). When the ASO was injected into the CNS of delta7-SMA mutant mice during the first 10 days after birth, the ASO distributed throughout the CNS, elevated levels of *SMN* protein, and improved early postnatal body weight gain and righting response (Williams et al., 2009), confirming the *in vivo* efficacy of ISS-N1-targeting ASOs.

Independent validation of the critical role of ISS-N1 in exon 7 skipping and intervention using ASOs was executed using an ASO "microwalk" along *SMN2*, identifying 2'-MOE ASOs that block heterogeneous nuclear ribonucleoprotein (hnRNP) A1/A2 motifs within the ISS-N1 sequence (Hua et al., 2008). This ASO, termed ASO 10-27, was first tested in hSMN2 mice, demonstrating robust exon 7 inclusion. As few as two intravenous injections of ASO 10-27 were able to significantly stimulate exon 7 inclusion (~50% of *SMN2* transcripts) in the liver and kidney of hSMN2 mice, and after 4 weeks of treatment, inclusion increased to over 90% (Hua et al., 2008). No effect was evident in spinal cord tissue, reflecting the poor ability of ASOs to cross the blood-brain barrier (BBB). In an effort to achieve central delivery, ASO 10-27 was infused directly into the lateral ventricle of hSMN2 mice, resulting in dose-dependent, long-lasting exon 7 inclusion and increased total *SMN* protein within the spinal cord (Hua et al., 2010). Remarkably, a single intracerebroventricular (ICV) injection of ASO was able to curb the necrotic tail and ear phenotype (Hua et al., 2010) and restored muscle physiology and motor function (Passini et al., 2011) in severe SMA mouse models (type III SMA mice and *Smn*^{-/-}, hSMN2^{+/+}, and SMN Δ 7^{+/+} mice, respectively). Because some *SMN* function is likely required in all cells, central and peripheral administration methods were compared to identify the best route for the most effective outcomes. Peripheral administration of ASO surprisingly provided substantial phenotypic rescue and significantly prolonged survival (Hua et al., 2011), suggesting a peripheral component of SMA is very important in mouse models. Whether peripheral administration will be a needed component in humans remains unknown.

Modulation of SMN splicing was separately identified with a morpholino oligonucleotide against the ISS-N1 *SMN* sequence, which differs from phosphorothioate ASOs in its backbone modification (Figure 2). Using a morpholino design, researchers identified a 3-fold increase in exon 7 inclusion and SMN protein expression within the brain and spinal cord after a single ICV injection in SMA model mice, conferring a dose-dependent, 97-day extension in survival (Porensky et al., 2012). Restoration of SMA expression also corrected electrophysiological and motor unit dysfunction in delta7-SMA mutant mice (Arnold et al., 2014, 2016). Collectively, ISS-N1-targeting studies agree on the early and central delivery of ASOs to not only correct *SMN2* splicing but also improve functional outcomes and survival. Preclinical research continues to adjust and improve ISS-N1-targeted ASO therapeutics to establish a more efficient and successful compound without adverse effects. In addition, newly developed mouse models have enabled delayed treatment paradigms in order to fully evaluate *SMN*-targeted ASOs (Bogdanik et al., 2015).

ASO 10-27, or nusinersen (previously IONIS-SMN_{FX}), achieved clinical trial status in December 2011 with the initiation of a phase I trial to determine the safety, tolerability, and dose range (NCT01494701 and NCT01780246; <https://clinicaltrials.gov>) followed by phase II testing in 2013 (NCT01839656; <https://clinicaltrials.gov>). In 2016, researchers reported on phase I results, citing preliminary clinical improvement without safety and tolerability concerns following nusinersen treatment in SMA patients aged 2–14 years old (Chiriboga et al., 2016). Only 6 years following its preclinical debut, nusinersen entered phase III trial testing (NCT02193074 and NCT02292537; <https://clinicaltrials.gov>), which speaks to both the urgent need for SMA therapies and the effectiveness of ASOs in treating SMA. Whereas the primary data have not been released at the time of this review, a joint press release on August 1, 2016 from Biogen Idec and Ionis Pharmaceuticals stated that the phase III ENDEAR trial, which was conducted in type 1 SMA patients, met its clinical endpoint. Infants treated with ASO targeting *SMN2* reached motor milestones on the Hammersmith infant neurological examination scale (BusinessWire, 2016). Whereas we are unable to judge the strength of these clinical data and, importantly, the magnitude of the effect at this time, meeting the primary endpoint in a phase III clinical study is a major accomplishment that has since assured the advancement of nusinersen in the market. Spinraza (nusinersen) was approved by the US Food and Drug Administration for use in December 2016, becoming the first drug available for the treatment of SMA in children and adults (FDA News Release, 2016a).

SMN Repressor Element 1 and Other Regulatory Elements. In addition to manipulating the missplicing of exon 7 via ISS-N1, alternative strategies seek to use ASOs to target other regulatory proteins to increase exon 7 inclusion. Exon 7 is surrounded by several splicing enhancers and silencers, exonic and intronic, to intricately regulate the splicing patterns. Baughan and colleagues (2009) confirmed the presence of an additional splicing repressor sequence upstream of exon 7. When this region, known as element 1 (E1), was deleted, potent increases in full-length *SMN* mRNA were noted, prompting the design of bifunctional RNAs to target the E1 sequence. In the case of *SMN*, the

bifunctional RNA consists of an antisense sequence complementary to E1 linked to an RNA segment that would recruit exonic splice enhancer proteins. A single ICV injection of E1 bifunctional RNA into *Smn*^{-/-}*SMN2*^{+/+} mice restored expression of SMN protein and improved weight gain (Baughan et al., 2009). Subsequently, morpholino ASOs were found to elevate SMN protein expression, drastically extend lifespan, and improve neuromuscular junction structure in two separate mouse models of varying SMA severity (Osman et al., 2014). This same group identified a separate target within the exon 8 splice site of *SMN* involved in recruitment of the splicing silencer, hnRNP A1, to stimulate upstream exon 7 inclusion (Dickson et al., 2008). A modest increase in SMN protein was achieved in the SMA mouse brain (Dickson et al., 2008). Interestingly, a dual treatment strategy targeted at both the ISS-N1 element and exon 8 regulatory site produced a 2-fold increase in *SMN* mRNA and protein levels compared to single ASOs given alone (Pao et al., 2014), suggesting that combinatorial therapy may result in even better outcomes than already successful single treatments. Other ASO targets, such as long non-coding RNA (lncRNA) *SMN* sequences, have also shown considerable promise in modulating SMN expression and extending survival in mice (d'Ydewalle et al., 2017).

Concluding Remarks. SMA mouse model experiments have paved the way for translation into human patients, with Spinraza at the forefront of clinical application. Notably, this therapy employs one of the remarkable features of ASOs—an alternative splicing manipulation to restore functional levels of the disrupted SMN protein. Whereas alternative strategies, including pharmaceutical inhibitors, viral *SMN* gene replacement, stem cells, and small molecules, are under current investigation (Duque et al., 2015; Van Meerbeke et al., 2013; Zanetta et al., 2014), the early success of ASOs in clinical studies will likely make this approach a benchmark to which others are compared. Whereas primary data from the pivotal phase III nusinersen trial are not yet available, this ASO has successfully transitioned from basic research to clinical trial. Now, with FDA approval, ASOs are a veritable treatment for SMA where none existed before. The success of Spinraza ensures that ASOs will likely remain a therapeutic option for SMA in the near future.

Huntington's Disease

Huntington's disease (HD) is an adult-onset, neurodegenerative condition characterized by both locomotor and cognitive decline. On average, patients are diagnosed between ages 30 and 40 based on symptoms that worsen over the course of disease. HD is inherited in an autosomal-dominant fashion and caused by abnormal expansion of a CAG repeat in the *huntingtin* (*Htt*) gene. Once mutant *Htt* is translated, the expanded polyglutamine (polyQ) protein likely mediates a toxic gain of function through the formation of intraneuronal aggregates or interaction with other proteins (Ordway et al., 1997; Rubinshtein, 2002). The medium spiny neurons of the striatum are particularly affected, yet neurons within other brain regions appear to be vulnerable (Rubinshtein, 2002), thus requiring a more global approach to treatment. Gene-silencing approaches, including those activating the RNAi pathway as well as by ASO, have been extensively researched to achieve reductions in mutant *Htt* and curb disease progression.

Huntingtin. A major breakthrough for an ASO therapy for HD reported on the in vivo effectiveness of ASOs targeted against mutant *Htt* mRNA by employing several mouse models of HD as well as testing intrathecal infusion in non-human primates (Kordasiewicz et al., 2012). Researchers exploited the RNase-H-mediated degradation properties of ASOs and 2'-MOE modifications for potent, stable, and tolerable ASO delivery. This study identified that a human *Htt*-targeted ASO afforded a significant decrease of Htt with lasting reduction for 3 months post-treatment. In addition, continuous infusion of the ASO resulted in its widespread distribution throughout the mouse brain, including deeper structures of the striatum and thalamus in neuronal and non-neuronal cells (Kordasiewicz et al., 2012), suggesting *Htt* ASOs are able to penetrate brain structures to reach the affected cellular populations. Remarkably, an ~50%–80% reduction of mutant human *Htt* mRNA was achieved in most brain regions. Pre-symptomatic and post-symptomatic ASO administration in an HD mouse model (YAC128) lessened disease phenotypes (Kordasiewicz et al., 2012; Stanek et al., 2013), and when applied to a more severe, quickly progressing HD mouse model (R6/2), ASO treatment afforded a significant extension in survival (Kordasiewicz et al., 2012). Whereas debate still exists regarding how well mouse models recapitulate human disease, ASO testing in multiple HD mouse models with repeated success helped to confirm *Htt*-targeted ASO efficacy. Finally, ASO infusion into non-human primates did not result in any adverse effects; however, a more limited distribution of the ASO was noted (Kordasiewicz et al., 2012). Overall, this study identified in vivo safety and good distribution of an *Htt*-targeted ASO, suggesting ASOs as applicable interventions for human studies. Behavioral improvements observed in mouse models show sustained therapeutic effect even after *Htt* target mRNA levels returned, underscoring the therapeutic potential for ASOs in HD. This success has recently sparked the initiation of clinical trial testing of *Htt*-targeted ASOs in Canada and Europe (PRSNNewswire, 2015).

One possible limitation associated with the above study is the targeting and reduction of both the normal and mutant *Htt* allele. Huntingtin has known physiological roles in neuronal survival (Rigamonti et al., 2000) and axon stability (Dragatsis et al., 2000). In order to address this, Kordasiewicz and colleagues (2012) demonstrated that reduction of endogenous mouse *Htt* along with mutant human *Htt* did not result in any adverse events, suggesting that reduction of *Htt* in the adult may be well tolerated. Nevertheless, enthusiasm remains for identifying a strategy that can specifically reduce the mutant allele. One possible mechanism to identify mutant Htt and therefore target it specifically would use genetic SNPs within the CAG repeat that distinguish the mutant allele from the normal allele. Recent studies have focused on the identification of several lead SNP-targeted compounds to distinguish mutant *Htt* allele. In particular, a lead candidate modified for increased potency and selectivity achieved significant *Htt* mRNA reductions and striatal delivery without overt inflammatory effects in mice. This ASO and others have undergone further development and improvement for human application to increase the specificity of the ASO to >100-fold for mutant *Htt* allele over normal allele (Ostergaard et al., 2013). When evaluated in Hu97/18 mice exhibiting expression

of both the mutant and wild-type *Htt* allele, specificity for the mutant allele was confirmed with specific reduction of mutant *Htt* without change to the normal mRNA or protein (Southwell et al., 2015) and demonstrated an extended duration of action from 16 to 32 weeks depending on the 2' modification (Skotte et al., 2014). Whether these impressive qualities afford a therapeutic effect for modulating locomotor or cognitive deficits in HD mouse models remains to be determined.

Concluding Remarks. Therapeutic strategies utilizing ASOs to reduce mutant *Htt* have shown great promise in preclinical Huntington's disease animal models, and *Htt* lowering ASOs have entered early-phase clinical studies in Europe (PRSNNewswire, 2015). A mutant *Htt* allele-specific design seems advantageous, as some evidence may point to the requirement of Htt in normal cellular function, though lowering of Htt in mice and non-human primates was well tolerated (Kordasiewicz et al., 2012) and may, in fact, not be a concern. Regardless, ASOs that target toxic *Htt* alone are currently undergoing development and comprehensive characterization. Additional ASO designs, including morpholino (Sun et al., 2014) or those targeting *Htt* by splicing manipulation (Evers et al., 2014), may provide alternative strategies to reduce Htt toxicity without lowering total protein.

Alzheimer's Disease and Primary Tauopathies

One of the most devastating and common neurodegenerative conditions is AD, which currently affects nearly 5.4 million individuals. As the population ages, the number of AD patients is expected to rise exponentially. The two major protein pathologies associated with AD are amyloid beta (A β) and tau. Abnormal aggregation or mutation of tau extends beyond AD to several neurodegenerative disorders, including corticobasal degeneration (CBD), frontotemporal dementia with parkinsonism-17 (FTDP-17), progressive supranuclear palsy (PSP), and Pick's disease, collectively known as tauopathies. Although the molecular cascades of disease pathogenesis are highly researched, there are currently no effective therapies to stop disease progression and prevent cognitive decline. ASO application in AD and tauopathies has, to this point, centered on preclinical models targeted against proteins involved in amyloid formation and tau deposition. In addition, several groups have recognized the upregulation of miRNAs in the pathogenesis of AD and have employed ASOs to target these molecules.

Amyloid Precursor Protein. The characteristic A β plaque pathology of AD arises through intracellular processing of the amyloid precursor protein (APP). APP normally exists as a transmembrane protein with known cleavage sites for α -, β -, and γ -secretases but unknown physiological function. When APP is cleaved by β - and γ -secretase, the resulting A β fragment can oligomerize and form the insoluble fibrils and plaques present in AD. APP mutations have been identified in a percentage of human AD patients, accounting for a rare, rapid onset familial form. Given its genetic link and originating role in A β production, APP can be overexpressed or mutated in mouse models to derive AD pathology and behavioral abnormalities and is a primary target for ASO development.

APP-targeting ASO research has initially focused on development of an APP-lowering ASO targeted against the region that will become the central portion of the A β protein (corresponding to A β amino acids 17–30), specifically between the sites of β - and

γ -secretase cleavage. Whereas these ASOs were modestly effective at lowering APP protein and generated cognitive improvements (Erickson et al., 2012; Kumar et al., 2000), both the mouse model used and the route of ASO administration limit interpretation of these results. Senescence-accelerated mouse-prone (SAMP8) mice (Erickson et al., 2012; Kumar et al., 2000) and, later, Tg2576 (K670M/N671L Swedish APP mutation; Farr et al., 2014) were treated with peripheral injection of the ASO. ASOs exhibit poor ability to cross the BBB, preventing clear interpretations of ASO-mediated effects in these studies. In addition, the behavioral changes observed in SAMP8 mice may be confounded by the naturally occurring neuropathological and behavioral phenotype likely resulting from age-related changes in numerous genes and proteins (Butterfield and Poon, 2005). To date, these limitations have slowed the advancement of an APP-targeted ASO strategy, and further studies with other mouse models or improved delivery methods are necessary.

An alternative approach to reducing levels of APP is targeting proteins involved in the production or clearance of A β . Pharmacological β -secretase (BACE) inhibitors have achieved preclinical success (Vassar, 2014) and have advanced into phase II/III clinical trials (NCT02245737 and NCT02783573; <https://clinicaltrials.gov>). Using an ASO, inhibition of BACE was effective at decreasing A β fragments in rodent brain (McMahon et al., 2003). Whereas exciting from a treatment perspective, BACE reduction by ASO may carry several downsides associated with altering normal BACE function, as several groups have reported negative phenotypes in BACE-null mice (Dominguez et al., 2006; Hu et al., 2008; Laird et al., 2005). Using a method that avoids issues associated with complete loss of BACE expression, researchers targeted the BACE cleavage site within APP, demonstrating that weekly intraventricular ASO injections in Tg2576 mice increased levels of soluble APP α and lowered levels of acetylcholinesterase, a marker of A β toxicity (Chauhan and Siegel, 2007). Whereas these results suggest that β -secretase activity was blocked, only modest effect sizes were reported, and key neurodegenerative features of Tg2576 mice, including plaque pathology and cognitive impairments, were not addressed. Thus, the effectiveness of BACE targeting by ASO is difficult to conclude at this stage.

Recently, ASO targeting of apolipoprotein E receptor 2 (ApoER2) has been tested, linking a potential ASO target to the known AD risk gene, APOE. It is well established that the apolipoprotein isoform ApoE- ϵ 4 confers a 2- to 12-fold risk for developing AD in humans, depending on the number of ϵ 4 alleles (Corder et al., 1993). Whereas the role of ApoER2 in mediating AD pathogenesis is not fully understood, the receptor may modulate APP localization and processing, resulting in increased A β production (Fuentealba et al., 2007; He et al., 2007), and its different splice variants confer its ligand binding and signaling function (Sun and Soutar, 1999). Specifically, inclusion of exon 19 in the ApoER2 gene enables interaction with NMDA receptors to facilitate synaptic function and plasticity (Volmer and Krieg, 2009). This splicing event at exon 19 appears deregulated in human AD and in aged mice (Hinrich et al., 2016). Restoration of full-length ApoER2 via ASO-mediated splicing correction afforded improvements in synaptic function and cognitive behavior in APP mutant mice (TgCRND8; Hinrich et al., 2016).

This study and those directed against APP or APP-processing genes highlight the flexibility of ASOs in identifying potential targets and the variety of mechanisms—degradation or splicing modulation—that can be harnessed for therapeutic gain.

Tau. The microtubule-associated protein, tau, is implicated in AD pathogenesis and other neurodegenerative diseases known as tauopathies. Under normal conditions, tau functions to stabilize microtubules and may also be involved in intracellular signaling, scaffolding, and neurogenesis (Morris et al., 2011). In humans, tau is expressed as six different isoforms that differ based on alternative splicing within the N terminus (0N, 1N, and 2N) and repeat domain region of the tau (*MAPT*) gene. This latter splicing event at exon 10 regulates expression of a three-repeat or 3R (– exon 10) transcript and a four-repeat or 4R (+ exon 10) transcript. In disease, tau becomes abnormally hyperphosphorylated and accumulates within neurons and is associated with neuronal dysfunction and cell death. Therefore, targeting of tau by itself with ASOs is a plausible therapeutic intervention for tauopathies, some of which have known mechanisms tied to the abnormal regulation *MAPT* mRNA via mutations and altered isoform expression. Given the aggregation of tau and recognized exon 10 splicing changes, there are two potential therapeutic strategies for tauopathies: (1) lowering of total tau by RNase-H-directed degradation and (2) lowering of 4R tau alone by an ASO splicing strategy. The advantage of lowering total tau is the development of one therapeutic approach that would work for all tauopathies. The advantage of specifically reducing 4R tau may be its selectivity, leaving total tau unaltered.

Genetic deletion of tau in mice has shown no abnormalities (Ittner et al., 2010; Roberson et al., 2007), and in adult mice, lowering mouse tau with ASO for 1 month did not demonstrate any behavioral defects (DeVos et al., 2013). This latter experiment compares most closely to how and when ASOs might be applied therapeutically and supports the safety of a total tau-lowering strategy. In contrast, there are some reports demonstrating synaptic abnormalities and cognitive impairment with genetic deletion of tau in mice (Lei et al., 2012; Ma et al., 2014). Overall, our interpretation of the current data is that lowering tau will be well tolerated in humans, but further toxicology data are needed in large animals to better assess this outcome.

In human mutant (E10+14) tau-expressing mice, use of a tau-targeted ASO significantly reduced total tau mRNA and protein (Sud et al., 2014), confirming in vivo application. Although this study did not assess pathological outcomes, an improvement might be expected in light of previous findings in which doxycycline-induced tau transgene suppression could halt tau aggregates (Mocanu et al., 2008; Polydoro et al., 2013) and arrest neuronal death and cognitive deficits (Santacruz et al., 2005). In support of this work, ASO-mediated total tau reduction was also highly effective in mitigating disease pathology. PS19 mice, expressing the P301S *MAPT* mutation, exhibit widespread phosphorylated tau, neuronal loss, and premature death (Yoshiyama et al., 2007). However, when a tau-lowering ASO was administered to aged PS19 mice, phosphorylated tau burden was prevented, hippocampal neurons were spared, and survival was significantly extended (DeVos et al., 2017). Furthermore, tau reduction was evident in non-human primates following ASO

administration (DeVos et al., 2017). This study is the first to apply a total tau-targeting ASO strategy to alter disease outcome and provides critical support for a therapeutic tau ASO. In addition to dementia, emerging evidence suggests tau may also mediate hyperexcitability in neurodegenerative states (Holtzman et al., 2013; Ittner et al., 2010; Roberson et al., 2007). To investigate this link, ASOs designed to degrade endogenous tau were used to investigate seizure outcomes in adult mice. Robust tau knockdown afforded significant reductions in hyperexcitability in two separate methods of seizure induction (DeVos et al., 2013). These results not only support the association between tau and hyperexcitability but also show that lowering tau could be an important therapeutic for both tauopathies and epilepsy.

In light of the *MAPT* mutations identified in FTDP-17 that alter splicing of exon 10, splicing alteration by ASO is an intriguing treatment strategy. The majority of primary tauopathies exhibit mutations within exon 10; thus, using splicing ASOs could be a way to excise an existing mutation and correct deficits without affecting total tau levels. Recently, ASOs were used to manipulate *MAPT* splicing in vivo, demonstrating that ASO-mediated splicing toward increased 4R tau elevated aggregated tau, increased seizure severity, and impaired nesting behavior (Schoch et al., 2016), suggesting greater 4R is detrimental. The reverse approach, an exon-10-skipping ASO to bias toward 3R tau expression was also evaluated. In two human tau-expressing mouse models, ASO treatment significantly shifted splicing patterns to lower 4R tau without change to total tau levels (Schoch et al., 2016). Tau-targeted splicing ASOs appear to be effective based on their mRNA target sites, which can interfere with splicing regulatory sequences or disruption of secondary structural components necessary for interactions with splicing factors. Whereas changes to neuronal cytoskeleton or other function will be a consideration for future application in vivo, these studies highlight the potential for corrections in tau splicing for therapeutic development, especially in diseases affected by exon/intron 10 mutations or splicing abnormalities.

miRNA Inhibition. Although tau- and A β -targeting strategies have dominated the field, research focused on miRNA changes in AD is an exciting new area and introduces potential targets for ASOs. Several regulatory miRNAs, including miR-34a and miR-206, were found to be highly expressed in mouse models of AD or postmortem human AD brain tissues (Faghini et al., 2010; Lee et al., 2012; Wang et al., 2009). ASOs targeted against miR-34a, a likely regulator of the anti-apoptotic Bcl-2, resulted in de-repression of Bcl-2 protein and decreased caspase-3 in APP/presenilin 1 mutant mice (Wang et al., 2009). Thus, miR-34a inhibition by ASO may block cell death pathways occurring in neurodegeneration. In the Tg2576 mouse model, ASO inhibition of miR-206, a predicted regulator of brain-derived neurotrophic factor (BDNF), resulted in de-repression and normalization of BDNF levels in multiple brain regions and improved memory function (Lee et al., 2012). Similar results were seen with intranasal delivery (Lee et al., 2012), highlighting this method as a possible noninvasive delivery route for ASOs. Overall, miRNA sequestration by ASO appears to modulate aspects of neurodegeneration that may prove beneficial as a treatment strategy.

Concluding Remarks. The various targets and pathogenic mechanisms that mediate AD and tauopathies offer multiple

opportunities for intervention in human dementias. The most straightforward approach, and one that can be achieved by ASOs, is to reduce levels of pathogenic protein itself, namely APP or tau. Whereas animal data thus far suggest that this will be well tolerated, issues associated with disruption of the normal protein function will remain a potential safety issue and will need to be weighed against the severity of the disease being treated. Creative developments in ASO design, including tau splicing intervention or targeting regulatory miRNA pathways, are also promising. ASO therapeutics for dementias are on a path toward human clinical trials and will be an exciting area of translation.

Promise for ASO Therapeutics

ASOs hold considerable promise for neurodegenerative disease therapies as evidenced by SMA and ALS human clinical trial successes, and preclinical efforts in other CNS disorders continue to progress. Although not discussed in detail here, ASOs against ataxin-2 (*ATNX2*), a gene implicated in both spinocerebellar ataxia (SCA) type 2 and TDP-43-driven ALS, were recently tested in vivo, generating promising results. ASO-mediated *ATNX2* reduction in two separate SCA mouse models improved motor performance, corrected abnormal gene expression, and restored normal Purkinje cell electrophysiology (Scoles et al., 2017). When tested in TDP-43 transgenic mice, *ATNX2*-targeted ASOs afforded motor function improvements and significantly extended survival (Secker et al., 2017). From a therapeutic perspective, the use of ASO technology extends far beyond the scope of neurodegenerative disorders. The ASOs mipomersen (Kynamro) for familial hypercholesterolemia (FDA News Release, 2013) and eteplirsen for Duchenne muscular dystrophy (FDA News Release, 2016b) have been approved by the FDA. In addition, ASOs are currently being investigated in models and human patients for multiple sclerosis, cancer, and cholesterol management. Even more targets for ASOs may become available as more information regarding the pathogenic role for specific genes or proteins emerges. Given their widespread application, advancements in design, and achievements in clinical application, ASOs continue to garner enthusiasm for the treatment of many human diseases.

With the newness of ASO application to CNS disorders, there are likely still many lessons to be learned regarding how to translate preclinical results to human clinical trials. Whereas intrathecal delivery is safe and well tolerated, identifying an even easier route of administration may be an important step to achieve more ubiquitous application of this approach. To this end, ASOs are constantly being modified and restructured in order to enhance their target potency and improve delivery. ASOs offer a possible treatment for neurodegenerative diseases, and given their specificity, flexibility, and overall safety, we anticipate a number of ASO clinical trials in the near future.

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neurodegenerative diseases. T.M.M. served on a medical advisory board for Biogen Idec and for Ionis Pharmaceuticals and currently serves as a consultant for Cytokinetics.

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