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Antisense oligonucleotides for modulating survival motor neuron 2 (SMN2) splicing

Field of the invention

The present invention relates to antisense oligonucleotides which modulate RNA splicing. In particular, the antisense oligonucleotides are for enhancing exon 7 inclusion in survival motor neuron 2 (SMN2) mRNA during splicing. The antisense oligonucleotides may be used for treating spinal muscular atrophy.

Background of the invention

Spinal muscular atrophy (SMA) is an autosomal recessive genetic disease. It affects infants and children from young and is characterized by progressive degeneration of the motor neurons, with consequent paralysis of the trunk and limbs and can result in premature death. The disease has an incidence of about 1:6000. About 1:50 among the general population are carriers of the disease. Three forms of the diseases are recognized – type I, a very severe and fatal form affecting infants, type II, affecting young children who do not achieve the ability to walk, and a relatively milder type III causing milder disability among older children with the ability to walk. Currently, there is no effective treatment for SMA.

SMA is linked to mutations in the survival motor neuron 1 gene (SMN1) on chromosome 5. There is also a second survival motor neuron (SMN) gene, SMN2 located on the same chromosome. While there is only one copy of SMN1 on chromosome 5, there may be 0 to 4 copies of SMN2. However, SMN2 differs from SMN1 by a single nucleotide in exon 7 and in a few intronic nucleotides. This exon 7 nucleotide polymorphism causes exon 7 to be spliced out during SMN2 precursor mRNA splicing, resulting in a truncated SMN2 mRNA. As full length SMN transcript is required to generate functional SMN protein, SMN2 can only generate a small proportion (about 15%) of functional

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SMN protein. In normal individuals, the great proportion of functional SMN protein is, therefore, derived from the SMN1 gene, which generates a full length SMN transcript. In SMA, mutations in SMN1 lead to a lack of functional SMN protein, which in turn results in the loss of motor neurons and the clinical features of SMA.

As the SMN2 gene is still capable of generating a small amount of functional SMN protein, one strategy for therapy of SMA is the up-regulation of SMN2 gene expression to increase the amount of functional SMN protein. There is evidence to support this strategy, as SMA patients and SMA mouse models with higher SMN2 gene copy numbers have milder disease. Various agents which are able to up-regulate SMN2 gene expression, eg. butyrate and valproate, have undergone or are undergoing clinical trials. Although preliminary reports were encouraging, the results of completed studies have been inconclusive or unhelpful. Furthermore, these agents which up-regulate SMN2 gene expression do not act specifically, as they may also up-regulate other genes. Thus, while trials of these agents are continuing, there is definitely scope and need to develop other potential therapeutic agents.

Antisense oligonucleotides (AONs) may offer a potentially effective and more specific therapeutic strategy for SMA. AONs designed to cause skipping of an exon during precursor mRNA splicing are currently undergoing clinical trials in Duchenne muscular dystrophy. For SMA, AONs may be designed to cause the inclusion, instead of skipping, of an exon during precursor mRNA splicing. AONs which are able to induce inclusion of exon 7 in SMN2 precursor mRNA to generate full length SMN2 mRNA may be candidates for use in therapy of SMA.

Summary of the invention

The present invention provides an isolated fusion antisense oligonucleotide comprising a first oligonucleotide sequence and a second oligonucleotide sequence, each of which is complementary to or targets a different region (or sequence) of the SMN precursor mRNA.

According to a first aspect, the present invention provides an isolated fusion antisense oligonucleotide comprising a first oligonucleotide sequence joined to a second oligonucleotide sequence, wherein the second oligonucleotide sequence comprises a sequence complementary to a first splicing regulatory element (SRE) of SMN precursor mRNA and the first oligonucleotide sequence comprises a sequence complementary to a sequence of the SMN precursor mRNA other than the said first SRE complementary to the second oligonucleotide sequence.

In particular, the first oligonucleotide sequence is complementary to or targets a sequence on the SMN precursor mRNA downstream of the said first SRE. The first oligonucleotide sequence may also be complementary to a second SRE of SMN precursor mRNA. The first and/or second SRE may comprise a splicing silencer (SS) or a splicing enhancer (SE). Further, the first and/or second SRE may either be intronic, exonic or located at the junction of intronic/exonic or exonic/intronic sequences.

The first and second oligonucleotide sequence of the fusion antisense oligonucleotide may be complementary to any combination of a first and second SRE, wherein the first and second SRE is from any intron, exon, exon/intron or intron/exon junction. In particular, the first and/or second SRE may be of close proximity to exon 7. An SRE may be considered of close proximity to exon 7 if the SRE is near enough to exon 7 and thus able to modulate splicing of exon 7. For example, the second oligonucleotide sequence may comprise a sequence complementary to a first SRE from either exon 6, intron 6, exon 7, intron 7, exon

8, intron 8 or any one of the junctions of exon6/intron6, intron6/exon 7, exon 7/intron 7, intron7/exon 8 or exon 8/intron 8 while the first oligonucleotide sequence may comprise a sequence complementary to a second SRE from either exon 6, intron 6, exon 7, intron 7, exon 8 or any one of the junctions of exon6/intron 6, intron6/exon 7, exon 7/intron 7, intron 7/exon 8 or exon8/intron 8.

In other examples, the first and/or second SRE may comprise an intronic splicing silencer (ISS), exonic splicing silencer (ESS), intronic splicing enhancer (ISE) or exonic splicing enhancer (ESE). Accordingly, the first and/or second SRE may comprise an intronic splicing silencer (ISS) or an exonic splicing silencer (ESS). In particular, the first and/or second SRE may be located in exon 6, intron 6, exon 7, intron 7, exon 8 or intron 8 or any one of the junctions of exon6/intron 6, intron6/exon 7, exon 7/intron 7, intron 7/exon 8 or exon8/intron 8.

The isolated fusion antisense oligonucleotide according to any aspect of the invention may be for use in modulating SMN2 mRNA splicing. In particular, the isolated fusion antisense oligonucleotide according to any aspect of the invention may be for use in enhancing exon 7 inclusion in SMN2 mRNA. The isolated fusion antisense oligonucleotide according to any aspect of the invention may also be for use in treating spinal muscular atrophy.

Brief description of the figures

Figure 1 shows the gel electrophoresis of raw data from the RT-PCR using exon 6 forward primer 5'-accacctcccatatgtccag-3' (SEQ ID NO: 50) and exon 8 reverse primer 5'-aactggcctcatttcttcaaa-3' (SEQ ID NO: 51) from cells transfected with 100 nM antisense oligonucleotides; A1: AON-1 (SEQ ID NO: 5); A7: AON-7 (SEQ ID NO: 44), A8: AON-8 (SEQ ID NO: 45), A9: AON-9 (SEQ ID NO: 21); A10: AON-10 (SEQ ID NO: 23); A13: AON-13 (SEQ ID NO: 25);

A14: AON-14 (SEQ ID NO: 17); Singh (SEQ ID NO: 46); Hua (SEQ ID NO: 47); Lim (SEQ ID NO: 11); SCMB (SEQ ID NO: 49) and NT: non-transfected control.

Figure 2 shows the densitometry image analysis from ImageJ software of the raw data of Figure 1. The figures below the lanes show the percentage of exon 7 inclusion computed as the ratio of the upper band densitometry value to the sum of the upper and lower band densitometry values.

Figure 3 shows the effect of 25 nM transfected antisense oligonucleotides on expression of full-length SMN2. The antisense oligonucleotides are: AON-1 (SEQ ID NO: 5); AON-7 (SEQ ID NO: 44), AON-8 (SEQ ID NO: 45), AON-9 (SEQ ID NO: 21); AON-10 (SEQ ID NO: 23); AON-13 (SEQ ID NO: 25); AON-14 (SEQ ID NO: 17); Singh (SEQ ID NO: 46); Hua (SEQ ID NO: 47); LimE8 (SEQ ID NO: 11); SCMB (SEQ ID NO: 49) and NT: non-transfected control. The number above each bar represents the fold increase over the NT control.

Figure 4 shows the effect of 50 nM transfected antisense oligonucleotides on expression of full-length SMN2. The antisense oligonucleotides are as for Figure 3. The number above each bar represents the fold increase over the NT control.

Figure 5 shows the effect of 100 nM transfected antisense oligonucleotides on expression of full-length SMN2. The antisense oligonucleotides are as for Figure 3. Three samples were analysed for each AON. The number above each bar represents the fold increase over the NT control.

Figure 6 shows the effect of 200 nM transfected antisense oligonucleotides on expression of full-length SMN2. The antisense oligonucleotides are as for Figure 3. Three samples were analysed for each AON. The number above the the three bars for each AON represent the average fold increase over the NT control for each AON.

Figure 7 shows the dose effect for different antisense oligonucleotide on expression of full-length SMN2, based on the data from Figures 3-6. The antisense oligonucleotides are as for Figure 3.

Figure 8 shows the Western blot of SMA fibroblast cells (GM03813) transfected with 100 nM of five AONs; A1: AON-1 (SEQ ID NO: 5); A13: AON-13 (SEQ ID NO: 25); Singh: SEQ ID NO: 46; Hua: SEQ ID NO: 47; LimE8: SEQ ID NO: 11 and NT: non-transfected control. The figures at the bottom of the blot refer to fold change of SMN protein relative to the non-transfected (NT) control.

Figure 9 shows the Western blot of SMA fibroblast cells (GM03813) transfected with 200 nM of various AONs; A1: AON-1 (SEQ ID NO: 5); A7: AON-7 (SEQ ID NO: 44); A9: AON-9 (SEQ ID NO: 21); A10: AON-10 (SEQ ID NO: 23); A13: AON-13 (SEQ ID NO: 25); Singh (SEQ ID NO: 46); Hua (SEQ ID NO: 47); LimE8 (SEQ ID NO: 11); SCMB (SEQ ID NO: 49) and NT: non-transfected control.

Figure 10 shows a graph of the number of cells detected with 1, 2 or = 3 nuclear GEMs for the SMA fibroblast cells transfected with 100 nM of three AONs; AON-1: SEQ ID NO: 5; AON-2: SEQ ID NO: 41; Hua27: SEQ ID NO: 47 and NT: non-transcribed control.

Figure 11 shows a graph of the number of cells detected with 1, 2 or = 3 GEMs for the SMA fibroblast cells transfected with the various AONs; A1: AON-1 (SEQ ID NO: 5); A7: AON-7 (SEQ ID NO: 44); A9: AON-9 (SEQ ID NO: 21); A10: AON-10 (SEQ ID NO: 23); A13: AON-13 (SEQ ID NO: 25); Singh (SEQ ID NO: 46); Hua (SEQ ID NO: 47); LimE8 (SEQ ID NO: 11); SCMB (SEQ ID NO: 49) and NT: non-transfected control.

Figure 12 illustrates the most stable predicted structure for the window consisting of Exon 7 (54 nt), Intron 7 (444 nt) and Exon 8 (first 502 nt of the 577 nt long exon 8) from Mfold.

Definitions

The terms “active substance” or “active ingredient” refer to a substance that is biologically active. For example, the “active substance” of a pharmaceutical composition is also known as the “active pharmaceutical ingredient” and refers to the component with pharmacological activity or other direct effect in the diagnosis, cure, mitigation, treatment or prevention of disease or to affect the structure and function of the body.

The term “antisense oligonucleotide” refers to a single-stranded oligonucleotide which is sufficiently complementary to a target sequence on DNA or RNA to base pair with the target sequence. Antisense oligonucleotides are capable of modulating splicing of precursor mRNA and/or silencing the expression of target genes.

As used herein, the terms "complementary" or “reverse complementary” are used with reference to base pairing of two nucleotide sequences (for example, a nucleotide sequence with its target sequence). While not limited to a particular mechanism, the most common mechanism of base pairing involves hydrogen pairing, which may be Watson-Crick, Hoogsteen or reverse Hoogsteen hydrogen bonding, between complementary nucleoside or nucleotide bases. For example, the sequence" 5'-A-C-G-T-3'" is completely complementary to the sequence" 3'-T-G-C-A-5'. "Complementarity” may be "partial," in which only some of the nucleic acids-bases are matched according to the base pairing rules. Typically, the nucleotide sequence and its target sequence should contain no more than about 1%, 2%, 5%, 10%, 15%, 20%, 25% or 30% base pairing mismatches. Or, there may be "complete" or "total" complementarity between the nucleic acids. The degree of complementarity between nucleic acid strands has significant effects on the efficiency and strength of hybridization between nucleic acid strands.

Messenger RNA (mRNA) is a polynucleotide molecule comprising ribonucleoside monomers which encodes for a protein product. mRNA is transcribed from a DNA template. Processing of precursor mRNA differs greatly among eukaryotes, bacteria and archea. Non-eukaryotic mRNA is essentially mature upon transcription and requires no processing, except in rare cases. In eukaroytes, precursor mRNA is first synthesised and processed extensively into a mature mRNA molecule.

Modulation of splicing refers to altering the processing of a precursor mRNA molecule such that the spliced mRNA molecule contains either a different combination of exons, as a result of exon skipping or exon inclusion, a deletion in one or more exons, or addition of sequences (e.g, intronic sequences) in the spliced mRNA.

The term "nucleoside" refers to a molecule having a purine or pyrimdiine base covalently linked to a ribose or deoxyribose sugar. Exemplary nucleosides include adenosine, guanosine, cytidine, uridine and thymidine.

The term "nucleotide" refers to a nucleoside having one or more phosphate groups joined in ester linkages to the sugar moiety. Exemplary nuclotides include nucleoside monophosphates, diphosphates and triphosphates.

The terms "nucleic acid molecule" and "polynucleotide" are used interchangeably and refers to a polymer of nucleotides joined together by 5'-3' phosphodiester bonding. The term DNA, "DNA molecule" or "deoxyribonucleic acid molecule" refers to a polymer of deoxyribonucleotides. The term "RNA", "RNA molecule" or "ribonucleic acid molecule" refers to a polymer of ribonucleotides.

Precursor mRNA (or pre-mRNA) refers to a single stranded ribonucleic acid before it has been processed into a messenger RNA (mRNA). Precursor mRNA is synthesized from a DNA template in the cell nucleus by transcription.

The term "oligonucleotide" refers to a short polymer of nucleotides and/or nucleotide analogs having a length between 5 and 150 nucleotides. For example, exemplary embodiments of such an oligonucleotide have a length of 15-50 nucleotides. The number of nucleotides and/or nucleotide analogs in the oligonucleotide may be any integer within any of the above ranges.

Splicing is the process by which precursor mRNA is modified to remove certain stretches of non-coding sequences called introns; the stretches that remain include protein-coding sequences and are called exons. Sometimes precursor mRNA may be spliced in several different ways, allowing a single gene to encode multiple proteins. This process is called alternative splicing.

Splicing regulatory elements (SREs) are sites or regulatory sequences on RNA which regulate splicing. For example, SREs are sites to which regulatory proteins (repressors and activators) or antisense oligonucleotides bind and regulate RNA splicing. SREs may be exonic, intronic or located at the junctions of exons/introns or introns/extrons. SREs may be either enhancers or silencers. Splicing enhancers are sites which enhance splicing at a site near to the splicing enhancer. Splicing activator proteins may bind to splicing enhancers, thus increasing the probability that a nearby site will be used as a splice junction. These also may occur in the introns (intronic splicing enhancers, ISE) or exons (exonic splicing enhancers, ESE) or at the junctions of exons/introns or introns/exons. Splicing silencers are sites which repress splicing at a site near to the splicing silencer. Splicing repressor proteins may bind to splicing silencers, thus reducing the probability that a nearby site will be used as a splice junction. These can be located in the intron itself (intronic splicing silencers, ISS) or in a neighboring exon (exonic splicing silencers, ESS) or at the junctions of exons/introns or introns/exons. They vary in sequence, as well as in the types of proteins that bind to them.

Survival motor neuron (SMN) gene refers to either SMN1 or SMN2. Similarly, survival motor neuron (SMN) precursor mRNA refers to either SMN1 or SMN2 precursor mRNA. Full-length SMN protein refers to the full-length protein translated from either full-length SMN1 or SMN2 mRNA.

A variant of a nucleic acid sequence comprises a sequence that is substantially homologous to the said nucleic acid sequence. Typically, a variant comprises a region of at least about 70%, 80%, 85%, 90%, 95%, 98% or 99% identity with the nucleic acid sequence.

Detailed description of the invention

The fusion antisense oligonucleotide of the present invention comprises a first oligonucleotide sequence and a second oligonucleotide sequence, each of which is complementary to or targets a different region (or sequence) of the SMN precursor mRNA. Accordingly, the first and second oligonucleotide sequences of the fusion antisense oligonucleotide are complementary to two different target regions (or sequences) of the SMN precursor mRNA. Typically, the first and second oligonucleotide sequences of the fusion antisense oligonucleotides are not substantially complementary to a continuous sequence (or continuous stretch) of nucleotide bases in the SMN precursor mRNA. . In particular, the first and/or second oligonucleotide sequences target SREs. For example, the first SRE complementary to the second oligonucleotide and the sequence complementary to the first oligonucleotide on the SMN precursor mRNA are separated by at least one intervening nucleotide or an intervening nucleotide sequence not complementary to the isolated fusion oligonucleotide. For example, the number of nucleotides in the intervening nucleotide sequence may be from 2 to 500.

These fusion antisense oligonucleotides were found to be more effective in modulating mRNA splicing than antisense oligonucleotides complementary only to a single region of SMN precursor mRNA.

According to one aspect, the second oligonucleotide sequence comprises a sequence complementary to an intronic splicing silencer sequence (ISS) of SMN precursor mRNA and the first oligonucleotide sequence comprises a sequence complementary to a sequence of the SMN precursor mRNA other than the said ISS complementary to the second oligonucleotide sequence. For example, the second oligonucleotide sequence comprises a sequence complementary to an intronic splicing silencer sequence (ISS) in intron 7 of SMN precursor mRNA and the first oligonucleotide sequence comprises a sequence complementary to a sequence of the SMN precursor mRNA other than the said ISS in intron 7.

The fusion antisense oligonucleotide may be an oligo-RNA (with uracil bases in the sequence) or an oligoDNA (with thymidine bases in the sequence).

According to one such embodiment, the second oligonucleotide sequence comprises a sequence complementary to 5'-GCCAGCAUU-3' (SEQ ID NO: 1). According to a second embodiment, the second oligonucleotide sequence comprises a sequence complementary to 5'-GCCAGCAUUAUGAAAGUGAAU-3' (SEQ ID NO: 2).

In a third embodiment, the second oligonucleotide sequence comprises the sequence 5'-AAUGCUGGC-3' (SEQ ID NO: 3), 5'-AATGCTGGC-3' (SEQ ID NO: 4) or a variant thereof. In a fourth embodiment, the second oligonucleotide sequence comprises the sequence 5'-AUUCACUUUCAUAAUGCUGGC-3' (SEQ ID NO: 5), 5'-ATTCACTTTCATAATGCTGGC-3' (SEQ ID NO: 6) or a variant thereof.

According to another aspect, the first oligonucleotide sequence may comprise a sequence complementary to a second SRE. This second SRE the first oligonucleotide sequence is complementary to or targets may be intronic, exonic or located at the junction of intronic/exonic or exonic/intronic sequences. This second SRE may be a splicing enhancer or a splicing silencer. For

example, this second SRE may comprise an ISS, ESS, ISE or ESE. According to a particular embodiment, the first oligonucleotide sequence comprises a sequence complementary to a sequence selected from the group consisting of:
5'-CUCCUUUGCAGGAAAUACUAG-3' (SEQ ID NO: 7);

5'-UUCUCAUUUGCAGGAAAU-3' (SEQ ID NO: 8);

5'-UGCAGGAAAUGCUGGCA-3' (SEQ ID NO: 9); and

5'-AGUUAGAAAGUUGAAAGGU-3' (SEQ ID NO: 10).

Accordingly, the first oligonucleotide sequence comprises a sequence selected from the group consisting of:

5'-CUAGUAUUUCCUGCAAAUGAG-3' (SEQ ID NO: 11) or
5'-CTAGTATTTCCTGCAAATGAG-3' (SEQ ID NO: 12);

5'-AUUUCCUGCAAAUGAGAA-3' (SEQ ID NO: 13) or
5'-ATTTCCTGCAAATGAGAA-3' (SEQ ID NO: 14);

5'-UGCCAGCAUUUCCUGCA -3' (SEQ ID NO: 15) or
5'-TGCCATCATTTCCTGCA-3' (SEQ ID NO: 16); and

5'-ACCUUUCAACUUUCUAACA-3' (SEQ ID NO: 17) or
5'-ACCTTTCAACTTTCTAACA-3' (SEQ ID NO: 18);

or a variant of each sequence thereof.

Accordingly, the isolated fusion antisense nucleotide may comprise a sequence or selected from the group consisting of:

5'-CUAGUAUUUCCUGCAAAUGAG AUUCACUUUCAUAAUGCUGGC-3'
(AON-3, SEQ ID NO: 19) or
5'-CTAGTATTTCCTGCAAAATGAG ATTCACTTTCATAATGCTGGC-3'
(SEQ ID NO: 20).

5'-AUUUCCUGCAAAUGAGAA AUUCACUUUCAUAAUGCUGGC-3'
(AON-9, SEQ ID NO: 21) or
5'-ATTTCCTGCAAATGAGAA ATTCACTTTCATAATGCTGGC-3'
(SEQ ID NO: 22)

5'-UGCCAGCAUUUCCUGCA AUUCACUUUCAUAAUGCUGGC-3'
(AON-10, SEQ ID NO: 23) or
5'-TGCCAGCATTTCCTGCA ATTCACTTTCATAATGCTGGC-3'
(SEQ ID NO: 24)

5'-ACCUUUCAACUUUCUAACA AUUCACUUUCAUAAUGCUGGC-3'
(AON-13, SEQ ID NO: 25) or
5'-ACCTTTCAACTTTCTAAACA ATTCACTTTCATAATGCTGGC-3' (SEQ ID NO: 26);

or a variant of each sequence thereof.

According to a further aspect, the second oligonucleotide sequence comprises a sequence complementary to an exonic silencer sequence (ESS) of SMN precursor mRNA and the first oligonucleotide sequence comprises a sequence complementary to a sequence of the SMN precursor mRNA other than the said ESS complementary to the second oligonucleotide sequence. For example, the second oligonucleotide sequence comprises a sequence complementary to an exonic splicing silencer sequence (ESS) in exon 7 of SMN precursor mRNA and the first oligonucleotide sequence comprises a sequence complementary to a sequence of the SMN precursor mRNA other than the ESS in exon 7. The first oligonucleotide sequence may also comprise a sequence complementary to an exonic splicing silencer. For example, the first oligonucleotide sequence may also comprise a sequence complementary to an exonic splicing silencer in exon 7.

Accordingly, in one such embodiment, the second oligonucleotide sequence comprises a sequence complementary to 5'-AGACAAAAUCAAAAA-3' (SEQ ID NO: 27). Accordingly, the second oligonucleotide sequence comprises the sequence 5'-UUUUUGAUUUUGUCU-3' (SEQ ID NO: 28), 5'-TTTTTGATTTTGTCT-3' (SEQ ID NO: 29) or a variant thereof.

5'-AGACAAAAUCAAAAA-3' (SEQ ID NO: 27) and 5' UCACAUUCCUUAAAU 3' (SEQ ID NO: 30) are proposed exonic splicing silencers in exon 7 (Hua *et al.*,

2002), with SEQ ID NO: 30 downstream of SEQ ID NO: 27 on SMN2 precursor mRNA.

The first oligonucleotide sequence may comprise a sequence complementary to an exonic splicing silencer (ESS) of SMN precursor mRNA. For example, the first oligonucleotide comprises a sequence complementary to an exonic splicing silencer (ESS) in exon 7 of SMN precursor mRNA. In particular, the first oligonucleotide sequence comprises a sequence complementary to 5'-UCACAUUCCUUAAAU-3' (SEQ ID NO: 30). Accordingly, the first oligonucleotide sequence may comprise the sequence 5'-AUUUAAGGAAUGUGA-3' (SEQ ID NO: 31), 5'-ATTTAAGGATGTGA-3' (SEQ ID NO: 32) or a variant thereof. In particular, the fusion antisense oligonucleotide comprises the sequence

5'-AUUUAAGGAAUGUGA UUUUUGAUUUUGUCU-3' (AON-4, SEQ ID NO: 33), 5'-ATTTAAGGAATGTGA TTTTTGATTTTGTCT-3' (SEQ ID NO: 34) or a variant thereof.

The length of each of the first and/or second oligonucleotide sequences of a fusion antisense oligonucleotide according to any aspect of the present invention may be at least 15 nucleotides (nt) long. For example, the length of each of the first and/or second oligonucleotide sequences may be about 15-35 nt. In particular, the length of each of the first and/or second oligonucleotide sequences is about 17-21 nt. The length of each of the first and/or second oligonucleotide sequences may be any integer within the above ranges.

The 5' end of the second oligonucleotide sequence may be joined to the 3' end of the first oligonucleotide sequence in the fusion antisense oligonucleotide. Alternatively, the first and second oligonucleotide sequences may be joined via a linker oligonucleotide $(N)_n$, wherein N is A, G, C or T and n comprises any integer from 1 to 20. For example, n may be any integer from 2 to 20.

The fusion antisense oligonucleotide may be about 30-80 nt long and the length of the fusion antisense oliognucleotide may be any integer within this range.

The antisense oligonucleotides are typically synthesised as modified oligonucleotides. Modified oligonucleotides are more resistant to degradation by nucleases. The antisense oligonucleotides may be modified in any conventional manner, including but not limited to 2'-O-methyl, 2'-O-methoxyethyl, phosphorothioate 2-O-methyl or morpholino modifications. In particular, the modification is phosphorothioate 2-O-methyl modified oligonucleotides.

According to another aspect, the invention provides a method of modulating SMN2 mRNA splicing. Modulating SMN2 mRNA splicing may be *in vitro* or *in vivo*. For modulating SMN2 mRNA splicing in a cell or cell extract, the cell or cell extract may be contacted with a fusion antisense oligonucleotide according to any aspect of the invention. SMN2 RNA splicing may also be modulated in an organism by administering to an organism a fusion antisense oligonucleotide according to any aspect of the invention. An effective amount of the antisense oligonucleotide sufficient to modulate SMN2 mRNA splicing is used.

In particular, the fusion antisense oligonucleotide according to any aspect of the invention is able to enhance exon 7 inclusion in SMN2 mRNA. Accordingly, the amount of SMN2 mRNA comprising exon 7 in a cell, cell extract or organism is increased in the presence of the fusion antisense oligonucleotide according to any aspect of the invention.

The invention also provides for the use of an isolated antisense oligonucleotide according to any aspect of the invention in the preparation of a composition for modulating SMN2 mRNA splicing. The composition may be a medicament. In particular, the composition may be for treating spinal muscular atrophy in a subject. The composition may comprise more than one antisense oligonucleotide of the invention. The composition may further comprise at least one other active substance. The composition may be prepared with any suitable

pharmaceutical excipient. The composition may be formulated for any mode of delivery, including but not limited to mucosal, oral, parenteral or topical delivery.

Accordingly, the invention provides a method of treating spinal muscular atrophy in a subject, comprising administering to the subject the isolated fusion antisense oligonucleotide according to any aspect of the invention. The isolated antisense nucleotide may be administered in an amount effective to modulate SMN2 mRNA splicing.

Also provided is a kit comprising at least one isolated fusion antisense oligonucleotide according to any aspect of the invention.

The isolated fusion antisense oligonucleotide according to the invention modulates SMN2 mRNA splicing. In particular, the isolated fusion antisense oligonucleotide enhances exon 7 inclusion in the spliced SMN2 mRNA. Various sequences of SMN2 are provided below.

SMN2 intron 6, exon 7, intron 7 and exon 8 sequence (SEQ ID NO: 35)

The sequence of intron 6, exon 7, intron 7 and intron 8 of SMN2 is shown. Intronic sequences are in small letters. Exon 7 sequence is in bold capital. Exon 8 sequence is in unbold capital.

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1    gtaagtaatc actcagcatc ttttcctgac aatttttttg tagttatgtg
     actttgtttt gtaaatttat aaaatactac ttgcttctct ctttatatta
     ctaaaaaata aaaataaaaa aatacaactg tctgaggctt aaattactct
     tgcattgtcc ctaagtataa ttttagttaa ttttaaaaag ctttcatgct
     attgttagat tattttgatt atacactttt gaattgaaat tatacttttt
251  ctaaataatg ttttaatctc tgatttgaaa ttgattgtag ggaatggaaa
     agatgggata atttttcata aatgaaaaat gaaattcttt ttttttttt
     ttttttttg agacggagtc ttgctctgtt gcccaggctg gagtgcaatg
     gcgtgatctt ggctcacagc aagctctgcc tcctggattc acgccattct
     cctgcctcag cctcagaggt agctgggact acaggtgcct gccaccacgc
501  ctgtctaatt ttttgtattt ttttgtaaag acagggtttc actgtgttag
     ccaggatggt ctcaatctcc tgaccccgtg atccacccgc ctcggccttc
     caagagaaat gaaatttttt taatgcacaa agatctgggg taatgtgtac
     cacattgaac cttggggagt atggcttcaa acttgtcact ttatacgtta
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      cctcccgggt tcacgccatt ctcctgcctc agcctctccg agtagctggg
      actacaggcg cccgccacca cgcccggcta atttttttct atttttagta
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      gtgatattgt aggcacttgt atttttagta aattaatata gaagaaacaa
      ctgacttaaa ggtgtatgtt tttaaatgta tcatctgtgt gtgcccccat
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      cagtgagttg agattgtgcc actgcactcc acccttggtg acagagtgag
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      ttcaggacca gcctggccaa catggtgact actccatttc tactaaatac
      acaaaactta gcccagtggc gggcagttgt aatcccagct acttgagagg
      ttgaggcagg agaatcactt gaacctggga ggcagaggtt gcagtgagcc
1751  gagatcacac cgctgcactc tagcctggcc aacagagtga gaatttgcgg
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      gtatgtatca tgaattcctc attttaatga ccaaaaagta ataaatcaac
      agcttgtaat ttgttttgag atcagttatc tgactgtaac actgtaggct
      tttgtgtttt ttaaattatg aaatatttga aaaaaataca taatgtatat
2001  ataaagtatt ggtataattt atgttctaaa taactttctt gagaaataat
      tcacatggtg tgcagtttac ctttgaaagt atacaagttg gctgggcaca
      atggctcacg cctgtaatcc cagcactttg ggaggccagg gcaggtggat
      cacgaggtca ggagatcgag accatcctgg ctaacatggt gaaaccccgt
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5   5501 ccctacctcc gcctcccaaa gttgtgggat tgtaggcatg agccactgca
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        aagtacatta aaagactatc aacttaattt ctgatcatat tttgttgaat
        aaaataagta aaatgtcttg tgaaacaaaa tgctttttaa catccatata
        aagctatcta tatatagcta tctatatcta tatagctatt ttttttaact
10  5751 tcctttattt tccttacagG GTTTTAGACA AAATCAAAAA GAAGGAAGGT
        GCTCACATTC CTTAAATTAA GGAgtaagtc tgccagcatt atgaaagtga
        atcttacttt tgtaaaactt tatggtttgt ggaaaacaaa tgtttttgaa
        catttaaaaa gttcagatgt tagaaagttg aaaggttaat gtaaaacaat
        caatattaaa gaattttgat gccaaaacta ttagataaaa ggttaatcta
15  6001 catccctact agaattctca tacttaactg gttggttgtg tggaagaaac
        atactttcac aataaagagc tttaggatat gatgccattt tatatcacta
        gtaggcagac cagcagactt ttttttattg tgatatggga taacctaggc
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        GTGGAATGGG TAACTCTTCT TGATTAAAAG TTATGTAATA ACCAAATGCA
        ATGTGAAATA TTTTACTGGA CTCTATTTTG AAAAACCATC TGTAAAAGAC
25  6501 TGAGGTGGGG GTGGGAGGCC AGCACGGTGG TGAGGCAGTT GAGAAAATTT
        GAATGTGGAT TAGATTTTGA ATGATATTGG ATAATTATTG GTAATTTTAT
        GAGCTGTGAG AAGGGTGTTG TAGTTTATAA AAGACTGTCT TAATTTGCAT
        ACTTAAGCAT TTAGGAATGA AGTGTTAGAG TGTCTTAAAA TGTTTCAAAT
        GGTTTAACAA AATGTATGTG AGGCGTATGT GGCAAAATGT TACAGAATCT
30  6751 AACTGGTGGA CATGGCTGTT CATTGTACTG TTTTTTTCTA TCTTCTATAT
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```

Intron 6 Sequence (SEQ ID NO: 36)

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        tgcattgtcc ctaagtataa ttttagttaa ttttaaaaag ctttcatgct
        attgttagat tattttgatt atacactttt gaattgaaat tatacttttt
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        ttttttttg agacggagtc ttgctctgtt gcccaggctg gagtgcaatg
        gcgtgatctt ggctcacagc aagctctgcc tcctggattc acgccattct
        cctgcctcag cctcagaggt agctgggact acaggtgcct gccaccacgc
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        gtctcctacg gacatgttct attgtatttt agtcagaaca tttaaaatta
  751   ttttatttta ttttattttt ttttttttt tgagacggag tctcgctctg
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        cctcccgggt tcacgccatt ctcctgcctc agcctctccg agtagctggg
        actacaggcg cccgccacca cgcccggcta attttttttt atttttagta
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        gtgatattgt aggcacttgt atttttagta aattaatata gaagaaacaa
        ctgacttaaa ggtgtatgtt tttaaatgta tcatctgtgt gtgcccccat
        taatattctt atttaaaagt taaggccaga catggtggct tacaactgta
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        cagtgagttg agattgtgcc actgcactcc acccttggtg acagagtgag
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        acaaaactta gcccagtggc gggcagttgt aatcccagct acttgagagg
        ttgaggcagg agaatcactt gaacctggga ggcagaggtt gcagtgagcc
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     aaaataagta aaatgtcttg tgaaacaaaa tgctttttaa catccatata
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5751 tcctttattt tccttacag
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SMN 2 Exon 7 sequence (SEQ ID NO: 37)

```
1    GGTTTTAGAC AAAATCAAAA AGAAGGAAGG TGCTCACATT CCTTAAATTA
51   AGGA
```

SMN2 Intron 7 sequence (SEQ ID NO: 38)

```
1    gtaagtctgc cagcattatg aaagtgaatc ttacttttgt aaaactttat
     ggtttgtgga aaacaaatgt ttttgaacat ttaaaaagtt cagatgttag
     aaagttgaaa ggttaatgta aaacaatcaa tattaaagaa ttttgatgcc
     aaaactatta gataaaaggt taatctacat ccctactaga attctcatac
     ttaactggtt ggttgtgtgg aagaaacata ctttcacaat aaagagcttt
251  aggatatgat gccattttat atcactagta ggcagaccag cagacttttt
     tttattgtga tatgggataa cctaggcata ctgcactgta cactctgaca
     tatgaagtgc tctagtcaag tttaactggt gtccacagag gacatggttt
     aactggaatt cgtcaagcct ctggttctaa tttctcattt gcag
```

SMN2 Exon 8 sequence (SEQ ID NO: 39)

```
     GAAATGCTGG CATAGAGCAG CACTAAATGA CACCACTAAA GAAACGATCA
     GACAGATCTG GAATGTGAAG CGTTATAGAA GATAACTGGC CTCATTTCTT
     CAAAATATCA AGTGTTGGGA AAGAAAAAAG GAAGTGGAAT GGGTAACTCT
     TCTTGATTAA AAGTTATGTA ATAACCAAAT GCAATGTGAA ATATTTTACT
     GGACTCtATT TTGAAAAACC ATCTGTAAAA GACTGAGGTG GGGGTGGGAG
251  GCCAGCACGG TGGTGAGGCA GTTGAGAAAA TTTGAATGTG GATTAGATTT
     TGAATGATAT TGGATAATTA TTGGTAATTT TATGAGCTGT GAGAAGGGTG
     TTGTAGTTTA TAAAAGACTG TCTTAATTTG CATACTTAAG CATTTAGGAA
     TGAAGTGTTA GAGTGTCTTA AAATGTTTCA AATGGTTTAA CAAAATGTAT
     GTGAGGCGTA TGTGGCAAAA TGTTACAGAA TCTAACTGGT GGACATGGCT
501  GTTCATTGTA CTGTTTTTTT CTATCTTCTA TATGTTTAAA AGTATATAAT
     AAAAATATTT AATTTTTTTT TAAATAA
```

SMN2 cDNA sequence (SEQ ID NO: 40)

The sequence of SMN2 cDNA is shown below. Exon 7 is from position 998 to 1051 (in bold).

```
1     CCACAAATGT GGGAGGGCGA TAACCACTCG TAGAAAGCGT GAGAAGTTAC
      TACAAGCGGT CCTCCCGGCC ACCGTACTGT TCCGCTCCCA GAAGCCCCGG
      GCGGCGGAAG TCGTCACTCT TAAGAAGGGA CGGGGCCCCA CGCTGCGCAC
      CCGCGGGTTT GCTATGGCGA TGAGCAGCGG CGGCAGTGGT GGCGGCGTCC
      CGGAGCAGGA GGATTCCGTG CTGTTCCGGC GCGGCACAGG CCAGAGCGAT
251   GATTCTGACA TTTGGGATGA TACAGCACTG ATAAAAGCAT ATGATAAAGC
      TGTGGCTTCA TTTAAGCATG CTCTAAAGAA TGGTGACATT TGTGAAACTT
      CGGGTAAACC AAAAACCACA CCTAAAAGAA AACCTGCTAA GAAGAATAAA
      AGCCAAAAGA AGAATACTGC AGCTTCCTTA CAACAGTGGA AAGTTGGGGA
      CAAATGTTCT GCCATTTGGT CAGAAGACGG TTGCATTTAC CCAGCTACCA
501   TTGCTTCAAT TGATTTTAAG AGAGAAACCT GTGTTGTGGT TTACACTGGA
      TATGGAAATA GAGAGGAGCA AAATCTGTCC GATCTACTTT CCCCAATCTG
      TGAAGTAGCT AATAATATAG AACAAAATGC TCAAGAGAAT GAAAATGAAA
      GCCAAGTTTC AACAGATGAA AGTGAGAACT CCAGGTCTCC TGGAAATAAA
      TCAGATAACA TCAAGCCCAA ATCTGCTCCA TGGAACTCTT TTCTCCCTCC
751   ACCACCCCCC ATGCCAGGGC CAAGACTGGG ACCAGGAAAG CCAGGTCTAA
      AATTCAATGG CCCACCACCG CCACCGCCAC CACCACCACC CCACTTACTA
      TCATGCTGGC TGCCTCCATT TCCTTCTGGA CCACCAATAA TTCCCCCACC
      ACCTCCCATA TGTCCAGATT CTCTTGATGA TGCTGATGCT TTGGGAAGTA
      TGTTAATTTC ATGGTACATG AGTGGCTATC ATACTGGCTA TTATATGGGT
1001  TTTAGACAAA ATCAAAAAGA AGGAAGGTGC TCACATTCCT TAAATTAAGG
      AGAAATGCTG GCATAGAGCA GCACTAAATG ACACCACTAA AGAAACGATC
      AGACAGATCT GGAATGTGAA GCGTTATAGA AGATAACTGG CCTCATTTCT
      TCAAAATATC AAGTGTTGGG AAAGAAAAAA GGAAGTGGAA TGGGTAACTC
      TTCTTGATTA AAAGTTATGT AATAACCAAA TGCAATGTGA AATATTTTAC
1251  TGGACTCTAT TTTGAAAAAC CATCTGTAAA AGACTGAGGT GGGGGTGGGA
      GGCCAGCACG GTGGTGAGGC AGTTGAGAAA ATTTGAATGT GGATTAGATT
      TTGAATGATA TTGGATAATT ATTGGTAATT TTATGAGCTG TGAGAAGGGT
      GTTGTAGTTT ATAAAAGACT GTCTTAATTT GCATACTTAA GCATTTAGGA
      ATGAAGTGTT AGAGTGTCTT AAAATGTTTC AAATGGTTTA ACAAAATGTA
1501  TGTGAGGCGT ATGTGGCAAA ATGTTACAGA ATCTAACTGG TGGACATGGC
      TGTTCATTGT ACTGTTTTTT TCTATCTTCT ATATGTTTAA AAGTATATAA
      TAAAAATATT TAATTTTTTT TTAAATTAAA AAAA
```

Having now generally described the invention, the same will be more readily understood through reference to the following examples which are provided by way of illustration, and are not intended to be limiting of the present invention.

EXAMPLES

Standard molecular biology techniques known in the art and not specifically described were generally followed as described in Sambrook and Russel, Molecular Cloning: A Laboratory Manual, Cold Springs Harbor Laboratory, New York (2001).

Example 1

AONs were designed to target sites in Survival Motor Neuron gene (SMN) precursor mRNA which were optimal for induction of exon 7 inclusion in the course of SMN2 precursor mRNA splicing. In designing the AONs, the following principles were considered:

1. The AON should target sequences which are critically involved in blocking splicing of an exon, specifically exonic splicing silencers and intronic splicing silencers. The AON should target sequences which are not involvd in secondary structure folding; i.e. "open" or available for AON binding. AONs should target sequences in SMN precursor mRNA intron 6 or 7 which:

(A) encompass in its entirety a putative splicing regulatory element (SRE). In particular, the SRE is an SRE with splicing silencing property.

The 4 SREs to be targeted by the AONs were:
(i) Intron 7 ISS-N1 (Singh *et al.*, 2006)
(ii) 3' Splice Site of Intron 7 (Lim and Kertel 2001)
(iii) Intron 7 +100 (Kashima *et al.*, 2007)
(iv) Intron 6 Element 1 (Miyajima *et al.*, 2002)

(B) are predicted to exhibit minimal secondary structure folding by avoiding all or having a minimum of "engaged" nucleotides, and selecting sequences with optimal access according to secondary structure predictions.

2. The lengths of the AONs were selected to be approximately 17-21 nucleotides long.

Predicted secondary structure folding of precursor mRNA and thermodynamic stability, as predicted by m-fold, were taken into account in designing all antisense oligonucleotides. As precursor mRNA transcription proceeds, the secondary structure of a newly generated precursor mRNA sequence continually changes as new nucleotides are added in the transcription process. The binding of AONs is likely to be co-transcriptional and likely to compete with endogenous splicing factors occurs as soon as the target site is transcribed. The approach used in determining secondary structures for design of AONs, takes into consideration such secondary structure.

Secondary structure prediction was conducted using open software created by Michael Zuker & Nick Markham available from the UNAFold web server hosted by the RNA Institute, State University of New York at Albany, USA from http://mfold.rna.albany.edu. A window of 1000 nucleotides, consisting of the last (3') 102 nucleotides of intron 6, all 54 nucleotides of exon 7, all 444 nucleotides of intron 7 and the first (5') 400 nucleotides of exon 8, was assigned empirically as the reference window of analysis. The Mfold software from the website http://mfold.rna.albany.edu/?q=mfold/RNA-folding-form was then directed to generate folding predictions of this reference window and an additional 19 randomly selected overlapping 1000 nucleotide windows on either 5' and 3' sides of the reference window which must include exon 7 (54 nt) and Intron 7 (444 nt) as part of its windows, with varying portions of intron 6 on the 5' side of exon 7 and varying portions of exon 8 on the 3' side of intron 7 that will add up to 1000 nt. This means the range will be from 502 nt upstream of exon 7 to 502 nt downstream of intron 7.

For each of the 20 windows, the 20 most thermodynamically stable structures, as indicated by their free Gibbs energy lost to determine stability (delta G), were

selected, giving a total of 400 predicted structures. From among these, the 40 most stable structures based on their delta G values were selected for use in designing the AONs. The 40 structures were used to identify bases within the precursor mRNA sequence which were "engaged", i.e. paired with another base in all 40 structures. The AONs are designed to bind near or to these structures.

Figure 12 illustrates the most stable predicted structure for the window consisting of Exon 7 (54 nt), Intron 7 (444 nt) and Exon 8 (first 502 nt of the 577 nt long Exon 8) from Mfold. This stemloop structure is formed mostly by AT base pairs, which may randomly denature and reanneal more frequently than high GC content stem loops (i.e. the stability of the stemloop structure depends on the base pairing, AT base pairing being less stable than GC base pairing). There is only 1 ideal GC base pair. There is also a GT base pair, which is held together by two hydrogen bonds instead of three hydrogen bonds for GC. On the other hand, if a structure is less stable, there is more opportunity for the AON to anneal to its target.

In addition to the AONs designed under these conditions with each targeting a single splicing regulatory cis-element (SRE) with putative silencing property ('single target' AONs), fusion AONs targeting two separate SREs with putative splicing silencing property were also designed. These fusion or "double-target" AONs essentially consist of combinations of 2 single target AONs, joined together. Consequently, these fusion or "double-target" AONs have lengths exceeding those of the single-target AONs, varying between 38 to 42 bases.

A selection of 14 AONs was designed for induction of SMN exon 7 inclusion:

AON-1 (SEQ ID NO: 5)
5'-AUUCACUUUCAUAAUGCUGGC-3' [21 nt, targets 9-29 of Intron 7 or 5832-5852 of SEQ ID NO: 35)]

AON-2 (SEQ ID NO: 41)
5'-GAUUCACUUUCAUAAUGCUGG-3' [21 nt, targets 10-30 of Intron 7, targets 5833-5853 of SEQ ID NO: 35)]

AON-3 (SEQ ID NO: 19)
5'-CUAGUAUUUCCUGCAAAUGAG AUUCACUUUCAUAAUGCUGGC-3' [42 nt, fusion of SEQ ID NO: 11 and AON-1 (SEQ ID NO: 5), double targets 9-29 of Intron 7 (5832-5852 of SEQ ID NO: 35) and junction of Intron 7/Exon 8 (imperfect match to 6257-6277 of SEQ ID NO: 35 (similar to antisense oligonucleotide $oligo_{junct}$ reported in Lim and Hertel 2001)].

AON-4 (SEQ ID NO: 33)
5'-AUUUAAGGAAUGUGA UUUUUGAUUUUGUCU-3' [30 nt, fusion of SEQ ID NO: 31 and SEQ ID NO: 28, double targets 7-21 of Exon 7 (5776-5790 of SEQ ID NO: 35) and 34-48 of Exon 7 (5803-5817 of SEQ ID NO: 35]. Each half of this fusion antisense oligonucleotide have been reported as two separate antisense oligonucleotides of 15 nt each (ASO 07-21 and ASO 35-48) in Hua *et al.*, 2007.

AON-5 (SEQ ID NO: 42)
5'-AUGGAUGUUAAAAAGUAUUUUGUUU-3' [25 nt target Intron6, Element 1 (-73-97 upstream of Exon 7 as reported in Miyajima *et al.*, 2002 or 5673 to 5697 of SEQ ID NO: 35)]

AON-6 (SEQ ID NO: 43)
5'-UAAAAAGCAUUUUGUUUCACAAGAC-3' [25 nt, targets Intron6, Element 1 (–81-105 upstream of Exon 7 as reported in Miyajima *et al.*, 2002 or 5965-5997 of SEQ ID NO: 35)]

AON-7 (SEQ ID NO: 44)
5'-AUUCACUUUCAUAAUGCUGGCA-3' [22nt, targets 8-29 of Intron 7 (5831-5852 of SEQ ID NO: 35)]

AON-8 (SEQ ID NO: 45)
5'-GAUUCACUUUCAUAAUGCUGGC-3' [22 nt, targets 9-30 of Intron 7 (5832-5853 of SEQ ID NO: 35)]

AON-9 (SEQ ID NO: 21)
5'-AUUUCCUGCAAAUGAGAA AUUCACUUUCAUAAUGCUGGC-3' [39 nt, fusion of AON-11 (SEQ ID NO: 13) and AON-1 (SEQ ID NO: 5), double targets 9-29 of Intron 7 (5832-5852 of SEQ ID NO: 35) and junction of Intron 7/Exon 8 (6255-6272 of SEQ ID NO: 35)].

AON-10 (SEQ ID NO: 23)
5'-UGCCAGCAUUUCCUGCA AUUCACUUUCAUAAUGCUGGC-3' [38 nt, fusion of AON-12 (SEQ ID NO: 15) and AON-1 (SEQ ID NO: 5), double targets 9-29 of Intron 7 (5832-5852 of SEQ ID NO: 35) and junction of Intron 7/Exon 8 (6263-6279 of SEQ ID NO: 35)].

AON-11 (SEQ ID NO: 13)
5'-AUUUCCUGCAAAUGAGAA-3' [18 nt, targets junction of Intron 7/Exon 8 (6265-6272 of SEQ ID NO: 35)]

AON-12 (SEQ ID NO: 15)
5'-UGCCAGCAUUUCCUGCA-3' [17 nt, targets junction of Intron 7/Exon 8 (6263-6279 of SEQ ID NO: 35)]

AON-13 (SEQ ID NO: 25)
5'-ACCUUUCAACUUUCUAACA AUUCACUUUCAUAAUGCUGGC-3' [40 nt, fusion of AON-14 (SEQ ID NO: 17) + AON-1 (SEQ ID NO: 5), double targets 9-29 of Intron 7 (5832-5852 of SEQ ID NO: 35) and junction of Intron 7) and +95 to 113 of Intron 7 (5818-5836 of SEQ ID NO: 35)]

AON-14 (SEQ ID NO: 17)
5'-ACCUUUCAACUUUCUAACA-3' [19 nt, targets +95 to 113 of intron 7 (5818-5836 of SEQ ID NO: 35)].

AON-1, AON-2, AON-5, AON-6, AON-7, AON-8, AON-11, AON-12 and AON-14 are complementary to or target single sites on SMN precursor mRNA.

AON-3, AON-4, AON-9, AON-10 and AON-13 are complementary to or target two sites on SMN precursor mRNA.

The above AON-1 to AON-14 were synthesised and analysed for enhancing exon 7 inclusion in SMN2 mRNA. For comparison, the following previously reported AONs were also synthesised.

Anti-N1 oligonucleotide reported in Singh *et al.*, (2009), ("Singh")
5'-AUUCACUUUCAUAAUGCUGG-3' (SEQ ID NO: 46).

Similar to ASO 10-27 reported in Hua *et al.*, (2008) ("Hua" or "Hua27")
5'- UCACUUUCAUAAUGCUGG –3' (SEQ ID NO: 47)

Oligo-element 1 reported in Miyajima *et al.*, (2002) ("Mi6")
5'-UGGAUGUUAAAAAGUA-3' (SEQ ID NO: 48)

$Oligo_{jnct}$ reported in Lim and Hertel (2001)("Lim" or "LimE8")
5'-CUAGUAUUUCCUGCAAAUGAG-3' (SEQ ID NO: 11)

A control scrambled oligonucleotide ("SCMB") with the sequence 5'-UAGUGAUGAUCCUACUCAUCAU -3' (SEQ ID NO: 49) was also synthesized. The sequence of this oligonucleotide was derived from randomly scrambling the antisense sequence complementary to ISS-N1 reported in Singh *et al.*, (2006).

The AONs were synthesised as phosphorothioate 2-O-methyl modified oligonucleotides by Sigma Proligo.

Example 2

Preliminary screening of the designed AONs with comparison against published and control AONs was conducted by lipofectamine-mediated transfection studies (as described below) of SMA1 patient fibroblasts at AON concentrations of 100 and 200 nM.

Cell culture

SMA Fibroblast cell line (GM03813) derived from a human donor with SMA type 1 and having two copies of the SMN2 gene was obtained from Coriell and used in transfection studies of the AONs. The SMA fibroblast cells were grown in

Basal Fibroblast Medium (Promocell) containing BFGF and insulin, with 15% FCS, 2mM L-Glutamine, 10% Penicillin/Streptomycin.

Transfection

Transfection was performed using reagents according to the manufacturer's recommendations. In brief, 5 µl of lipotectamine 2000 (Invitrogen) was added to a round bottom tube and 95 µl of Opti-Mem I reduced serum medium (Invitrogen). 98 µl of Opti-Mem I reduced serum medium was mixed with 2 µl of 100 µM AON to be transfected in an eppendorf and allowed to stand for 5 minutes. This mixture was then added to the contents of the round bottom tube, mixed and allowed to stand for 30 minutes. This solution was topped up to 2 ml with Opti-Mem I reduced serum medium with 5% serum (i.e. the "transfection mixture").

24 hours prior to transfection, the SMA fibroblast cells were seeded into 6 well plates. On the day of transfection, the cells were at a density of ~ 80%. After removal of culture medium, 2 ml aliquots of the transfection mixture containing the antisense oligonucleotide to be transfected were added to separate wells. The transfection was allowed to take place for 5 hours. Thereafter, the transfection mixture was replaced by cell culture medium. Cells were harvested for RNA extraction at 24 hours after addition of the transfection mixture. Transfection was also carried out with AON concentrations of 25 nM, 50 nM, 100 nM and 200 nM to evaluate dose effect.

RNA extraction

RNA was extracted using the Nucleospin RNA II kit (Macherey-Nagel) according to the manufacturer's recommendations. Trizol-based RNA extraction may also be used. RNA concentration was measured with uv spectrometer from Nanodrop, Thermo Scientific.

Reverse transcription-polymerase chain reaction (RT-PCR)

Reverse transcription was performed with a SuperScript III cDNA synthesis kit (Invitrogen) according to the manufacturer's protocol, using an oligo(dT) primer. Primers for the PCR were: exon 6 forward primer 5'-accacctcccatatgtccag-3' (SEQ ID NO: 50) and exon 8 reverse primer 5'-aactggcctcatttcttcaaa-3' (SEQ ID NO: 51). PCR cycling conditions were: 94 °C for 5 minutes; 35 cycles of 94 °C for 30 seconds (denaturation); 60 °C for 30 seconds (annealing); 72 °C for 1 minute (extension); 72 °C for 10 minutes and hold at 4 °C. The PCR amplicons were separated by standard gel electrophoresis. ImageJ software was used for densitometry analysis of the separated PCR amplicons.

Figure 1 shows the gel electrophoresis showing raw data from the RT-PCR using exon 6 forward primer 5'-accacctcccatatgtccag-3' (SEQ ID NO: 50) and exon 8 reverse primer 5'-aactggcctcatttcttcaaa-3' (SEQ ID NO: 51) from cells transfected with antisense oligonucleotides. A1: AON-1 (SEQ ID NO: 5); A7: AON-7 (SEQ ID NO: 44), A8: AON-8 (SEQ ID NO: 45), A9: AON-9 (SEQ ID NO: 21); A10: AON-10 (SEQ ID NO: 23); A13: AON-13 (SEQ ID NO: 25); A14: AON-14 (SEQ ID NO: 17); Singh (SEQ ID NO: 46); Hua (SEQ ID NO: 47); Lim (SEQ ID NO: 11); SCMB (SEQ ID NO: 49) and NT: non-transfected control.

Figure 2 shows the densitometry image analysis from ImageJ software of the raw data of Figure 1. The figures below the lanes show the percentage of exon 7 inclusion computed as ratio of upper band densitometry value upon sum of upper and lower band densitometry values. Transfections with A1: AON-1 (SEQ ID NO: 5), A7: AON-7 (SEQ ID NO: 44), A8: AON-8 (SEQ ID NO: 45), A9: AON-9 (SEQ ID NO: 21), A10: AON-10 (SEQ ID NO: 23) and A13: AON-13 (SEQ ID NO: 25) show 90, 89, 85, 95, 96 AND 92 % exon 7 inclusion, respectively, which were at least equal or higher than the antisense AONs Singh (SEQ ID NO: 46); Hua (SEQ ID NO: 47) and Lim (SEQ ID NO: 11) reported in the literature while A14: AON-14 (SEQ ID NO: 17) shows 76% exon 7 inclusion. In

particular, it is noted that the fusion AONs, A9: AON-9 (SEQ ID NO: 21), A10: AON-10 (SEQ ID NO: 23) and A13: AON-13 (SEQ ID NO: 25) showed the highest % exon 7 inclusion.

Example 3 Quantitative Real-time PCR (qPCR) to evaluate dose effect of transfected AON concentration

Antisense oligonucleotides [AON-1 (SEQ ID NO: 5); AON-7 (SEQ ID NO: 44); AON-8 (SEQ ID NO: 45); AON-9 (SEQ ID NO: 21),; AON-10 (SEQ ID NO: 23); AON-13 (SEQ ID NO: 25); AON-14 (SEQ ID NO: 17); Singh (SEQ ID NO: 46), Hua (SEQ ID NO: 47), LimE8 (SEQ ID NO: 11) and SCRMB (SEQ ID NO: 49)] were transfected at 25 nM, 50 nM, 100 nM and 200 nM to evaluate does effect, as described above. Following transfection, qPCR was performed to quantitate exon 7 inclusion at the different doses.

Reverse transcription was performed with a SuperScript III cDNA synthesis kit (Invitrogen) according to the manufacturer's protocol, using an oligo(dT) primer, as described above. Primers for the qPCR were: exon 7 forward primer 5'-aatcaaaaagaaggaaggtgct-3' (SEQ ID NO: 52) and exon 8 reverse primer 5'-aactggcctcatttcttcaaa-3' (SEQ ID NO: 51). Primers for qPCR were diluted to 10μM. The qPCR reaction mixture was prepared in optical tubes using the Roche Light Cycler DNA Plus Master Mix as follows:

4μl SYBR Green Mix (5x) (FastStart DNA MasterPlus SYBR Green – Roche)
4μl cDNA
2μl primer pair mix (1μl each primer)
10μl H2O
20μl Total

The PCR program was performed on a BioRad CFX96 Real Time PCR machine with the cycling conditions: 94°C 4 min; 38 cycles of 95 °C for 10 seconds (denaturation); 60 °C for 10 seconds (annealing), primer extension at 72 °C for 10 seconds (extension). Melting curve was performed from 65°C to

95 °C. qPCR specificity was confirmed by 2% agarose gel using 10µl of qPCR product. qPCRs were performed in triplicates for each AON treatment trial.

Figure 3 shows the effect of 25 nM transfected antisense oligonucleotides on expression of full-length SMN2.

Figure 4 shows the effect of 50 nM transfected antisense oligonucleotides on expression of full-length SMN2.

Figure 5 shows the effect of 100 nM transfected antisense oligonucleotides on expression of full-length SMN2.

Figure 6 shows the effect of 200 nM transfected antisense oligonucleotides on expression of full-length SMN2.

Figure 7 shows the dose effect for these different antisense oligonucleotides on expression of full-length SMN2. In general, exon 7 inclusion increases for all AONs when the dose is increased, except for SCRMB (SEQ ID NO: 49). The non-transfected sample also did not show a dosage effect. In particular, the greatest increase in exon 7 inclusion with increasing dosage was for the fusion antisense oligonucleotides AON-9 (SEQ ID NO: 21), AON-10 (SEQ ID NO: 23) and AON-13 (SEQ ID NO: 25). Interestingly, AON-14 (SEQ ID NO: 17) showed almost a three-fold increase in exon 7 inclusion when the transfected dose was increased from 100nM to 200 nM.

Example 4 Protein extraction and Western blotting

Cells were lysed after AON transfection with reporter lysis buffer with protease inhibitor cocktail (Promega Corporation) and used according to the manufacturer's instructions. Average concentration of protein in each sample was determined using the Bradford protein quantification method. The proteins in the samples were separated on 12% SDS-PAGE and electroblotted onto nitrocellulose membranes.

The membranes were probed for SMN protein with monoclonal anti-SMN antibody (BD transduction Laboratories) at 1:1000 dilution as the primary antibody. Monoclonal anti-α-tubulin antibody (Sigma) at 1:10000 dilution was used to probe α-tubulin as a control. The secondary antibody was horseradish peroxidase-conjugated goat anti-mouse polyclonal antibody (BioRad) at a dilution of 1:5000. Detection was performed with the ECL Western blotting detection reagents (GE Healthcare) and ECL Hyperfilm (GE Healthcare). Densitrometry analysis of the protein bands was performed using ImageJ software.

Figure 8 shows the Western blot of SMA fibroblast cells (GM03813) transfected with 100 nM of five different AONs. The figures at the bottom of the blot refer to fold change of SMN protein relative to the non-transfected (NT) control. The A1: AON-1 (SEQ ID NO: 5) and A13: AON-13 (SEQ ID NO: 25) samples were both found to have higher expression of SMN compared to the AONS, Singh (SEQ NO: 48)' Hua (SEQ ID NO: 47) and Lim E8 (SEQ ID NO: 11).

Figure 9 shows the Western blot of SMA fibroblast cells (GM03813) transfected with 200 nM of various AONs [A1: AON-1 (SEQ ID NO: 5); A7: AON-7 (SEQ ID NO: 44); A8: AON-8 (SEQ ID NO: 45); A9: AON-9 (SEQ ID NO: 21); A10: AON-10 (SEQ ID NO: 23); A13: AON-13 (SEQ ID NO: 25); A14: AON-14 (SEQ ID NO: 17); Singh (SEQ ID NO: 46), Hua (SEQ ID NO: 47), LimE8 (SEQ ID NO: 11) and SCRMB (SEQ ID NO: 49)]. The figures at the bottom of the blot refer to fold change of SMN protein relative to the non-transfected (NT) control. The fusion antisense oligonucleotides A9: AON-9, A10: AON-10 and A13: AON-13 showed the highest full-length SMN2 expression.

Example 5 Immunofluorescence and Nuclear GEM quantification.

SMA fibroblast cells (GM03813) were plated onto 4 chamber slides and transfected separately with various 100 nM AONs, essentially as described above. At 48 hours after addition of the transfection mixture, the cells were washed in phosphate-buffered saline (PBS), fixed with 4% paraformaldehyde for 15 minutes, blocked with 1 mg/ml bovine serum albumin (BSA) and incubated with monoclonal anti-SMN antibody (BD Transduction Laboratories) as the primary anti-SMN antibody at a dilution of 1:50 or 1:100. The cells were washed to remove unbound antibody before incubation with secondary antibody Alexa Fluor 594 conjugated goat anti-mouse antibody (Invitrogen) at a dilution of 1:1000. Nuclei were counterstained with DAPI. The cells were washed again and examined under confocal laser-scanning microsope (Nikon A1) for Gemini of coiled body (GEMs) counting. GEMs were first reported using SMN antibodies by Liu and Dreyfuss (1996). For each AON transfection, 120 consecutive cells were counted to determine the number of nuclear gems In each cell. Cytoplasmic gems were disregarded. The results were expressed as number of cells expressing 3 or more nuclear gems, 2 nuclear gems, 1 nuclear gem and no nuclear gems.

A normal fibroblast cell line (GM05758), also from Coriell, was also cultured for use as a control in the immunohistochemical studies. The normal fibroblast cells were grown in DMEM with 15% FCS, 2mM L-Glutamine, 10% Penicillin/Streptomycin.

Table 1 shows the nuclear GEMs count in SMA fibroblast cells (GM03813) transfected with 100 nM of different AONs.

Table 1: Nuclear GEMs count in SMA fibroblast cells (GM03813) transfected with 100 nM of different AONs.

No. of nuclear GEMs	AON-1	AON-7	AON-9	AON-10	AON-13	Singh	Hua	LimE8	SCRMB	NT
= 3	9	11	30	23	23	12	10	7	0	0
2	13	12	15	12	15	10	12	14	1	0
1	27	21	31	29	20	16	18	20	0	2
0	71	76	44	56	62	82	80	79	119	118
Total number of cells	120	120	120	120	120	120	120	120	120	120

Representing the data from Table 1 graphically, Figure 11 shows a graph of the number of cells detected with 1, 2 or 3 or more GEMs for the SMA fibroblast cells transfected with the various AONs. It can be observed that the fusion antisense oligonucleotides AON-9 (SEQ ID NO: 21), AON-10 (SEQ ID NO: 23) and AON-13 (SEQ ID NO: 25) increased the number of GEMs in the transfected SMA fibroblast cells.

References

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Claims

1. An isolated fusion antisense oligonucleotide comprising a first oligonucleotide sequence joined to a second oligonucleotide sequence, wherein the second oligonucleotide sequence comprises a sequence complementary to a first splicing regulatory element (SRE) of SMN precursor mRNA and the first oligonucleotide sequence comprises a sequence complementary to a sequence of the SMN precursor mRNA other than the said first SRE complementary to the second oligonucleotide sequence.

2. The isolated fusion antisense oligonucleotide according to claim 1, wherein the first oligonucleotide sequence is complementary to a sequence of the SMN precursor mRNA downstream of the said first SRE.

3. The isolated fusion antisense oligonucleotide according to claim 1 or 2, wherein the first SRE comprises a splicing silencer (SS) or a splicing enhancer (SE).

4. The isolated fusion antisense oligonucleotide according to any one of the preceding claims, wherein the first SRE is intronic, exonic or located at the junction of intronic/exonic or exonic/intronic sequences.

5. The isolated fusion antisense oligonucleotide according to any one of the preceiding claims, wherein the first oligonucleotide sequence is complementary to a second SRE of SMN precursor mRNA.

6. The isolated fusion antisense oligonucelotide according to claim 5, wherein the second SRE comprises a splicing silencer (SS) or a splicing enhancer (SE).

7. The isolated fusion antisense oligonucleotide according to claim 5 or 6, wherein the second SRE is intronic, exonic or located at the junction of intronic/exonic or exonic/intronic sequences.

8. The isolated fusion antisense oligonucleotide according to any one of the preceding claims, wherein the first and/or second SRE is of close proximity to exon 7.

9. The isolated fusion antisense oligonucleotide according to any one of the preceding claims, wherein the second oligonucleotide sequence comprises a sequence complementary to a first SRE from either exon 6, intron 6, exon 7, intron 7, exon 8, intron 8 or any one of the junctions of exon6/intron6, intron6/exon 7, exon 7/intron 7, intron7/exon 8 or exon 8/intron 8 while the first oligonucleotide sequence comprises a sequence complementary to a second SRE from either exon6, intron 6, exon 7, intron 7, exon 8, intron 8 or any one of the junctions of exon6/intron6, intron6/exon 7, exon 7/intron 7, intron 7/exon 8 or exon 8/intron 8.

10. The isolated fusion antisense oligonucleotide according to any one of claims 1 to 4, wherein the second oligonucleotide sequence comprises a sequence complementary to an intronic splicing silencer sequence (ISS) of SMN precursor mRNA and the first oligonucleotide sequence comprises a sequence complementary to a sequence of the SMN precursor mRNA other than the said ISS complementary to the second oligonucleotide sequence.

11. The isolated fusion antisense oligonucleotide according to any one of claims 1 to 4 or 10, wherein the second oligonucleotide sequence comprises a sequence complementary to an intronic splicing silencer sequence (ISS) in intron 7 of SMN precursor mRNA and the first oligonucleotide sequence comprises a sequence complementary to a

sequence of the SMN precursor mRNA other than the said ISS in intron 7.

12. The isolated fusion antisense oligonucleotide according to any one of the preceding claims, wherein the second oligonucleotide sequence comprises a sequence complementary to 5'-GCCAGCAUU-3' (SEQ ID NO: 1).

13. The isolated fusion antisense oligonucleotide according to any one of the preceding claims, wherein the second oligonucleotide sequence comprises a sequence complementary to 5'-GCCAGCAUUAUGAAAGUGAAU-3' (SEQ ID NO: 2).

14. The isolated fusion antisense oligonucleotide according to any one of the preceding claims, wherein the second oligonucleotide sequence comprises the sequence 5'-AAUGCUGGC-3' (SEQ ID NO: 3), 5'-AATGCTGGC-3' (SEQ ID NO: 4) or a variant thereof.

15. The isolated fusion antisense oligonucleotide according to any one of the preceding claims, wherein the second oligonucleotide sequence comprises the sequence 5'-AUUCACUUUCAUAAUGCUGGC-3' (SEQ ID NO: 5), 5'-ATTCACTTTCATAATGCTGGC-3' (SEQ ID NO: 6) or a variant thereof.

16. The isolated fusion antisense oligonucleotide according to any one of the preceding claims, wherein the first oligonucleotide sequence comprises a sequence complementary to a sequence selected from the group consisting of:

5'CUCCUUUGCAGGAAAUACUAG-3' (SEQ ID NO: 7);

5'- UUCUCAUUUGCAGGAAAU -3' (SEQ ID NO: 8);

5'- UGCAGGAAAUGCUGGCA -3' (SEQ ID NO: 9); and

5'- AGUUAGAAAGUUGAAAGGU -3' (SEQ ID NO: 10).

17. The isolated fusion antisense oligonucleotide according to any one of the preceding claims, wherein the first oligonucleotide sequence comprises a sequence selected from the group consisting of:

5'-CUAGUAUUUCCUGCAAAUGAG-3' (SEQ ID NO: 11) or
5'-CTAGTATTTCCTGCAAATGAG-3' (SEQ ID NO: 12);

5'-AUUUCCUGCAAAUGAGAA-3' (SEQ ID NO: 13) or
5'-ATTTCCTGCAAATGAGAA-3' (SEQ ID NO: 14);

5'-UGCCAGCAUUUCCUGCA -3' (SEQ ID NO: 15) or
5'-TGCCATCATTTCCTGCA-3' (SEQ ID NO: 16); and

5'-ACCUUUCAACUUUCUAACA-3' (SEQ ID NO: 17) or
5'-ACCTTTCAACTTTCTAACA-3' (SEQ ID NO: 18);

or a variant of each sequence thereof.

18. The isolated fusion antisense oligonucleotide according to any one of the preceding claims comprising a sequence selected from the group consisting of:

5'-CUAGUAUUUCCUGCAAAUGAG AUUCACUUUCAUAAUGCUGGC-3' (AON-3, SEQ ID NO: 19) or
5'-CTAGTATTTCCTGCAAAATGAG ATTCACTTTCATAATGCTGGC-3' (SEQ ID NO: 20).

5'-AUUUCCUGCAAAUGAGAA AUUCACUUUCAUAAUGCUGGC-3' (AON-9, SEQ ID NO: 21) or
5'-ATTTCCTGCAAATGAGAA ATTCACTTTCATAATGCTGGC-3' (SEQ ID NO: 22)

5'-UGCCAGCAUUUCCUGCA AUUCACUUUCAUAAUGCUGGC-3' (AON-10, SEQ ID NO: 23) or
5'-TGCCAGCATTTCCTGCA ATTCACTTTCATAATGCTGGC-3' (SEQ ID NO: 24)

5'-ACCUUUCAACUUUCUAACA AUUCACUUUCAUAAUGCUGGC-3' (AON-13, SEQ ID NO: 25) or
5'-ACCTTTCAACTTTCTAAACA ATTCACTTTCATAATGCTGGC-3' (SEQ ID NO: 26);

or a variant of each sequence thereof.

19. The isolated fusion antisense oligonucleotide according to any one of claims 14-15 or 17-18, wherein the variant comprises a region of at least about 70%, 80%, 85%, 90%, 95%, 98% or 99% identity with the sequence.

20. The isolated fusion antisense oligonucleotide according to any one of claims 1 to 4, wherein the second oligonucleotide sequence comprises a sequence complementary to an exonic splicing silencer (ESS) of SMN precursor mRNA and the first oligonucleotide sequence comprises a sequence complementary to a sequence of the SMN precursor mRNA other than the said ESS complementary to the second oligonucleotide sequence.

21. The isolated fusion antisense oligonucleotide according to any one of claims 1 to 9 or 20, wherein the second oligonucleotide sequence comprises a sequence complementary to an exonic splicing silencer sequence (ESS) in exon 7 of SMN precursor mRNA and the first oligonucleotide sequence comprises a sequence complementary to a sequence of the SMN precursor mRNA other than the ESS in exon 7.

22. The isolated fusion antisense oligonucleotide according to any one of claims 1 to 9 or 20 to 21, wherein the second oligonucleotide sequence comprises a sequence complementary to 5'-AGACAAAAUCAAAAA-3' (SEQ ID NO: 27).

23. The isolated fusion antisense oligonucleotide according to any one of claims 1 to 9 or 20 to 22, wherein the second oligonucleotide sequence comprises the sequence 5'-UUUUUGAUUUUGUCU-3' (SEQ ID NO: 28), 5'-TTTTTGATTTTGTCT-3' (SEQ ID NO: 29) or a variant thereof.

24. The isolated fusion antisense oligonucleotide according to any one of claims 1 to 9 or 20 to 23, wherein the first oligonucleotide sequence comprises a sequence complementary to an exonic splicing silencer (ESS) of SMN precursor mRNA.

25. The isolated fusion antisense oligonucleotide according to any one of claims 1 to 9 or 20 to 24, wherein the first oligonucleotide sequence comprises a sequence complementary to an ESS in exon 7 of SMN precursor mRNA.

26. The isolated fusion antisense oligonucleotide according to any one of claims 1 to 9 or 20 to 25, wherein the first oligonucleotide sequence comprises a sequence complementary to 5'-UCACAUUCCUUAAAU-3' (SEQ ID NO: 30).

27. The isolated fusion antisense oligonucleotide according to any one of claims 1 to 9 or 20 to 26, wherein the first oligonucleotide sequence comprises the sequence 5'-AUUUAAGGAAUGUGA-3' (SEQ ID NO: 31), 5'ATTTAAGGATGTGA-3' (SEQ ID NO: 32) or a variant thereof.

28. The isolated fusion antisense oligonucleotide according to any one of claims 1 to 9 or 20 to 27, comprising the sequence 5'-AUUUAAGGAAUGUGA UUUUUGAUUUUGUCU-3' (AON-4, SEQ ID NO: 33), 5'-ATTTAAGGAATGTGA TTTTTGATTTTGTCT-3' (SEQ ID NO: 34) or a variant thereof.

29. The isolated fusion antisense oligonucleotide according to any one of claims 23 or 27 to 28, wherein the variant comprises a region of at least

about 70%, 80%, 85%, 90%, 95%, 98% or 99% identity with the sequence.

30. The isolated fusion antisense oligonucleotide according to any one of the preceding claims, for use in modulating SMN2 mRNA splicing.

31. The isolated fusion antisense oligonucleotide according to any one of the preceding claims, for use in modulating SMN2 mRNA splicing in a cell or cell extract, wherein the fusion antisense oligonucleotide is for contacting a cell or cell extract.

32. The isolated fusion antisense oligonucleotide according to any one of the preceding claims, for use in modulating SMN2 mRNA splicing in an organism, wherein the fusion antisense oligonucleotide is for administering to the organism,

33. The isolated fusion antisense oligonucleotide, according to any one of the preceding claims, for use in treating spinal muscular atrophy in a subject.

34. The isolated fusion antisense oligonucleotide according to claim 33, wherein the isolated fusion antisense oligonucleotide is for administering to the subject in an effective amount to modulate SMN2 splicing.

35. The isolated fusion antisense oligonucleotide according to any one of claims 30 to 32 or 34, wherein modulating SMN2 mRNA splicing comprises enhancing exon 7 inclusion in the SMN2 mRNA.

36. A method of modulating SMN2 mRNA splicing in a cell or cell extract, comprising contacting the cell or cell extract with the isolated fusion antisense oligonucleotide according to any one of claims 1 to 31.

37. The method according to claim 36, wherein the method comprises an *in vitro* method.

38. A method of modulating SMN2 mRNA splicing in an organism, comprising administering to the organism the isolated fusion antisense oligonucleotide according to any one of claims 1 to 32.

39. A method of treating spinal muscular atrophy in a subject, comprising administering to the subject the isolated fusion antisense oligonucleotide according to any one of claims 1 to 33.

40. The method according to claim 39, wherein an amount effective to modulate SMN2 RNA splicing is administered.

41. The method according to any one of claims 36 to 38 or 40, wherein modulating SMN2 mRNA comprises enhancing exon 7 inclusion in the SMN2 mRNA.

42. Use of the isolated fusion antisense oligonucleotide according to any one of claims 1 to 29 in the preparation of a composition for modulating SMN2 mRNA splicing.

43. Use according to claim 42, wherein the composition is for modulating SMN2 mRNA splicing in a cell or a cell extract.

44. Use according to claim 43, wherein the composition is for contacting the cell or cell extract.

45. Use according to claim 44, for modulating SMN2 mRNA splicing in an organism, wherein the composition is for administering to the organism.

46. Use of the fusion antisense oligonucleotide according to any one of claims 1 to 29 in the preparation of a composition for treating spinal muscular atrophy in a subject.

47. Use according to claim 46, wherein the composition is for administering to the subject.

48. Use according to claim 47, wherein the composition is for administering in an amount effective to modulate SMN2 RNA splicing in the subject.

49. Use according to any one of claims 42 to 44 or 47, wherein modulating SMN2 splicing comprises enhancing exon 7 inclusion in the SMN2 mRNA.

50. A kit comprising at least one isolated fusion oligonucleotide according to any one of claims 1 to 35.

SEQUENCE LISTING

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<120> Antisense oligonucleotides for modulating survival motor neuron 2 (SMN2) splicing

<130> SG1985

<160> 52

<170> PatentIn version 3.5

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tccttacag 5769

<210> 37
<211> 54
<212> DNA
<213> artificial

<220>
<223> SEQ ID NO: 37 (SMN2 exon 7)

<400> 37
ggttttagac aaaatcaaaa agaaggaagg tgctcacatt ccttaaatta agga 54

<210> 38
<211> 444
<212> DNA
<213> artificial

<220>
<223> SEQ ID NO: 38 (SMN2 intron 7)

<400> 38
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aaacaatcaa tattaaagaa ttttgatgcc aaaactatta gataaaaggt taatctacat 180

ccctactaga attctcatac ttaactggtt ggttgtgtgg aagaaacata ctttcacaat 240

aaagagcttt aggatatgat gccattttat atcactagta ggcagaccag cagacttttt 300

tttattgtga tatgggataa cctaggcata ctgcactgta cactctgaca tatgaagtgc 360

tctagtcaag tttaactggt gtccacagag gacatggttt aactggaatt cgtcaagcct 420

ctggttctaa tttctcattt gcag 444

<210> 39
<211> 577
<212> DNA

<213> artificial

<220>
<223> SEQ ID NO: 39 (SMN2 exon 8)

<400> 39
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gaatgtgaag cgttatagaa gataactggc ctcatttctt caaaatatca agtgttggga 120

aagaaaaaag gaagtggaat gggtaactct tcttgattaa aagttatgta ataaccaaat 180

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taaaagactg tcttaatttg catacttaag catttaggaa tgaagtgtta gagtgtctta 420

aaatgtttca aatggtttaa caaaatgtat gtgaggcgta tgtggcaaaa tgttacagaa 480

tctaactggt ggacatggct gttcattgta ctgtttttt ctatcttcta tatgtttaaa 540

agtatataat aaaaatattt aatttttttt taaataa 577

<210> 40
<211> 1634
<212> DNA
<213> Homo sapiens

<400> 40
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tgtggcttca tttaagcatg ctctaaagaa tggtgacatt tgtgaaactt cgggtaaacc 360

aaaaaccaca cctaaaagaa aacctgctaa gaagaataaa agccaaaaga agaatactgc 420

agcttcctta caacagtgga aagttgggga caaatgttct gccatttggt cagaagacgg 480

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atctgctcca tggaactctt ttctccctcc accacccccc atgccagggc caagactggg 780

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ttaaattaaa aaaa 1634

<210> 41
<211> 21
<212> RNA
<213> artificial

<220>
<223> SEQ ID NO: 41 (AON-2)

<400> 41
gauucacuuu cauaaugcug g 21

<210> 42
<211> 25
<212> RNA
<213> artificial

<220>
<223> SEQ ID NO: 42 (AON-5)

<400> 42
auggauguua aaaaguauuu uguuu 25

<210> 43
<211> 25
<212> RNA
<213> artificial

<220>
<223> SEQ ID NO: 43 (AON-6)

<400> 43
uaaaaagcau uuuguuucac aagac 25

<210> 44
<211> 22
<212> RNA
<213> artificial

<220>
<223> SEQ ID NO: 44 (AON-7)

<400> 44
auucacuuuc auaaugcugg ca 22

<210> 45
<211> 22
<212> RNA
<213> artificial

<220>
<223> SEQ ID NO: 45 (AON-8)

<400> 45
gauucacuuu cauaaugcug gc 22

<210> 46
<211> 20
<212> RNA
<213> artificial

<220>
<223> SEQ ID NO: 46 (Anti-N1 oligonucleotide reported in Singh et al 2009)

<400> 46
auucacuuuc auaaugcugg 20

<210> 47
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<212> RNA
<213> artificial

<220>
<223> SEQ ID NO: 47 (ASO 10-27 reported in Hua et al 2008)

<400> 47
ucacuuucau aaugcugg 18

<210> 48
<211> 16
<212> RNA
<213> artificial

<220>
<223> SEQ ID NO: 48 (Oligo-element 1 reported in Miyajima et al 2002

<400> 48
uggauguuaa aaagua 16

<210> 49
<211> 22
<212> RNA
<213> artificial

<220>
<223> SEQ ID NO: 49 (SCMB)

<400> 49
uagugaugau ccuacucauc au 22

<210> 50
<211> 20
<212> DNA
<213> artificial

<220>
<223> SEQ ID NO: 50 (SMN2 exon 6 forward primer)

<400> 50
accacctccc atatgtccag 20

<210> 51
<211> 21
<212> DNA
<213> artificial

<220>
<223> SEQ ID NO: 51 (SMN2 exon 8 reverse primer)

<400> 51
aactggcctc atttcttcaa a 21

<210> 52
<211> 22
<212> DNA
<213> artificial

<220>
<223> SEQ ID NO: 52 (SMN2 exon 7 forward primer)

<400> 52
aatcaaaaag aaggaaggtg ct 22

2

2