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

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
21 October 2010 (21.10.2010)

PCT

(10) International Publication Number
WO 2010/120820 A1

(51) International Patent Classification:
C07H 21/00 (2006.01) *C12P 19/34* (2006.01)

(21) International Application Number:
PCT/US2010/030940

(22) International Filing Date:
13 April 2010 (13.04.2010)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
61/168,885 13 April 2009 (13.04.2009) US

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

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

Published:

— *with international search report (Art. 21(3))*

— *with sequence listing part of description (Rule 5.2(a))*

WO 2010/120820 A1

(54) **Title**: COMPOSITIONS AND METHODS FOR MODULATION OF SMN2 SPLICING

(57) **Abstract**: Disclosed herein are compounds, compositions and methods for modulating splicing of SMN2 mRNA in a cell, tissue or animal. Also provided are uses of disclosed compounds and compositions in the manufacture of a medicament for treatment of diseases and disorders, including spinal muscular atrophy.

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COMPOSITIONS AND METHODS FOR MODULATION OF SMN2 SPLICING

SEQUENCE LISTING

The present application is being filed along with a Sequence Listing in electronic format. The Sequence Listing is provided as a file entitled CORE0084WOSEQ.txt, created April 13, 2010, which is 28 Kb in size. The information in the electronic format of the sequence listing is incorporated herein by reference in its entirety.

BACKGROUND OF THE INVENTION

Newly synthesized eukaryotic mRNA molecules, also known as primary transcripts or pre-mRNA, made in the nucleus, are processed before or during transport to the cytoplasm for translation. Processing of the pre-mRNAs includes addition of a 5' methylated cap and an approximately 200-250 base poly(A) tail to the 3' end of the transcript.

The next step in mRNA processing is splicing of the pre-mRNA, which occurs in the maturation of 90-95% of mammalian mRNAs. Introns (or intervening sequences) are regions of a primary transcript (or the DNA encoding it) that are not included in the coding sequence of the mature mRNA. Exons are regions of a primary transcript that remain in the mature mRNA when it reaches the cytoplasm. The exons are spliced together to form the mature mRNA sequence. Splice junctions are also referred to as splice sites with the 5' side of the junction often called the "5' splice site," or "splice donor site" and the 3' side the "3' splice site" or "splice acceptor site." In splicing, the 3' end of an upstream exon is joined to the 5' end of the downstream exon. Thus the unspliced RNA (or pre-mRNA) has an exon/intron junction at the 5' end of an intron and an intron/exon junction at the 3' end of an intron. After the intron is removed, the exons are contiguous at what is sometimes referred to as the exon/exon junction or boundary in the mature mRNA. Cryptic splice sites are those which are less often used but may be used when the usual splice site is blocked or unavailable. Alternative splicing, defined as the splicing together of different combinations of exons, often results in multiple mRNA transcripts from a single gene.

Up to 50% of human genetic diseases resulting from a point mutation are caused by aberrant splicing. Such point mutations can either disrupt a current splice site or create a new splice site, resulting in mRNA transcripts comprised of a different combination of exons or with deletions in exons. Point mutations also can result in activation of a cryptic splice site or disrupt regulatory *cis*

elements (i.e. splicing enhancers or silencers) (Cartegni et al., Nat. Rev. Genet., 2002, 3, 285-298; Drawczak et al., Hum. Genet., 1992, 90, 41-54).

Antisense oligonucleotides have been used to target mutations that lead to aberrant splicing in several genetic diseases in order to redirect splicing to give a desired splice product (Kole, *Acta Biochimica Polonica*, **1997**, *44*, 231-238). Such diseases include β-thalassemia (Dominski and Kole, *Proc. Natl. Acad. Sci.* USA, **1993**, *90*, 8673-8677; Sierakowska et al., Nucleosides & Nucleotides, **1997**, *16*,1173-1182; Sierakowska et al., *Proc. Natl. Acad. Sci.* USA, **1996**, *93*, 12840-44; Lacerra et al., Proc. Natl. Acad. Sci. USA, 2000, 97, 9591-9596); dystrophin Kobe (Takeshima et al., *J. Clin. Invest.*, **1995**, *95*, 515-520); Duchenne muscular dystrophy (Dunckley et al. *Nucleosides & Nucleotides*, **1997**, *16*, 1665-1668; Dunckley et al. *Human Mol. Genetics*, **1998**, *5*, 1083-90); osteogenesis imperfecta (Wang and Marini, *J. Clin Invest.*, **1996**, *97*, 448-454); and cystic fibrosis (Friedman et al., J. Biol. Chem., 1999, 274, 36193-36199).

Antisense compounds have also been used to alter the ratio of the long and short forms of Bcl-x pre-mRNA (U.S. Patent 6,172,216; U.S. Patent 6,214,986; Taylor et al., Nat. Biotechnol. 1999, 17, 1097-1100) or to force skipping of specific exons containing premature termination codons (Wilton et al., Neuromuscul. Disord., 1999, 9, 330-338). U.S. Patent 5,627,274 and WO 94/26887 disclose compositions and methods for combating aberrant splicing in a pre-mRNA molecule containing a mutation using antisense oligonucleotides which do not activate RNAse H.

Proximal spinal muscular atrophy (SMA) is a genetic, neurodegenerative disorder characterized by the loss of spinal motor neurons. SMA is an autosomal recessive disease of early onset and is currently the leading cause of death among infants. The severity of SMA varies among patients and has thus been classified into three types. Type I SMA is the most severe form with onset at birth or within 6 months and typically results in death within 2 years. Children with type I SMA are unable to sit or walk. Type II SMA is the intermediate form and patients are able to sit, but cannot stand or walk. Patients with type III SMA, a chronic form of the disease, typically develop SMA after 18 months of age (Lefebvre et al., Hum. Mol. Genet., 1998, 7, 1531-1536).

SMA is caused by the loss of both copies of survival of motor neuron 1 (SMN1), a protein that is part of a multi-protein complex thought to be involved in snRNP biogenesis and recycling. A nearly identical gene, SMN2, exists in a duplicated region on chromosome 5q13. Although SMN1 and SMN2 have the potential to code for the same protein, SMN2 contains a translationally silent mutation at position +6 of exon 7, which results in inefficient inclusion of exon 7 in SMN2

transcripts. Thus, the predominant form of SMN2 is a truncated version, lacking exon 7, which is unstable and inactive (Cartegni and Krainer, Nat. Genet., 2002, 30, 377-384).

Chimeric peptide nucleic acid molecules designed to modulate splicing of SMN2 have been described (WO 02/38738; Cartegni and Krainer, Nat. Struct. Biol., 2003, 10, 120-125).

Antisense technology is an effective means for modulating the expression of one or more specific gene products, including alternative splice products, and is uniquely useful in a number of therapeutic, diagnostic, and research applications. The principle behind antisense technology is that an antisense compound, which hybridizes to a target nucleic acid, modulates gene expression activities such as transcription, splicing or translation through one of a number of antisense mechanisms. The sequence specificity of antisense compounds makes them extremely attractive as tools for target validation and gene functionalization, as well as therapeutics to selectively modulate the expression of genes involved in disease.

Disclosed herein are antisense compounds useful for modulating gene expression and associated pathways via antisense mechanisms, which may include antisense mechanisms based on target occupancy. Provided herein are antisense compounds targeting SMN2 for use in modulation of SMN2 splicing. One having skill in the art, once armed with this disclosure will be able, without undue experimentation, to identify, prepare and exploit antisense compounds for these uses.

Certain antisense compounds complementary to SMN2 are known in the art. See for example, WO 2007/002390. Certain antisense compounds and methods discloses herein posses desirable characteristics compared to such compounds and methods known in the art.

SUMMARY OF THE INVENTION

The present invention is directed to antisense compounds targeted to and hybridizable with a nucleic acid molecule encoding SMN2. Provided are antisense compounds targeted to intron 7 of SMN2 which modulate splicing of SMN2 pre-mRNAs. In one embodiment, modulation of splicing results in an increase in exon 7 inclusion. In another embodiment, modulation of splicing results in a decrease in exon 7 inclusion. Contemplated and provided herein are antisense compounds 16 to 19 nucleotides in length targeted to intron 7 of SMN2, wherein the compounds comprise 2'-O-methoxyethyl sugar modifications.

In one aspect of the invention, the antisense compounds are targeted to *cis* splicing regulatory elements. Regulatory elements include exonic splicing enhancers, exonic splicing

silencers, intronic splicing enhancers and intronic splicing silencers. Exonic and intronic splicing silencers are preferred targets.

In one embodiment, the antisense compounds comprise at least an 8-nucleobase portion of one of the exemplary compounds provided herein.

Also provided are methods for modulating splicing of SMN2 mRNA in a cell, tissue or organ using one or more of the compounds of the invention. In one embodiment, modulation of splicing is exon inclusion. In another embodiment, modulation of splicing is exon skipping. In one aspect, the compound is targeted to an intronic splicing silencer element. In another aspect, the compound is targeted to an exonic splicing silencer element.

Also provided are pharmaceutical compositions comprising one or more of the compounds of the invention. Use of an antisense oligonucleotide provided herein for the preparation of a medicament for modulating splicing of an SMN2 pre-mRNA is also provided. In one aspect, modulation of splicing results in an increase in exon 7 inclusion. Use of an antisense oligonucleotide provided herein for the preparation of a medicament for the treatment of spinal muscular atrophy is further provided.

DETAILED DESCRIPTION OF THE INVENTION

Antisense technology is an effective means for modulating the expression of one or more specific gene products and is uniquely useful in a number of therapeutic, diagnostic, and research applications. Provided herein are antisense compounds useful for modulating gene expression via antisense mechanisms of action, including antisense mechanisms based on target occupancy. In one aspect, the antisense compounds provided herein modulate splicing of a target gene. Such modulation includes promoting or inhibiting exon inclusion. Further provided herein are antisense compounds targeted to *cis* splicing regulatory elements present in pre-mRNA molecules, including exonic splicing enhancers, exonic splicing silencers, intronic splicing enhancers and intronic splicing silencers. Disruption of *cis* splicing regulatory elements is thought to alter splice site selection, which may lead to an alteration in the composition of splice products.

Processing of eukaryotic pre-mRNAs is a complex process that requires a multitude of signals and protein factors to achieve appropriate mRNA splicing. Exon definition by the spliceosome requires more than the canonical splicing signals which define intron-exon boundaries. One such additional signal is provided by *cis*-acting regulatory enhancer and silencer sequences.

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Exonic splicing enhancers (ESE), exonic splicing silencers (ESS), intronic splicing enhancers (ISE) and intron splicing silencers (ISS) have been identified which either repress or enhance usage of splice donor sites or splice acceptor sites, depending on their site and mode of action (Yeo *et al.* 2004, *Proc. Natl. Acad. Sci. U.S.A. 101(44)*:15700-15705). Binding of specific proteins (*trans* factors) to these regulatory sequences directs the splicing process, either promoting or inhibiting usage of particular splice sites and thus modulating the ratio of splicing products (Scamborova *et al.* 2004, *Mol. Cell. Biol. 24(5)*:1855-1869; Hovhannisyan and Carstens, 2005, *Mol. Cell. Biol. 25(1)*:250-263; Minovitsky *et al.* 2005, *Nucleic Acids Res. 33(2)*:714-724). Little is known about the *trans* factors that interact with intronic splicing elements; however, several studies have provided information on exonic splicing elements. For example, ESEs are known to be involved in both alternative and constitutive splicing by acting as binding sites for members of the SR protein family. SR proteins bind to splicing elements via their RNA-binding domain and promote splicing by recruiting spliceosomal components with protein-protein interactions mediated by their RS domain, which is comprised of several Arg-Ser dipeptides (Cartegni and Krainer, 2003, *Nat. Struct. Biol. 10(2)*:120-125; Wang *et al.* 2005, *Nucleic Acids Res. 33(16)*:5053-5062). ESEs have been found to be enriched in regions of exons that are close to splice sites, particularly 80 to 120 bases from the ends of splice acceptor sites (Wu *et al.* 2005, *Genomics 86*:329-336). Consensus sequences have been determined for four members of the SR protein family, SF2/ASF, SC35, SRp40 and SRp55 (Cartegni *et al.* 2003, *Nucleic Acids Res. 31(13)*:3568-3571).

Although the *trans* factors that bind intronic splicing regulatory elements have not been extensively studied, SR proteins and heterogeneous ribonucleoproteins (hnRNPs) have both been suggested to interact with these elements (Yeo *et al.* 2004, *Proc. Natl. Acad. Sci. U.S.A. 101(44)*:15700-15705). Two intronic splicing enhancer elements (ISEs) have been identified in SMN2, one in intron 6 and the other in intron 7 (Miyajima *et al.* 2002, *J. Biol. Chem. 22*:23271-23277). Gel shift assays using the ISE in intron 7 showed formation of RNA-protein complexes, which suggests these *trans* proteins may be important for regulation of splicing (Miyaso *et al.* 2003, *J. Biol. Chem. 278(18)*:15825-15831).

The role of SMN2 in diseases such as spinal muscular atrophy (SMA) makes it an important therapeutic target. SMA is a genetic disorder characterized by degeneration of spinal motor neurons. SMA is caused by the loss of both functional copies of SMN1. However, SMN2 has the potential to code for the same protein as SMN1 and thus overcome the genetic defect of SMA patients. SMN2

contains a translationally silent mutation (C→T) at position +6 of exon 7 (nucleotide 66 of SEQ ID NO: 1), which results in inefficient inclusion of exon 7 in SMN2 transcripts. Therefore, the predominant form of SMN2, one which lacks exon 7, is unstable and inactive. Thus, therapeutic compounds capable of modulating SMN2 splicing such that the percentage of SMN2 transcripts containing exon 7 is increased, would be useful for the treatment of SMA.

Overview

Disclosed herein are oligomeric compounds, including antisense oligonucleotides and other antisense compounds for use in modulating the expression of nucleic acid molecules encoding SMN2. This is accomplished by providing oligomeric compounds which hybridize with one or more target nucleic acid molecules encoding SMN2. As used herein, the terms "target nucleic acid" and "nucleic acid molecule encoding SMN2" have been used for convenience to encompass DNA encoding SMN2, RNA (including pre-mRNA and mRNA or portions thereof) transcribed from such DNA, and also cDNA derived from such RNA.

Provided herein are antisense compounds for use in modulation of SMN2 pre-mRNA splicing. In one embodiment, the disclosed antisense compounds are targeted to exon 7 of SMN2 such that SMN mRNA splicing is modulated. In another embodiment, the antisense compounds are targeted to intron 6 of SMN2. In another embodiment, the antisense compounds are targeted to intron 7 of SMN2. Modulation of splicing may result in exon 7 inclusion or exon 7 skipping. See Hua et al., The American Journal of Human Genetics 82, 1-15 (April 2008), doi:10.1016/j.ajhg.2008.01.014, which is hereby incorporated by reference in its entirety for any purpose.

Also provided are antisense compounds targeted to *cis* regulatory elements. In one embodiment, the regulatory element is in an exon. In another embodiment, the regulatory element is an in intron.

Modulation of splicing

As used herein, modulation of splicing refers to altering the processing of a pre-mRNA transcript 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 additional sequence not normally found in the spliced mRNA (e.g., intron sequence). In the context of the present invention, modulation of splicing refers to altering splicing of SMN2 pre-mRNA to achieve exon skipping or exon inclusion. In one embodiment, exon skipping results in an SMN2 mRNA transcript

lacking exon 7 and exon inclusion results in an SMN2 mRNA transcript containing exon 7.

As used herein, alternative splicing is defined as the splicing together of different combinations of exons, which may result in multiple mRNA transcripts from a single gene. In the context of the present invention, an SMN2 mRNA transcript containing exon 7 and an SMN2 mRNA transcript lacking exon 7 are two products of alternative splicing.

Compounds

The term "oligomeric compound" refers to a polymeric structure capable of hybridizing to a region of a nucleic acid molecule. This term includes oligonucleotides, oligonucleosides, oligonucleotide analogs, oligonucleotide mimetics and chimeric combinations of these. An "antisense compound" or "antisense oligomeric compound" refers to an oligomeric compound that is at least partially complementary to the region of a nucleic acid molecule to which it hybridizes and which modulates its expression. Antisense compounds may modulate expression by modulating transcription, RNA proscessin, and/or translation. In certain embodiments, antisense compounds modulate splicing of a pre-mRNA. An "antisense oligonucleotide" is an antisense compound that is a nucleic acid-based oligomer. An antisense oligonucleotide can be chemically modified. Nonlimiting examples of oligomeric compounds include primers, probes, antisense compounds, antisense oligonucleotides, external guide sequence (EGS) oligonucleotides, alternate splicers, and siRNAs. As such, these compounds can be introduced in the form of single-stranded, double-stranded, circular, branched or hairpins and can contain structural elements such as internal or terminal bulges or loops. Oligomeric double-stranded compounds can be two strands hybridized to form double-stranded compounds or a single strand with sufficient self complementarity to allow for hybridization and formation of a fully or partially double-stranded compound.

The oligomeric compounds in accordance with this invention may comprise a complementary oligomeric compound having from about 15 to about 25 linked nucleosides. One having ordinary skill in the art will appreciate that this embodies antisense compounds of 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25 nucleosides.

In one embodiment, the antisense compounds of the invention have antisense portions of 16 nucleosides.

In one embodiment, the antisense compounds of the invention have antisense portions of 17 nucleosides.

In one embodiment, the antisense compounds of the invention have antisense portions of 18

nucleosides.

In one embodiment, the antisense compounds of the invention have antisense portions of 19 nucleosides.

Antisense compounds 10-50 nucleobases in length comprising a stretch of at least eight (8) consecutive nucleobases selected from within the illustrative antisense compounds are considered to be suitable antisense compounds as well.

Compounds of the invention include oligonucleotide sequences that comprise at least the 8 consecutive nucleobases from the 5'-terminus of one of the illustrative antisense compounds (the remaining nucleobases being a consecutive stretch of nucleobases continuing upstream of the 5'-terminus of the antisense compound until the oligonucleotide contains about 10 to about 50 nucleobases). Other compounds are represented by oligonucleotide sequences that comprise at least the 8 consecutive nucleobases from the 3'-terminus of one of the illustrative antisense compounds (the remaining nucleobases being a consecutive stretch of nucleobases continuing downstream of the 3'-terminus of the antisense compound and continuing until the oligonucleotide contains about 10 to about 50 nucleobases). It is also understood that compounds may be represented by oligonucleotide sequences that comprise at least 8 consecutive nucleobases from an internal portion of the sequence of an illustrative compound, and may extend in either or both directions until the oligonucleotide contains about 10 to about 50 nucleobases. The compounds described herein are specifically hybridizable to the target nucleic acid.

One having skill in the art armed with the antisense compounds illustrated herein will be able, without undue experimentation, to identify further antisense compounds.

Hybridization

As used herein, "hybridization" means the pairing of complementary strands of antisense compounds to their target sequence. While not limited to a particular mechanism, the most common mechanism of pairing involves hydrogen bonding, which may be Watson-Crick, Hoogsteen or reversed Hoogsteen hydrogen bonding, between complementary nucleoside or nucleotide bases (nucleobases). For example, the natural base adenine is complementary to the natural nucleobases thymidine and uracil which pair through the formation of hydrogen bonds. The natural base guanine is complementary to the natural bases cytosine and 5-methyl cytosine. Hybridization can occur under varying circumstances.

An antisense compound is specifically hybridizable when there is a sufficient degree of complementarity to avoid non-specific binding of the antisense compound to non-target nucleic acid sequences under conditions in which specific binding is desired, i.e., under physiological conditions in the case of *in vivo* assays or therapeutic treatment, and under conditions in which assays are performed in the case of *in vitro* assays.

As used herein, "stringent hybridization conditions" or "stringent conditions" refers to conditions under which an antisense compound will hybridize to its target sequence, but to a minimal number of other sequences. Stringent conditions are sequence-dependent and will be different in different circumstances, and "stringent conditions" under which antisense compounds hybridize to a target sequence are determined by the nature and composition of the antisense compounds and the assays in which they are being investigated.

Complementarity

"Complementarity," as used herein, refers to the capacity for precise pairing between two nucleobases on either two oligomeric compound strands or an antisense compound with its target nucleic acid. For example, if a nucleobase at a certain position of an antisense compound is capable of hydrogen bonding with a nucleobase at a certain position of a target nucleic acid, then the position of hydrogen bonding between the oligonucleotide and the target nucleic acid is considered to be a complementary position.

"Complementarity" can also be viewed in the context of an antisense compound and its target, rather than in a base by base manner. The antisense compound and the further DNA or RNA are complementary to each other when a sufficient number of complementary positions in each molecule are occupied by nucleobases which can hydrogen bond with each other. Thus, "specifically hybridizable" and "complementary" are terms which are used to indicate a sufficient degree of precise pairing or complementarity over a sufficient number of nucleobases such that stable and specific binding occurs between the antisense compound and a target nucleic acid. One skilled in the art recognizes that the inclusion of mismatches is possible without eliminating the activity of the antisense compound. The invention is therefore directed to those antisense compounds that may contain up to about 20% nucleotides that disrupt base pairing of the antisense compound to the target. Preferably the compounds contain no more than about 15%, more preferably not more than about 10%, most preferably not more than 5% or no mismatches. The remaining nucleotides do not disrupt hybridization (e.g., universal bases).

It is understood in the art that incorporation of nucleotide affinity modifications may allow for a greater number of mismatches compared to an unmodified compound. Similarly, certain oligonucleotide sequences may be more tolerant to mismatches than other oligonucleotide sequences. One of the skill in the art is capable of determining an appropriate number of mismatches between oligonucleotides, or between an oligonucleotide and a target nucleic acid, such as by determining melting temperature.

Identity

Antisense compounds, or a portion thereof, may have a defined percent identity to a SEQ ID NO, or a compound having a specific Isis number. As used herein, a sequence is identical to the sequence disclosed herein if it has the same nucleobase pairing ability. For example, a RNA which contains uracil in place of thymidine in the disclosed sequences of the instant invention would be considered identical as they both pair with adenine. This identity may be over the entire length of the oligomeric compound, or in a portion of the antisense compound (e.g., nucleobases 1-20 of a 27-mer may be compared to a 20-mer to determine percent identity of the oligomeric compound to the SEQ ID NO.) It is understood by those skilled in the art that an antisense compound need not have an identical sequence to those described herein to function similarly to the antisense compound described herein. Shortened versions of antisense compound taught herein, or non-identical versions of the antisense compound taught herein fall within the scope of the invention. Non-identical versions are those wherein each base does not have the same pairing activity as the antisense compounds disclosed herein. Bases do not have the same pairing activity by being shorter or having at least one abasic site. Alternatively, a non-identical version can include at least one base replaced with a different base with different pairing activity (e.g., G can be replaced by C, A, or T). Percent identity is calculated according to the number of bases that have identical base pairing corresponding to the SEQ ID NO or antisense compound to which it is being compared. The non-identical bases may be adjacent to each other, dispersed through out the oligonucleotide, or both.

For example, a 16-mer having the same sequence as nucleobases 2-17 of a 20-mer is 80% identical to the 20-mer. Alternatively, a 20-mer containing four nucleobases not identical to the 20-mer is also 80% identical to the 20-mer. A 14-mer having the same sequence as nucleobases 1-14 of an 18-mer is 78% identical to the 18-mer. Such calculations are well within the ability of those skilled in the art.

The percent identity is based on the percent of nucleobases in the original sequence present

in a portion of the modified sequence. Therefore, a 30 nucleobase antisense compound comprising the full sequence of the complement of a 20 nucleobase active target segment would have a portion of 100% identity with the complement of the 20 nucleobase active target segment, while further comprising an additional 10 nucleobase portion. In the context of the invention, the complement of an active target segment may constitute a single portion. In a preferred embodiment, the oligonucleotides of the instant invention are at least about 80%, more preferably at least about 85%, even more preferably at least about 90%, most preferably at least 95% identical to at least a portion of the complement of the active target segments presented herein.

It is well known by those skilled in the art that it is possible to increase or decrease the length of an antisense compound and/or introduce mismatch bases without eliminating activity. For example, in Woolf et al. (Proc. Natl. Acad. Sci. USA 89:7305-7309, 1992, incorporated herein by reference), a series of ASOs 13-25 nucleobases in length were tested for their ability to induce cleavage of a target RNA. ASOs 25 nucleobases in length with 8 or 11 mismatch bases near the ends of the ASOs were able to direct specific cleavage of the target mRNA, albeit to a lesser extent than the ASOs that contained no mismatches. Similarly, target specific cleavage was achieved using a 13 nucleobase ASOs, including those with 1 or 3 mismatches. Maher and Dolnick (Nuc. Acid. Res. 16:3341-3358,1988, incorporated herein by reference) tested a series of tandem 14 nucleobase ASOs, and a 28 and 42 nucleobase ASOs comprised of the sequence of two or three of the tandem ASOs, respectively, for their ability to arrest translation of human DHFR in a rabbit reticulocyte assay. Each of the three 14 nucleobase ASOs alone were able to inhibit translation, albeit at a more modest level than the 28 or 42 nucleobase ASOs. It is understood that antisense compounds of the instant invention can vary in length and percent complementarity to the target provided that they maintain the desired activity. Methods to determine desired activity are disclosed herein and well known to those skilled in the art.

Target Nucleic Acids

As used herein, "targeting" or "targeted to" refer to the process of designing an oligomeric compound such that the compound specifically hybridizes with a selected nucleic acid molecule.

"Targeting" an oligomeric compound to a particular target nucleic acid molecule can be a multistep process. The process usually begins with the identification of a target nucleic acid whose expression is to be modulated. As used herein, the terms "target nucleic acid" and "nucleic acid encoding SMN2" encompass DNA encoding SMN2, RNA (including pre-mRNA and mRNA)

transcribed from such DNA, and also cDNA derived from such RNA. For example, the target nucleic acid can be a cellular gene (or mRNA transcribed from the gene) whose expression is associated with a particular disorder or disease state, or a nucleic acid molecule from an infectious agent. As disclosed herein, the target nucleic acid encodes SMN2. In one preferred embodiment, the target nucleic acid is SMN2 pre-mRNA.

Target Regions, Segments, and Sites

The targeting process usually also includes determination of at least one target region, segment, or site within the target nucleic acid for the antisense interaction to occur such that the desired effect (e.g., modulation of splicing) will result. "Region" is defined as a portion of the target nucleic acid having at least one identifiable structure, function, or characteristic. Target regions may include an exon or an intron. Within regions of target nucleic acids are segments. "Segments" are defined as smaller or sub-portions of regions within a target nucleic acid. "Sites," as used in the present invention, are defined as unique nucleobase positions within a target nucleic acid.

Kits, Research Reagents and Diagnostics

The antisense compounds of the present invention can be utilized for diagnostics, and as research reagents and kits. Furthermore, antisense compounds, which are able to inhibit gene expression or modulate gene expression (e.g., modulation of splicing) with specificity, are often used by those of ordinary skill to elucidate the function of particular genes or to distinguish between functions of various members of a biological pathway.

For use in kits and diagnostics, the antisense compounds of the present invention, either alone or in combination with other compounds or therapeutics, can be used as tools in differential and/or combinatorial analyses to elucidate expression patterns of a portion or the entire complement of genes expressed within cells and tissues. Methods of gene expression analysis are well known to those skilled in the art.

Therapeutics

Antisense compounds of the invention can be used to modulate the expression of SMN2 in an animal, such as a human. In one non-limiting embodiment, the methods comprise the step of administering to said animal in need of therapy for a disease or condition associated with SMN2 an effective amount of an antisense compound that modulates expression of SMN2 (e.g. modulates splicing of SMN2). A disease or condition associated with SMN2 includes, but is not limited to, spinal muscular atrophy. In one embodiment, the antisense compounds of the present invention

effectively modulate splicing of SMN2, resulting in an increase in exon 7 inclusion. Antisense compounds of the present invention that effectively modulate expression of SMN2 RNA or protein products of expression are considered active antisense compounds.

For example, modulation of expression of SMN2 can be measured in a bodily fluid, which may or may not contain cells; tissue; or organ of the animal. Methods of obtaining samples for analysis, such as body fluids (e.g., sputum, serum), tissues (e.g., biopsy), or organs, and methods of preparation of the samples to allow for analysis are well known to those skilled in the art. Methods for analysis of RNA and protein levels are discussed above and are well known to those skilled in the art. The effects of treatment can be assessed by measuring biomarkers associated with the target gene expression in the aforementioned fluids, tissues or organs, collected from an animal contacted with one or more compounds of the invention, by routine clinical methods known in the art. These biomarkers include but are not limited to: liver transaminases, bilirubin, albumin, blood urea nitrogen, creatine and other markers of kidney and liver function; interleukins, tumor necrosis factors, intracellular adhesion molecules, C-reactive protein, chemokines, cytokines, and other markers of inflammation.

The antisense compounds of the present invention can be utilized in pharmaceutical compositions by adding an effective amount of a compound to a suitable pharmaceutically acceptable diluent or carrier. Acceptable carriers and diluents are well known to those skilled in the art. In certain embodiments, an acceptable carriers or diluent is sterile pharmaceutical grade saline. Selection of a diluent or carrier is based on a number of factors, including, but not limited to, the solubility of the compound and the route of administration. Such considerations are well understood by those skilled in the art. In one aspect, the antisense compounds of the present invention modulate splicing of SMN2. The compounds of the invention can also be used in the manufacture of a medicament for the treatment of diseases and disorders related to SMN2.

Methods whereby bodily fluids, organs or tissues are contacted with an effective amount of one or more of the antisense compounds or compositions of the invention are also contemplated. Bodily fluids, organs or tissues can be contacted with one or more of the compounds of the invention resulting in modulation of SMN2 expression in the cells of bodily fluids, organs or tissues. An effective amount can be determined by monitoring the modulatory effect of the antisense compound or compounds or compositions on target nucleic acids or their products by methods routine to the skilled artisan.

Thus, provided herein is the use of an isolated antisense compound targeted to SMN2 in the manufacture of a medicament for the treatment of a disease or disorder by means of the method described above. In one embodiment, the antisense compound is targeted to exon 7 of SMN2. In another embodiment, the antisense compound is targeted to intron 6 of SMN2. In yet another embodiment, the antisense compound is targeted to intron 7 of SMN2.

Chemical Modifications

As is known in the art, a nucleoside is a base-sugar combination. The base portion of the nucleoside is normally a heterocyclic base (sometimes referred to as a "nucleobase" or simply a "base"). The two most common classes of such heterocyclic bases are the purines and the pyrimidines. Nucleotides are nucleosides that further include a phosphate group covalently linked to the sugar portion of the nucleoside. For those nucleosides that include a pentofuranosyl sugar, the phosphate group can be linked to the 2', 3' or 5' hydroxyl moiety of the sugar. In forming oligonucleotides, the phosphate groups covalently link adjacent nucleosides to one another to form a linear polymeric compound. Within oligonucleotides, the phosphate groups are commonly referred to as forming the internucleoside backbone of the oligonucleotide. The normal linkage or backbone of RNA and DNA is a 3' to 5' phosphodiester linkage. It is often preferable to include chemical modifications in oligonucleotides to alter their activity. Chemical modifications can alter oligonucleotide activity by, for example: increasing affinity of an antisense oligonucleotide for its target RNA, increasing nuclease resistance, and/or altering the pharmacokinetics of the oligonucleotide. The use of chemistries that increase the affinity of an oligonucleotide for its target can allow for the use of shorter oligonucleotide compounds.

The term "nucleobase" or "heterocyclic base moiety" as used herein, refers to the heterocyclic base portion of a nucleoside. In general, a nucleobase is any group that contains one or more atom or groups of atoms capable of hydrogen bonding to a base of another nucleic acid. In addition to "unmodified" or "natural" nucleobases such as the purine nucleobases adenine (A) and guanine (G), and the pyrimidine nucleobases thymine (T), cytosine (C) and uracil (U), many modified nucleobases or nucleobase mimetics known to those skilled in the art are amenable to the present invention. The terms modified nucleobase and nucleobase mimetic can overlap but generally a modified nucleobase refers to a nucleobase that is fairly similar in structure to the parent nucleobase, such as for example a 7-deaza purine, a 5-methyl cytosine, or a G-clamp , whereas a nucleobase mimetic would include more complicated structures, such as for example a tricyclic

phenoxazine nucleobase mimetic. Methods for preparation of the above noted modified nucleobases are well known to those skilled in the art.

Antisense compounds of the present invention may also contain one or more nucleosides having modified sugar moieties. The furanosyl sugar ring of a nucleoside can be modified in a number of ways including, but not limited to, addition of a substituent group, bridging of two non-geminal ring atoms to form a bicyclic nucleic acid (BNA) and substitution of an atom or group such as -S-, -N(R)- or -C(R_1)(R_2) for the ring oxygen at the 4'-position. Modified sugar moieties are well known and can be used to alter, typically increase, the affinity of the antisense compound for its target and/or increase nuclease resistance. A representative list of preferred modified sugars includes but is not limited to bicyclic modified sugars (BNA's), including LNA and ENA (4'-(CH_2)$_2$-O-2' bridge); and substituted sugars, especially 2'-substituted sugars having a 2'-F, 2'-OCH_2 or a 2'-O(CH_2)$_2$-OCH_3 substituent group. Sugars can also be replaced with sugar mimetic groups among others. Methods for the preparations of modified sugars are well known to those skilled in the art.

The present invention includes internucleoside linking groups that link the nucleosides or otherwise modified monomer units together thereby forming an antisense compound. The two main classes of internucleoside linking groups are defined by the presence or absence of a phosphorus atom. Representative phosphorus containing internucleoside linkages include, but are not limited to, phosphodiesters, phosphotriesters, methylphosphonates, phosphoramidate, and phosphorothioates. Representative non-phosphorus containing internucleoside linking groups include, but are not limited to, methylenemethylimino (-CH_2-N(CH_3)-O-CH_2-), thiodiester (-O-C(O)-S-), thionocarbamate (-O-C(O)(NH)-S-); siloxane (-O-Si(H)2-O-); and N,N'-dimethylhydrazine (-CH_2-N(CH_3)-N(CH_3)-). Antisense compounds having non-phosphorus internucleoside linking groups are referred to as oligonucleosides. Modified internucleoside linkages, compared to natural phosphodiester linkages, can be used to alter, typically increase, nuclease resistance of the antisense compound. Internucleoside linkages having a chiral atom can be prepared racemic, chiral, or as a mixture. Representative chiral internucleoside linkages include, but are not limited to, alkylphosphonates and phosphorothioates. Methods of preparation of phosphorous-containing and non-phosphorous-containing linkages are well known to those skilled in the art.

As used herein the term "mimetic" refers to groups that are substituted for a sugar, a nucleobase, and/ or internucleoside linkage. Generally, a mimetic is used in place of the sugar or

sugar-internucleoside linkage combination, and the nucleobase is maintained for hybridization to a selected target. Representative examples of a sugar mimetic include, but are not limited to, cyclohexenyl or morpholino. Representative examples of a mimetic for a sugar-internucleoside linkage combination include, but are not limited to, peptide nucleic acids (PNA) and morpholino groups linked by uncharged achiral linkages. In some instances a mimetic is used in place of the nucleobase. Representative nucleobase mimetics are well known in the art and include, but are not limited to, tricyclic phenoxazine analogs and universal bases (Berger et al., Nuc Acid Res. 2000, 28:2911-14, incorporated herein by reference). Methods of synthesis of sugar, nucleoside and nucleobase mimetics are well known to those skilled in the art.

As used herein the term "nucleoside" includes, nucleosides, abasic nucleosides, modified nucleosides, and nucleosides having mimetic bases and/or sugar groups.

In the context of this invention, the term "oligonucleotide" refers to an oligomeric compound which is an oligomer or polymer of ribonucleic acid (RNA) or deoxyribonucleic acid (DNA). This term includes oligonucleotides composed of naturally- and non-naturally-occurring nucleobases, sugars and covalent internucleoside linkages, possibly further including non-nucleic acid conjugates.

The present invention provides compounds having reactive phosphorus groups useful for forming internucleoside linkages including for example phosphodiester and phosphorothioate internucleoside linkages. Methods of preparation and/or purification of precursors or antisense compounds of the instant invention are not a limitation of the compositions or methods of the invention. Methods for synthesis and purification of DNA, RNA, and the antisense compounds of the instant invention are well known to those skilled in the art.

As used herein the term "chimeric antisense compound" refers to an antisense compound, having at least one sugar, nucleobase and/or internucleoside linkage that is differentially modified as compared to the other sugars, nucleobases and internucleoside linkages within the same oligomeric compound. The remainder of the sugars, nucleobases and internucleoside linkages can be independently modified or unmodified. In general a chimeric oligomeric compound will have modified nucleosides that can be in isolated positions or grouped together in regions that will define a particular motif. Any combination of modifications and or mimetic groups can comprise a chimeric oligomeric compound of the present invention.

Chimeric oligomeric compounds typically contain at least one region modified so as to confer increased resistance to nuclease degradation, increased cellular uptake, and/or increased binding affinity for the target nucleic acid. An additional region of the oligomeric compound may serve as a substrate for enzymes capable of cleaving RNA:DNA or RNA:RNA hybrids. By way of example, RNase H is a cellular endonuclease that cleaves the RNA strand of an RNA:DNA duplex. Activation of RNase H, therefore, results in cleavage of the RNA target, thereby greatly enhancing the efficiency of inhibition of gene expression. Consequently, comparable results can often be obtained with shorter oligomeric compounds when chimeras are used, compared to for example phosphorothioate deoxyoligonucleotides hybridizing to the same target region. Cleavage of the RNA target can be routinely detected by gel electrophoresis and, if necessary, associated nucleic acid hybridization techniques known in the art.

As used in the present invention the term "fully modified motif" refers to an antisense compound comprising a contiguous sequence of nucleosides wherein essentially each nucleoside is a sugar modified nucleoside having uniform modification.

The compounds described herein contain one or more asymmetric centers and thus give rise to enantiomers, diastereomers, and other stereoisomeric configurations that may be defined, in terms of absolute stereochemistry, as (R) or (S), α or ß, or as (D) or (L) such as for amino acids et al. The present invention is meant to include all such possible isomers, as well as their racemic and optically pure forms.

In one aspect of the present invention antisense compounds are modified by covalent attachment of one or more conjugate groups. Conjugate groups may be attached by reversible or irreversible attachments. Conjugate groups may be attached directly to antisense compounds or by use of a linker. Linkers may be mono- or bifunctional linkers. Such attachment methods and linkers are well known to those skilled in the art. In general, conjugate groups are attached to antisense compounds to modify one or more properties. Such considerations are well known to those skilled in the art.

Oligomer Synthesis

Oligomerization of modified and unmodified nucleosides can be routinely performed according to literature procedures for DNA (Protocols for Oligonucleotides and Analogs, Ed. Agrawal (1993), Humana Press) and/or RNA (Scaringe, Methods (2001), 23, 206-217. Gait et al.,

Applications of Chemically synthesized RNA in RNA: Protein Interactions, Ed. Smith (1998), 1-36. Gallo et al., Tetrahedron (2001), 57, 5707-5713).

Antisense compounds of the present invention can be conveniently and routinely made through the well-known technique of solid phase synthesis. Equipment for such synthesis is sold by several vendors including, for example, Applied Biosystems (Foster City, CA). Any other means for such synthesis known in the art may additionally or alternatively be employed. It is well known to use similar techniques to prepare oligonucleotides such as the phosphorothioates and alkylated derivatives. The invention is not limited by the method of antisense compound synthesis.

Oligomer Purification and Analysis

Methods of oligonucleotide purification and analysis are known to those skilled in the art. Analysis methods include capillary electrophoresis (CE) and electrospray-mass spectroscopy. Such synthesis and analysis methods can be performed in multi-well plates. The method of the invention is not limited by the method of oligomer purification.

Salts, prodrugs and bioequivalents

The antisense compounds of the present invention comprise any pharmaceutically acceptable salts, esters, or salts of such esters, or any other functional chemical equivalent which, upon administration to an animal including a human, is capable of providing (directly or indirectly) the biologically active metabolite or residue thereof. Accordingly, for example, the disclosure is also drawn to prodrugs and pharmaceutically acceptable salts of the antisense compounds of the present invention, pharmaceutically acceptable salts of such prodrugs, and other bioequivalents.

The term "prodrug" indicates a therapeutic agent that is prepared in an inactive or less active form that is converted to an active form (i.e., drug) within the body or cells thereof by the action of endogenous enzymes, chemicals, and/or conditions. In particular, prodrug versions of the oligonucleotides of the invention are prepared as SATE ((S-acetyl-2-thioethyl) phosphate) derivatives according to the methods disclosed in WO 93/24510 or WO 94/26764. Prodrugs can also include antisense compounds wherein one or both ends comprise nucleobases that are cleaved (e.g., by incorporating phosphodiester backbone linkages at the ends) to produce the active compound.

The term "pharmaceutically acceptable salts" refers to physiologically and pharmaceutically acceptable salts of the compounds of the invention: i.e., salts that retain the desired biological activity of the parent compound and do not impart undesired toxicological effects thereto.

Sodium salts of antisense oligonucleotides are useful and are well accepted for therapeutic administration to humans. In another embodiment, sodium salts of dsRNA compounds are also provided.

Formulations

The antisense compounds of the invention may also be admixed, encapsulated, conjugated or otherwise associated with other molecules, molecule structures or mixtures of compounds.

The present invention also includes pharmaceutical compositions and formulations which include the antisense compounds of the invention. The pharmaceutical compositions of the present invention may be administered in a number of ways depending upon whether local or systemic treatment is desired and upon the area to be treated. In a preferred embodiment, administration is topical to the surface of the respiratory tract, particularly pulmonary, e.g., by nebulization, inhalation, or insufflation of powders or aerosols, by mouth and/or nose.

The pharmaceutical formulations of the present invention, which may conveniently be presented in unit dosage form, may be prepared according to conventional techniques well known in the pharmaceutical industry. Such techniques include the step of bringing into association the active ingredients with the pharmaceutical carrier(s) or excipient(s). In general, the formulations are prepared by uniformly and intimately bringing into association the active ingredients with liquid carriers, finely divided solid carriers, or both, and then, if necessary, shaping the product (e.g., into a specific particle size for delivery). In a preferred embodiment, the pharmaceutical formulations of the instant invention are prepared for pulmonary administration in an appropriate solvent, e.g., water or normal saline, possibly in a sterile formulation, with carriers or other agents to allow for the formation of droplets of the desired diameter for delivery using inhalers, nasal delivery devices, nebulizers, and other devices for pulmonary delivery. Alternatively, the pharmaceutical formulations of the instant invention may be formulated as dry powders for use in dry powder inhalers.

A "pharmaceutical carrier" or "excipient" can be a pharmaceutically acceptable solvent, suspending agent or any other pharmacologically inert vehicle for delivering one or more nucleic acids to an animal and are known in the art. The excipient may be liquid or solid and is selected, with the planned manner of administration in mind, so as to provide for the desired bulk, consistency, etc., when combined with a nucleic acid and the other components of a given pharmaceutical composition.

Combinations

Compositions of the invention can contain two or more antisense compounds. In another related embodiment, compositions of the present invention can contain one or more antisense compounds, particularly oligonucleotides, targeted to a first nucleic acid and one or more additional antisense compounds targeted to a second nucleic acid target. Alternatively, compositions of the present invention can contain two or more antisense compounds targeted to different regions of the same nucleic acid target. Two or more combined compounds may be used together or sequentially. Compositions of the instant invention can also be combined with other non-antisense compound therapeutic agents.

Nonlimiting disclosure and incorporation by reference

While certain compounds, compositions and methods of the present invention have been described with specificity in accordance with certain embodiments, the following examples serve only to illustrate the compounds of the invention and are not intended to limit the same. Each of the references, GenBank accession numbers, and the like recited herein is incorporated herein by reference in its entirety.

Example 1

Design of modified antisense compounds targeting SMN2

In accordance with the present invention, antisense compounds were designed to target intron 6, exon 7 or intron 7 of SMN2 (SEQ ID NO: 1). In reference to SEQ ID NO:1, nucleotides 61-114 represent exon 7, while nucleotides 1-60 and 115-174 represent portions of intron 6 and intron 7, respectively. The compounds, listed in Table 1, are either 12, 15, 16 or 18 nucleotides in length and are composed of 2'-O-methoxyethyl nucleotides, also known as 2'-MOE nucleotides. The internucleoside (backbone) linkages are phosphodiester throughout the oligonucleotide. All cytidine residues are 5-methylcytidines. Target site indicates the first (5'-most) nucleotide number of the target sequence (SEQ ID NO: 1) to which the oligonucleotide binds.

Table 1

2'-MOE Compounds Targeting SMN2

ISIS #	Target Site	Target Region	Length	Sequence (5' to 3')	SEQ ID NO
390645	1	Intron 6	15	TAGATAGCTATATAT	2
393593	2	Intron 6	15	ATAGATAGCTATATA	3
393592	3	Intron 6	15	TATAGATAGCTATAT	4
393591	4	Intron 6	15	ATATAGATAGCTATA	5

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ISIS #	Target Site	Target Region	Length	Sequence (5' to 3')	SEQ ID NO
393590	5	Intron 6	15	GATATAGATAGCTAT	6
393602	5	Intron 6	12	ATAGATAGCTAT	7
390644	6	Intron 6	15	AGATATAGATAGCTA	8
393601	6	Intron 6	12	TATAGATAGCTA	9
393589	7	Intron 6	15	TAGATATAGATAGCT	10
393600	7	Intron 6	12	ATATAGATAGCT	11
393588	8	Intron 6	15	ATAGATATAGATAGC	12
393599	8	Intron 6	12	GATATAGATAGC	13
393587	9	Intron 6	15	TATAGATATAGATAG	14
393598	9	Intron 6	12	AGATATAGATAG	15
393586	10	Intron 6	15	ATATAGATATAGATA	16
393597	10	Intron 6	12	TAGATATAGATA	17
390643	11	Intron 6	15	TATATAGATATAGAT	18
393596	11	Intron 6	12	ATAGATATAGAT	19
393595	12	Intron 6	12	TATAGATATAGA	20
393594	13	Intron 6	12	ATATAGATATAG	21
390642	16	Intron 6	15	ATAGCTATATAGATA	22
390641	21	Intron 6	15	AAAAAATAGCTATAT	23
390640	26	Intron 6	15	GTTAAAAAAAATAGC	24
390639	31	Intron 6	15	AGGAAGTTAAAAAAA	25
390638	36	Intron 6	15	AATAAAGGAAGTTAA	26
390637	41	Intron 6	15	AGGAAAATAAAGGAA	27
390636	46	Intron 6	15	CTGTAAGGAAAATAA	28
372641	61	Exon 7	15	ATTTTGTCTAAAACC	29
385909	62	Exon 7	15	GATTTTGTCTAAAAC	30
383497	63	Exon 7	12	TTTTGTCTAAAA	31
385908	63	Exon 7	15	TGATTTTGTCTAAAA	32
383496	64	Exon 7	12	ATTTTGTCTAAA	33
385907	64	Exon 7	15	TTGATTTTGTCTAAA	34
383495	65	Exon 7	12	GATTTTGTCTAA	35
385906	65	Exon 7	15	TTTGATTTTGTCTAA	36
385910	65	Exon 7	16	TTTTGATTTTGTCTAA	37
372642	66	Exon 7	15	TTTTGATTTTGTCTA	38
383494	66	Exon 7	12	TGATTTTGTCTA	39
383493	67	Exon 7	12	TTGATTTTGTCT	40
385905	67	Exon 7	15	TTTTTGATTTTGTCT	41
383492	68	Exon 7	12	TTTGATTTTGTC	42
385904	68	Exon 7	15	CTTTTTGATTTTGTC	43
383491	69	Exon 7	12	TTTTGATTTTGT	44
383490	70	Exon 7	12	TTTTTGATTTTG	45
372643	71	Exon 7	15	CTTCTTTTTGATTTT	46
383489	71	Exon 7	12	CTTTTTGATTTT	47
383488	72	Exon 7	12	TCTTTTTGATTT	48
372644	76	Exon 7	15	CCTTCCTTCTTTTTG	49
372645	81	Exon 7	15	GAGCACCTTCCTTCT	50
372646	86	Exon 7	15	AATGTGAGCACCTTC	51

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ISIS #	Target Site	Target Region	Length	Sequence (5' to 3')	SEQ ID NO
372647	91	Exon 7	15	TAAGGAATGTGAGCA	52
383470	92	Exon 7	18	AATTTAAGGAATGTGAGC	53
383477	92	Exon 7	15	TTAAGGAATGTGAGC	54
383469	93	Exon 7	18	TAATTTAAGGAATGTGAG	55
383476	93	Exon 7	15	TTTAAGGAATGTGAG	56
383487	93	Exon 7	12	AAGGAATGTGAG	57
383468	94	Exon 7	18	TTAATTTAAGGAATGTGA	58
383475	94	Exon 7	15	ATTTAAGGAATGTGA	59
383486	94	Exon 7	12	TAAGGAATGTGA	60
383467	95	Exon 7	18	CTTAATTTAAGGAATGTG	61
383474	95	Exon 7	15	AATTTAAGGAATGTG	62
383485	95	Exon 7	12	TTAAGGAATGTG	63
372648	96	Exon 7	15	TAATTTAAGGAATGT	64
383466	96	Exon 7	18	CCTTAATTTAAGGAATGT	65
383484	96	Exon 7	12	TTTAAGGAATGT	66
383473	97	Exon 7	15	TTAATTTAAGGAATG	67
383483	97	Exon 7	12	ATTTAAGGAATG	68
383472	98	Exon 7	15	CTTAATTTAAGGAAT	69
383482	98	Exon 7	12	AATTTAAGGAAT	70
383471	99	Exon 7	15	CCTTAATTTAAGGAA	71
383481	99	Exon 7	12	TAATTTAAGGAA	72
372649	100	Exon 7	15	TCCTTAATTTAAGGA	73
383480	100	Exon 7	12	TTAATTTAAGGA	74
383479	101	Exon 7	12	CTTAATTTAAGG	75
383478	102	Exon 7	12	CCTTAATTTAAG	76
390646	115	Intron 7	15	TGCTGGCAGACTTAC	77
390647	120	Intron 7	15	CATAATGCTGGCAGA	78
393610	121	Intron 7	15	TCATAATGCTGGCAG	79
393609	122	Intron 7	15	TTCATAATGCTGGCA	80
393608	123	Intron 7	15	TTTCATAATGCTGGC	81
387949	124	Intron 7	20	ATTCACTTTCATAATGCTGG	82
393607	124	Intron 7	15	CTTTCATAATGCTGG	83
393619	124	Intron 7	12	TCATAATGCTGG	84
390648	125	Intron 7	15	ACTTTCATAATGCTG	85
393618	125	Intron 7	12	TTCATAATGCTG	86
393606	126	Intron 7	15	CACTTTCATAATGCT	87
393617	126	Intron 7	12	TTTCATAATGCT	88
393605	127	Intron 7	15	TCACTTTCATAATGC	89
393616	127	Intron 7	12	CTTTCATAATGC	90
393604	128	Intron 7	15	TTCACTTTCATAATG	91
393615	128	Intron 7	12	ACTTTCATAATG	92
393603	129	Intron 7	15	ATTCACTTTCATAAT	93
393614	129	Intron 7	12	CACTTTCATAAT	94
390649	130	Intron 7	15	GATTCACTTTCATAA	95
393613	130	Intron 7	12	TCACTTTCATAA	96
393612	131	Intron 7	12	TTCACTTTCATA	97

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ISIS #	Target Site	Target Region	Length	Sequence (5' to 3')	SEQ ID NO
393611	132	Intron 7	12	ATTCACTTTCAT	98
390650	135	Intron 7	15	AGTAAGATTCACTTT	99
390651	140	Intron 7	15	ACAAAAGTAAGATTC	100
390652	145	Intron 7	15	GTTTTACAAAAGTAA	101
390653	150	Intron 7	15	ATAAAGTTTTACAAA	102
390654	155	Intron 7	15	AAACCATAAAGTTTT	103
390655	160	Intron 7	15	TCCACAAACCATAAA	104

Other nucleic acid sequences for SMN genes are publicly available and well known in the art. For example, Genbank Accession Nos. NM_000344, NM_022874, NM_022875, U43883, AC140134, AC139778, AC010237, AC022119 and AC004999 provide nucleotide sequences of SMN1 or SMN2.

Example 2

Treatment with oligomeric compounds

When cells reach appropriate confluency, they are treated with oligonucleotide using a transfection method as described.

LIPOFECTIN™

When cells reach 65-75% confluency, they are treated with oligonucleotide. Oligonucleotide is mixed with LIPOFECTIN™ Invitrogen Life Technologies, Carlsbad, CA) in Opti-MEM™-1 reduced serum medium (Invitrogen Life Technologies, Carlsbad, CA) to achieve the desired concentration of oligonucleotide and a LIPOFECTIN ™ concentration of 2.5 or 3 μg/mL per 100 nM oligonucleotide. This transfection mixture is incubated at room temperature for approximately 0.5 hours. For cells grown in 96-well plates, wells are washed once with 100 μL OPTI-MEM™-1 and then treated with 130 μL of the transfection mixture. Cells grown in 24-well plates or other standard tissue culture plates are treated similarly, using appropriate volumes of medium and oligonucleotide. Cells are treated and data are obtained in duplicate or triplicate. After approximately 4-7 hours of treatment at 37°C, the medium containing the transfection mixture is replaced with fresh culture medium.

Electroporation

When cells reach approximately 80% confluency, oligonucleotide is introduced via electroporation. Oligonucleotide concentrations used in electroporation experiments range from 0.1

to 40 μM. Cells are harvested by routine trypsinization to produce a single cell suspension. Following cell counting using a hemocytometer and pelleting by centrifugation, cells are resuspended in OPTI-MEM™-1 reduced serum medium (Invitrogen Life Technologies, Carlsbad, CA) to achieve a density of 1 x 10^7 cells/mL. Cells are mixed with the desired concentration of oligonucleotide and transferred to a 0.1 cm electroporation cuvette (BTX Molecular Delivery Systems, Hollister, MA). Cells are subjected to a single pulse using an electroporation apparatus (for example, the BTX Electro Square Porator T820 or the BTX HT300, BTX Molecular Delivery Systems, Hollister, MA), diluted into culture medium and plated into 24-well plates. Cells are treated and data were obtained in duplicate or triplicate.

Example 3

Minigenes for SMN2 splicing studies

All SMN constructs are derivatives of pCITel (Lorson and Androphy, Hum. Mol. Genet., 2000, 9, 259-265). The primers used to generate each SMN2 construct are shown in Table 2. Using a Quickchange kit (Stratagene, La Jolla, CA), an *XbaI* site was inserted by site-directed mutagenesis at nucleotide 7170 (in intron 7) to generate pCI-SMNx-wt. For in vitro transcription studies, intron 6 was shortened by overlap-extension PCR to generate pCISMNxΔ6-wt, deleting 5,570 nt from position 1235 to the *BclI* site at nt 6805. Two sets of PCR were performed with Pfu polymerase and pCISMNx-wt as template. The first PCR was carried out with primers CIF1 and Δ6-bclR, the second with primers smnΔ6-vrlp and CIR. The PCR products were purified, combined and reamplified with the outer primers (CIF1 and CIR). The final product was digested with *Xho*I and *Not*I and subcloned it into pCISMNx-wt digested with the same enzymes. All the constructs were verified by direct sequencing. Templates were generated for in vitro transcription by PCR amplification of pCISMNxΔ6-wt using primers CIF2 and smn8-75+5R. The final products contained a T7 promoter, exon 6 (124 nt), a shortened intron 6 (200 nt), exon 7 (54 nt), intron 7 (444 nt), and 75 nt of exon 8 followed by a consensus 5' splice site (GTAAGTACTT; SEQ ID NO: 22) (Cartegni and Krainer, Nature Genet., 2002, **30**, 377-384; WO 02/38738).

Table 2

Primers used to generate SMN2 minigenes and templates

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Construct	Primer Name	Primer Sequence	SEQ ID NO
pCI-SMNx-wt	smnI7xbaF	AGATAAAAGGTTAATCTAGATCCCTACTAGAATTCTC	106
pCI-SMNx-wt	smnI7xbaR	GAGAATTCTAGTAGGGATCTAGATTAACCTTTTATCT	107
pCISMNxΔ6-wt	CIF1	AATTGCTAACGCAGTCAGTGCTTC	108
pCISMNxΔ6-wt	Δ6-bclR	AATATGATCAGCAAAACAAAGTCACATAACTAC	109
pCISMNxΔ6-wt	smnΔ6-vrlp	GTGACTTTGTTTTGCTGATCATATTTTGTTGAATAAAATAAG	110
pCISMNxΔ6-wt	CIR	AATGTATCTTATCATGTCTGCTCG	111
In vitro templates	CIF2	AATGTATCTTATCATGTCTGCTCG	112
In vitro templates	Smn8-75+5'R	AAGTACTTACCTGTAACGCTTCACATTCCAGATCTGTC	113

Example 4

Effect of antisense compounds on SMN2 splicing in cell-free extracts

2'-MOE antisense compounds designed to target exon 7 of SMN2 were evaluated for their effect on splicing of SMN2. Templates for in vitro SMN2 splicing studies were generated as described in Example 3. 5'-capped T7 runoff transcripts from purified PCR products were uniformly labeled with [α-^{32}P]-UTP and purified by denaturing polyacrylamide gel electrophoresis. Labeled in vitro transcripts were spliced in HeLa cell nuclear or S100 extracts (Mayeda and Krainer, Methods Mol. Biol., 1999, 118, 315-321; Mayeda and Krainer, Methods Mol. Biol., 1999, 118, 309-314) by incubating 10 fmol of transcript in 12.5 µl standard splicing reactions containing 3 µl of nuclear extract or 2 µl of S100 extract. Extracts either contained no antisense oligonucleotide or were complemented with 1, 5, 10, 25, 50, 100, 200 or 400 nM of ISIS 372641, ISIS 372642, ISIS 372643, ISIS 372644, ISIS 372645, ISIS 372646, ISIS 372647, ISIS 372648 or ISIS 372649. Control oligonucleotide ISIS 372693 was also used in this study (TTGTATTCTATGTTT; SEQ ID NO: 114). The $MgCl_2$ concentration of the splicing mixture was 1.6 mM. After incubation at 30°C for 4 h, RNA was extracted and analyzed on 8% denaturing polyacrylamide gels, followed by autoradiography and phosphorimager analysis. Exon inclusion was calculated as a percentage of the total amount of spliced mRNA (included mRNA x 100/(included mRNA + skipped mRNA).

The results showed that several of the SMN2 antisense oligonucleotides altered splicing of SMN2 exon 7, while control oligonucleotide ISIS 372693 had no effect. ISIS 372641 promoted skipping of SMN2 exon 7 in a dose-dependent manner. Exon 7 was included in only 2% of SMN2 spliced transcripts incubated with 400 nM of ISIS 372641, compared with 26% of transcripts incubated with no oligonucleotide. Similarly, ISIS 372646 inhibited inclusion of exon 7 in a dose-dependent manner with 16% of SMN2 spliced transcripts containing exon 7, compared with 32% of

transcripts incubated without oligonucleotide. In contrast, ISIS 372642 inhibited skipping of exon 7 in a dose-dependent manner. The percentage of SMN2 spliced transcripts containing exon 7 increased from 28% when incubated without oligonucleotide to 40% when incubated with 400 nM of ISIS 372642. ISIS 372648 also increased inclusion of exon 7 with 69% of SMN2 transcripts containing exon 7 when incubated with the highest concentration of oligonucleotide, compared with 42% of transcripts when incubated without oligonucleotide. Extracts containing ISIS 372643 also showed a slight increase in exon 7 inclusion at the higher oligonucleotide concentrations. Taken together, these results illustrate that antisense oligonucleotides targeting exon 7 of SMN2 are capable of altering splicing of transcripts to either promote or inhibit inclusion of exon 7.

Example 5

Effect of antisense compounds on SMN2 splicing in HEK293 cells

Antisense compounds targeting SMN2 exon 7 were evaluated for their effects on SMN2 splicing in cultured cells. HEK293 cells were electroporated with 10 μg SMN2 minigene and 10 μM of either SMN2 antisense oligonucleotide ISIS 372641, ISIS 372642, ISIS 372643, ISIS 372644, ISIS 372645, ISIS 372646, ISIS 372647, ISIS 372648 or ISIS 372649, or control oligonucleotide ISIS 372693. Sixty hours after transfection, total RNA was isolated using Trizol Reagent (Invitrogen, Carlsbad, CA) following the manufacturer's directions. One μg of DNAse-treated total RNA was used to generate first-strand cDNA sequences with oligo(dT) and Superscript II reverse transcriptase (Invitrogen), and the cDNA was amplified semi-quantitatively by 16 PCR cycles (94°C for 30 s, 57.5°C for 30 s, 72°C for 90 s) in the presence of [α-^{32}P]dCTP (Lorson and Androphy, Hum. Mol. Genet., 2000, 9, 259-265). PCR products were analyzed by electrophoresis on 6% denaturing polyacrylamide gels, followed by autoradiography and phosphorimager analysis. Exon inclusion was calculated as a percentage of the total amount of spliced mRNA (included mRNA x 100/(included mRNA + skipped mRNA). The percentage of SMN2 spliced transcripts containing exon 7 (% inclusion) is shown in Table 3. The target site of each oligonucleotide relative to SEQ ID NO: 1 is also indicated.

Table 3

Effect of SMN2 antisense compounds on exon 7 inclusion

ISIS #	Target Site	% Inclusion
372641	61	6.4
372642	66	67.4

ISIS #	Target Site	% Inclusion
372643	71	34.9
372644	76	12.9
372645	81	7.8
372646	86	11.8
372647	91	9.5
372648	96	75.2
372649	100	55.1
372693	Control	57.7

Compared to control oligonucleotide, transfection with either ISIS 372642 or 372648 resulted in a greater percentage of SMN2 transcripts with exon 7 included, which is consistent with results obtained from in vitro assays. Treatment with ISIS 372641, ISIS 372644, ISIS 372645, ISIS 372646 and ISIS 372647 resulted in the most significant increase in exon 7 skipping.

SMN2 antisense oligonucleotides were further evaluated for their effects on endogenous SMN1 and SMN2 pre-mRNA splicing in cultured cells. HEK293 cells were electroporated with 10 μM of either SMN2 antisense oligonucleotide ISIS 372641, ISIS 372642, ISIS 372643, ISIS 372644, ISIS 372645, ISIS 372646, ISIS 372647, ISIS 372648 or ISIS 372649, or control oligonucleotide ISIS 372693. Sixty hours after transfection, RNA was isolated and RT-PCR was performed as described above to examine splicing changes of both SMN1 and SMN2 pre-mRNAs. PCR products were digested with *DdeI* to distinguish between SMN1 and SMN2, separated by electrophoresis on 6% denaturing polyacrylamide gels and analyzed by autoradiography. The percentage of SMN1 and SMN2 spliced transcripts containing exon 7 (% inclusion) is shown in Table 4.

Table 4

Effect of SMN2 antisense oligonucleotides on SMN1 and SMN2 pre-mRNA splicing

ISIS #	% Inclusion SMN1	% Inclusion SMN2
372641	82.5	11.1
372642	96.2	69.5
372643	94.1	28.8
372644	68.5	23.8
372645	47.3	15.2
372646	57.7	20.2
372647	58.8	12.8
372648	93.1	52.2
372649	94.8	49.3
372693	95.1	50.1

In accordance with previous results, transfection with ISIS 372642 and ISIS 372648 led to

the greatest level of exon 7 inclusion in SMN2 pre-mRNA transcripts. ISIS 372641, ISIS 372644, ISIS 372645, ISIS 372646 and ISIS 372647 significantly reduced the percentage of SMN1 transcripts containing exon 7. These oligonucleotides, along with ISIS 372643, also reduced exon 7 inclusion in SMN2 mRNAs.

Additional antisense oligonucleotides targeting the 3' end of SMN2 exon 7 (see Table 1) were evaluated for their effects on SMN2 pre-mRNA splicing. HEK293 cells were electroporated with 10 μM of either SMN2 antisense oligonucleotide ISIS 383466, ISIS 383467, ISIS 383468, ISIS 383469, ISIS 383470, ISIS 383471, ISIS 383472, ISIS 383473, ISIS 383474, ISIS 383475, ISIS 383476, ISIS 383477 or ISIS 372648, or a control oligonucleotide. Fifty hours after transfection, RNA was isolated and RT-PCR was performed as described above to examine splicing changes of SMN2 pre-mRNA. PCR products were digested with *DdeI* to distinguish between SMN1 and SMN2, separated by electrophoresis on 6% denaturing polyacrylamide gels and analyzed by autoradiography. The percentage of SMN2 spliced transcripts containing exon 7 (% inclusion) is shown in Table 5. The length and target site of each oligonucleotide relative to SEQ ID NO: 1 are also indicated.

Table 5

Effect of SMN2 antisense oligonucleotides on SMN2 pre-mRNA splicing

ISIS #	Target Site	Length	% Inclusion
383470	92	18	4.9
383477	92	15	5.3
383469	93	18	18.9
383476	93	15	32.8
383468	94	18	18.7
383475	94	15	84.8
383467	95	18	8.1
383474	95	15	77.0
372648	96	15	59.6
383466	96	18	37.5
383473	97	15	42.2
383472	98	15	45.0
383471	99	15	37.1
Control	N/A	N/A	41.3
Vehicle	N/A	N/A	41.4

The results demonstrate that a number of SMN2 antisense oligonucleotides can alter splicing of SMN2 pre-mRNAs. ISIS 383467, ISIS 383468, ISIS 383469, ISIS 383470, ISIS 383476 and ISIS 383477 inhibited inclusion of exon 7; ISIS 383474, ISIS 383475 and ISIS 372648 significantly

increased inclusion of exon 7; and ISIS 383466, ISIS 383471, ISIS 383472 and ISIS 383473 appeared to have little effect on SMN2 splicing, relative to oligonucleotide and vehicle controls. These results suggest that SMN2 oligonucleotides with a target site between nucleotides 94-96 are particularly effective at achieving inclusion of exon 7 during SMN2 pre-mRNA splicing, and further suggests oligonucleotides 15 nucleotides in length are more effective than those 18 nucleotides in length.

Example 6

Effect of antisense compounds on SMN2 splicing in SMA fibroblast cells

In accordance with the present invention, SMN2 antisense oligonucleotides were tested in fibroblast cells derived from a patient with type I SMA (3813 cell line; Coovert et al., Human Mol. Genet., 1997, 6, 1205-1214). SMA fibroblasts contain SMN2, but do not express SMN1. SMA fibroblasts were lipofected with 200 nM of either SMN2 antisense oligonucleotide ISIS 372641, ISIS 372642, ISIS 372643, ISIS 372644, ISIS 372645, ISIS 372646, ISIS 372647, ISIS 372648 or ISIS 372649, or control oligonucleotide ISIS 372693. Seventy hours after transfection, RNA was isolated and RT-PCR was performed as described above to examine splicing changes of endogenous SMN2 pre-mRNAs. PCR products were separated by electrophoresis and analyzed by autoradiography. The percentage of SMN2 spliced transcripts containing exon 7 (% inclusion) is shown in Table 6. The target site of each oligonucleotide relative to SEQ ID NO: 1 is also indicated.

Table 6

Effect of SMN2 antisense oligonucleotides on exon 7 inclusion in SMA fibroblasts

ISIS #	Target Site	% Inclusion
372641	61	41.8
372642	66	55.2
372643	71	40.9
372644	76	43.4
372645	81	43.7
372646	86	38.8
372647	91	43.6
372648	96	49.8
372649	100	48.8
372693	Control	48.7
PBS	N/A	48.8

In accordance with previous findings, treatment with ISIS 372642 and ISIS 372648 generated a greater percentage of SMN2 splicing products containing exon 7.

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A second experiment to further evaluate ISIS 372642 and ISIS 383475 in SMA fibroblasts was performed. SMA fibroblasts were lipofected with either 200 nM ISIS 372642, 200 nM ISIS 383475, or 100 nM ISIS 372642 in combination with 100 nM ISIS 383475. ISIS 372693 (200 nM) and vehicle only were also used as controls. Fifty hours after transfection, RNA was isolated and RT-PCR was performed. PCR products were separated by electrophoresis and analyzed by autoradiography. The percentage of SMN2 spliced transcripts containing exon 7 (% inclusion) is shown in Table 7.

Table 7

Effect of ISIS 372642 and ISIS 383475 on exon 7 inclusion in SMA fibroblasts

Treatment (ISIS #)	% Inclusion
372642	47.8
383475	53.9
372642 & 383475	49.2
372693	36.3
Vehicle	35.0

The results demonstrate that treatment with ISIS 372642 or ISIS 383475, either alone or in combination, leads to greater inclusion of exon 7 in SMA transcripts.

Example 7

Microwalk of ISIS 372642 and ISIS 372648 Target Sites

The studies shown above demonstrated that both ISIS 372642 and ISIS 372648 were effective in promoting SMN2 exon 7 inclusion. To further evaluate the target sites surrounding these compounds, additional compounds were designed as 1 nucleotide microwalks around each site (see Table 1 for sequences and target sites). Ten compounds 12 nucleotides in length were designed for each microwalk. Seven additional compounds 15 or 16 nucleotides in length were designed to target the region of ISIS 372642. The antisense compounds targeting the 3' end of exon 7 (ISIS 383466-382477), described above in Example 5, were included for comparison with the ISIS 372648 microwalk compounds. Each compound was evaluated in the SMN2 minigene splicing assay and the endogenous SMN1/SMN2 splicing assay in HEK293 cells. Both assays are described in previous examples herein. The results are in shown in Tables 8 and 9.

Table 8

ISIS 372642 Microwalk Compounds: Effect on Exon 7 Inclusion

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ISIS #	Target Site	Length	% Inclusion SMN2 Minigene	% Inclusion Endogenous SMN2	% Inclusion Endogenous SMN1
385909	62	15	44	43	81
383497	63	12	55	51	86
385908	63	15	49	53	85
383496	64	12	32	36	81
385907	64	15	56	54	86
383495	65	12	54	49	85
385906	65	15	56	57	87
385910	65	16	60	66	89
372642	66	15	66	74	89
383494	66	12	57	51	86
383493	67	12	57	52	85
385905	67	15	74	85	89
383492	68	12	60	56	85
385904	68	15	9	6	19
383491	69	12	38	41	81
383490	70	12	51	49	84
383489	71	12	13	27	76
383488	72	12	24	38	82
Control	N/A	N/A	52	51	86
Control	N/A	N/A	53	50	86

Table 9

ISIS 372648 Microwalk Compounds: Effect on Exon 7 Inclusion

ISIS #	Target Site	Length	% Inclusion SMN2 Minigene	% Inclusion Endogenous SMN2	% Inclusion Endogenous SMN1
383470	92	18	8	12	63
383477	92	15	6	7	38
383469	93	18	29	26	89
383476	93	15	36	31	87
383487	93	12	11	16	79
383468	94	18	31	26	88
383475	94	15	85	88	96
383486	94	12	44	41	91
383467	95	18	14	13	82
383474	95	15	79	71	94
383485	95	12	63	60	93
372648	96	15	70	57	93
383466	96	18	38	43	92
383484	96	12	65	56	92
383473	97	15	62	53	94
383483	97	12	63	56	94
383472	98	15	41	46	93
383482	98	12	59	45	94

ISIS #	Target Site	Length	% Inclusion SMN2 Minigene	% Inclusion Endogenous SMN2	% Inclusion Endogenous SMN1
383471	99	15	38	47	92
383481	99	12	42	46	92
383480	100	12	39	48	91
383479	101	12	44	44	92
383478	102	12	47	41	93
Control	N/A	N/A	41	44	93
Control	N/A	N/A	44	48	92

In accordance with previous results, treatment with ISIS 372642, ISIS 372648 or ISIS 383475 led to a significant increase in exon 7 inclusion. In addition, ISIS 385905 was identified as a particularly effective compound for promoting exon 7 inclusion. To further evaluate ISIS 385905 and ISIS 383475, dose-response and duration of action studies were performed. To determine the effect of oligonucleotide dose, HEK293 cells were electroporated with either ISIS 385905 or ISIS 383475 at a concentration of 0, 0.2, 0.5, 1, 2, 5, 10 or 20 μM. Sixty hours after electroporation, RNA was isolated and RT-PCR was performed as described above to determine the extent of exon 7 inclusion. The results, expressed as % inclusion of exon 7, are shown in Table 10.

Table 10

Dose-response of ISIS 385905 and ISIS 383475

ISIS #	0 μM	0.2 μM	0.5 μM	1.0 μM	2.0 μM	5.0 μM	10 μM	20 μM
385905	50	53	61	65	74	78	82	87
383475	51	57	65	72	77	83	87	89

The results show a dose-dependent increase in exon 7 inclusion following treatment with either compound. To assess duration of action, HEK293 cells were electroporated with 10 μM of either compound and RNA was isolated and subjected to RT-PCR at day 0, 1, 2, 3, 4 and 5. The results, expressed as % inclusion of exon 7, are shown in Table 11.

Table 11

Duration of Action of ISIS 385905 and ISIS 383475

ISIS #	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5
385905	49	80	83	82	85	79
383475	48	84	89	88	89	83

These results demonstrate a significant increase in exon 7 inclusion following treatment of either compound, and further show the compounds are effective for at least five days.

Taken together, the results of the experiments detailed above demonstrate that antisense compounds having a target site (5'-most nucleotide to which the compound binds) of nucleotides 64-68 or 94-97 of SEQ ID NO: 1 are most effective at promoting exon 7 inclusion in SMN2 transcripts. The target sites of these compounds overlap with predicted ESS (exonic splicing silencer) elements, without having significant overlap with predicted ESE (exonic splicing enhancer) elements. Thus, the antisense compounds described herein may function by blocking binding of *trans* splicing factors to particular *cis* regulatory elements, thereby influencing splice site selection and specifically, inclusion or exclusion of exon 7 from SMN2 mRNAs.

Example 8

Effect of compounds targeting exon 7 or intron 7 on inclusion of exon 7

In previous examples herein, antisense compounds ISIS 372642, ISIS 385905 and ISIS 383475, each of which target exon 7, were shown to significantly increase inclusion of SMN2 exon 7. These exon 7-targeted compounds and a compound targeting SMN2 intron 7 (ISIS 387949) were compared for their capacity to promote exon 7 inclusion. As described in previous examples herein, HEK293 cells were electroporated with 10 μM of oligonucleotide and RT-PCR was performed after two days to examine splicing changes of endogenous SMN1 and SMN2. In comparison to control oligonucleotide, the compounds targeted to exon 7 exhibited a significant increase in exon 7 inclusion, as expected. In addition, the intron 7 targeted compound led to incorporation of exon 7 in nearly all SMN1 and SMN2 mRNAs. These results suggest antisense compounds targeted to intronic sequences also contribute to incorporation of SMN2 exon 7. Intronic sequences also are known to contain splicing regulatory elements (*i.e.* intronic splicing enhancers and intronic splicing silencers), providing a possible mechanism of action for ISIS 387949.

Example 9

Systematic Mapping of Intronic Splicing Silencers (ISSs)

To further investigate whether antisense compounds targeting the introns flanking exon 7 of SMN2 could alter inclusion of exon 7, such as by interfering with intronic splicing silencers, compounds were designed to target the 60 nucleotides of intron 6 (nucleotides 1-60 of SEQ ID NO: 1) or the 60 nucleotides of intron 7 (nucleotides 115-174 of SEQ ID NO: 1) immediately adjacent to exon 7. Antisense compounds targeting intron 6 (ISIS 390636, ISIS 390637, ISIS 390638, ISIS

390639, ISIS 390640, ISIS 390641, ISIS 390642, ISIS 390643, ISIS 390644 and ISIS 390645) or intron 7 (ISIS 390646, ISIS 390647, ISIS 390648, ISIS 390649, ISIS 390650, ISIS 390651, ISIS 390652, ISIS 390653, ISIS 390654 and ISIS 390655) are show above in Table 1. Each compound was tested in three different assays to evaluate their effect on exon 7 inclusion: SMN2 minigene splicing in cell-free extracts, SMN2 minigene splicing in transfected HEK293 cells and splicing of endogenous SMN2 in HEK293 cells. The results obtained from the three assays demonstrated that several antisense compounds were able to increase inclusion of exon 7. In particular, ISIS 390644 and ISIS 390648 were the most effect intron 6 and intron 7-targeted compounds, respectively.

To further investigate the regions targeted by ISIS 390644 and ISIS 390648, additional compounds were designed as microwalks around these target sequences (see Table 1 for sequences). For these experiments, compounds 12 and 15 nucleotides in length were designed and tested in accordance with the procedures detailed in previous examples herein. Compounds targeting the region of ISIS 390644 (intron 6) were tested in the in vitro SMN2 minigene assay and endogenous SMN1/SMN2 assay in HEK293 cells. The results are shown in Table 12.

Table 12

Results of Microwalk of Intron 6 Compound ISIS 390644

ISIS #	Target Site	Length	% Inclusion SMN2 minigene	% Inclusion Endogenous SMN2
393586	10	15	10	32
393587	9	15	18	44
393588	8	15	32	60
393589	7	15	59	79
390644	6	15	65	75
393590	5	15	49	67
393591	4	15	20	46
393592	3	15	22	44
393593	2	15	29	50
393594	13	12	20	44
393595	12	12	13	39
393596	11	12	15	44
393597	10	12	13	39
393598	9	12	17	48
393599	8	12	30	64
393600	7	12	28	62
393601	6	12	44	63
393602	5	12	29	46
Control	N/A	N/A	22	43

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As shown in Table 12, antisense compounds having a target site of nucleotides 5-8 (SEQ ID NO: 1) results in the greatest percentage of transcripts containing exon 7. These findings suggest this region of intron 6 contains an intronic splicing silencer, which normally functions to inhibit inclusion of exon 7. Upon blockade of this regulatory element, splice site selection is altered to promote exon 7 inclusion.

Compounds targeting the region of 390648 (intron 7) were assayed using the SMN2 minigene in vitro and in transfected HEK293 cells and tested in the endogenous SMN2 splicing assay in HEK293 cells. The results are shown in Table 13.

Table 13

Results of Microwalk of Intron 7 Compound ISIS 390648

ISIS #	Target Site	Length	% Inclusion SMN2 in vitro minigene	% Inclusion SMN2 minigene	% Inclusion Endogenous SMN2
393603	129	15	43	51	76
393604	128	15	46	75	97
393605	127	15	57	97	100
393606	126	15	56	97	100
390648	125	15	53	98	100
393607	124	15	67	100	100
393608	123	15	75	100	100
393609	122	15	60	97	100
393610	121	15	54	58	78
393611	132	12	41	17	40
393612	131	12	39	30	58
393613	130	12	42	43	64
393614	129	12	48	43	64
393615	128	12	38	36	44
393616	127	12	38	30	90
393617	126	12	36	30	92
393618	125	12	44	71	97
393619	124	12	69	92	97
Control	N/A	N/A	28	23	44

While all compounds led to an increase in exon 7 inclusion, compounds with target sites between nucleotides 121 and 129 (SEQ ID NO: 1) were most effective.

Select compounds targeting intron 7 were further evaluated for SMN2 exon 7 inclusion following transfection at a low oligonucleotide dose of 0.1 μM. As previously described herein, HEK293 cells were electroporated with ISIS 393605, ISIS 393606, ISIS 390648, ISIS 393607, ISIS

393608, ISIS 393609, ISIS 393617, ISIS 393618 or ISIS 393619 and levels of endogenous SMN2 splice products were determined. The results are shown in Table 14.

Table 14

Effect on SMN2 Exon 7 Incorporation Following Low-Dose Treatment

ISIS #	Target Site	Length	% Inclusion Endogenous SMN2
393605	127	15	56
393606	126	15	58
390648	125	15	60
393607	124	15	60
393608	123	15	63
393609	122	15	53
393617	126	12	51
393618	125	12	51
393619	124	12	57
Control	N/A	N/A	49

As shown in Table 14, even at a very low dose, antisense compounds targeting intron 7 are effective at promoting inclusion of exon 7. Taken together, these results suggest the region near the 5' end of intron 7 (encompassing nucleotides 121-129 of SEQ ID NO: 1) contains an intronic splicing silencer.

Example 10 – Additional antisense compounds targeting intron 7.

Additional antisense oligonucleotides (ASOs) targeting intron 7 were synthesized. The ASOs are summarized in Table 15. All ASOs were full 2'-MOE. Internucleoside linkages are indicated in Table 15 (PO = phosphodiester, PS = phosphorothioate).

Table 15
ASOs targeted to intron 7 of SMN2

ISIS#	ASO sequence	target	Linkage	SEQ ID
396441	ACTTTCATAATGCTGGCA	8 to 25	PS	115
396442	CACTTTCATAATGCTGGC	9 to 26	PS	116
396443	TCACTTTCATAATGCTGG	10 to 27	PS	117
396444	TTCACTTTCATAATGCTG	11 to 28	PS	118

396449	TTTCATAATGCTGGC	9 to 23	PS	119
393603	ATTCACTTTCATAAT	15 to 29	PO	120
393604	TTCACTTTCATAATG	14 to 28	PO	121
393605	TCACTTTCATAATGC	13 to 27	PO	122
393606	CACTTTCATAATGCT	12 to 26	PO	123
393607	CTTTCATAATGCTGG	10 to 24	PO	124
393608	TTTCATAATGCTGGC	9 to 23	PO	125
393609	TTCATAATGCTGGCA	8 to 22	PO	126
393610	TCATAATGCTGGCAG	7 to 21	PO	127
393611	ATTCACTTTCAT	18 to 29	PO	128
393612	TTCACTTTCATA	17 to 28	PO	129
393613	TCACTTTCATAA	16 to 27	PO	130
393614	CACTTTCATAAT	15 to 26	PO	131
393615	ACTTTCATAATG	14 to 25	PO	132
393616	CTTTCATAATGC	13 to 24	PO	133
393617	TTTCATAATGCT	12 to 23	PO	134
393618	TTCATAATGCTG	11 to 22	PO	135
393619	TCATAATGCTGG	10 to 21	PO	136
399752	TGCATCTCATTGTAG	None[a]	PS	137

[a]ISIS 399752 is a scrambled control

The ASOs were tested for their ability to induce exon 7 inclusion in HEK293 cells as described above. Treatment with any of the above ASOs resulted in exon inclusion. Four 18mers (ISIS396443, ISISI396441, ISISI396442, and ISIS396444) demonstrated the strongest effect, with ISIS396443 slightly more active than the other three. ISIS396449 was the most effective 15mer.

Example 11 – Antisense compounds *in vivo*

Antisense compounds ISIS396443 and ISIS396449 were tested for their ability to induce exon 7 inclusion in transgenic mice expressing human SMN2. Thirty-two adult human-*SMN2*-transgenic mice, male or female, hemizygote or WT at the mouse *Smn* locus, were divided into four treatment groups: saline control, scrambled control oligo (ISIS399752), ISIS396449 (15mer), and ISIS396443 (18mer). ASO's were dissolved in 0.9% saline solution. Each ASO or saline control

was injected through the tail vein at a dose of 25 mg/kg, twice a week for each mouse. Two mice from each group were sacrificed after one week, two weeks, three weeks, and four weeks. Mouse tissues and organs, including liver, thigh muscles, kidney, and spinal cord, were snap-frozen in liquid N_2 and kept at -70 °C. For extraction of RNA samples, 0.1 g of mouse tissue was pulverized in liquid N_2 using mortar and pestle, and homogenized with 1 mL of Trizol (Invitrogen). Total RNA was then isolated according to the manufacturer's directions.

The greatest *hSMN2* exon 7 inclusion effect was observed in the liver, followed by kidney. A weak effect was observed in muscle and no effect was detected in spinal cord. These tissue-specific effects are consistent with previous reports that ASOs comprising 2'-MOE modified nucleosides preferentially distribute to peripheral tissues, and that hepatocytes spontaneously take up these compounds.

Example 12 – Activity in SMA type III mice

Two antisense compounds and one control compound were tested in a mouse model of SMA. The compounds are described in Table 16, below.

Table 16

Compounds Tested in Taiwan Strain SMA Mice

ISIS#	Sequence	Description	SEQ ID
396443	TCACTTTCATAATGCTGG	Uniform 2'-MOE, full PS; 18-mer; complementary to intron 7 of human SMN2	117
449220	ATTCACTTTCATAATGCTGG	Uniform 2-OMe; full PS; 20-mer; complementary to intron 7 of human SMN2	82
439272	TTAGTTTAATCACGCTCG	Uniform 2'-MOE; full PS; 18-mer; control sequence	138

Taiwan strain of SMA type III mice were obtained from The Jackson Laboratory (Bar Harbor, Maine). These mice lack mouse SMN and are homozygous for human SMN2 (mSMN -/-; hSMN2 +/+). These mice have been described in Hsieh-Li HM, et al., *Nature Genet.* 24, 66-70 2000.

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Mice were treated with 3, 10, 30, or 100 μg of ISIS396443 or ISIS449220 per day or with 30 or 100 μg of control compound ISIS439272 per day in phosphate buffered saline (PBS). Control mice were treated with PBS alone (dose of 0). All treatments were administered by intracerebroventricular (ICV) infusion using an Azlet 1007D osmotic pump. There were five animals for each dose, however, two of the mice from the highest dose of ISIS449220 died prior to completion of the study. Animals were sacrificed on day 9 (two days after final dose) and brain and lumbar sections of the spinal cords were collected from each animal. Real time PCR was performed on each sample to determine the amount of human SMN2 message including exon 7 ((+)exon 7) and the amount of human SMN2 message lacking exon 7((-)exon 7). Real time PCR was also performed to determine the expression levels of allograft inflammatory factor (AIF1) and glyceraldehyde 3-phosphate dehyrogenase (GADPH).

Expression levels for (+)exon 7 and (-)exon 7were normalized to GADPH levels. Those normalized expression levels were then divided by the GADPH-normalized levels from the PBS treated control mice. The resulting fold-control values are reported in Table 17, below. Data represent mean fold of control for all five mice in each group, except the highest dose of ISIS449220, which represent the 3 surviving mice.

Administration of ISIS396443 resulted in a striking increase in inclusion of exon 7. At 10 μg/day, ISIS396443 resulted in nearly twice as much (1.8 fold) exon 7 retained SMN2 message in brain, and in lumbar spinal cord it was more than twice as much compared to untreated control.

Table 17

Ability of Antisense Compounds to Alter Splicing in SMA Mice

Compound	Dose (μg/day)	Brain		Lumbar Cord	
		(+)exon 7	(-)exon 7	(+)exon 7	(-)exon 7
396443 (2'-MOE)	0	1.0	1.0	1.0	1.0
	3	1.3	1.0	1.4	1.0
	10	1.8	0.7	2.1	0.6
	30	2.4	0.6	3.4	0.3
	100	3.0	0.3	3.8	0.1

449220 (2'-OMe)	0	1.0	1.0	1.0	1.0
	3	0.9	1.1	1.0	1.1
	10	1.0	1.1	1.0	1.2
	30	1.0	1.2	1.1	1.2
	100*	1.0	1.0	1.2	1.1
439272 Control	0	1.0	1.0	1.0	1.0
	30	1.0	1.1	0.9	1.1
	100	1.0	1.0	1.0	1.0

* data from only 3 mice for this dose

Expression of allograft inflammatory factor (AIF1) was tested as a measure of inflammation. After normalization of all samples to (GADPH), the ratio of AIF1 for each treatment group was divided by the value for the PBS control. ISIS396443 resulted in no increase in AIF1, even at the highest dose. ISIS449220 resulted in increased AIF1 in both brain and lumbar spinal cord. Data in Table 18 represent mean fold of control for all five mice in each group, except the highest dose of ISIS449220, which represent the 3 surviving mice.

Table 18

Toxicity of antisense compounds in SMA Mice

Compound	Dose (μg/day)	AIF-1/GAPDH	
		Brain	Lumbar
396443 (2'-MOE)	0	1.0	1.0
	3	10	1.0
	10	1.1	1.2
	30	1.0	1.0
	100	0.9	1.0
449220 (2'-OMe)	0	1.0	1.0
	3	1.0	1.0
	10	1.0	1.8
	30	1.2	2.9
	100*	1.8	3.3

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439272 Control	0	0.9	0.9
	30	0.9	1.0
	100	0.9	1.2

* data from only 3 mice for this dose

CORE0084WO

What is claimed is:

1. An antisense oligonucleotide targeted to intron 7 of a nucleic acid molecule encoding SMN2, wherein the oligonucleotide is 16 to 19 linked nucleosides in length, and wherein each nucleoside comprises a 2'-O-methoxyethyl sugar modification.

2. The antisense oligonucleotide of claim 1 which is 16 nucleotides in length

3. The antisense oligonucleotide of claim 1 which is 17 nucleotides in length.

4. The antisense oligonucleotide of claim 1 which is 18 nucleotides in length.

5. The antisense oligonucleotide of claim 1 which is 19 nucleotides in length

6. The antisense oligonucleotide of claim 4 having the nucleobase sequence:

TCACTTTCATAATGCTGG.

7. The antisense oligonucleotide of any of claims 1-7 wherein at least one internucleoside linkage is a modified internucleoside linkage.

8. The antisense oligonucleotide of claim 7, wherein each internucleoside linkage is a modified internucleoside linkage.

9. The antisense oligonucleotide of claim 7 or 8, wherein each modified internucleoside linkage is a phosphorothioate linkage.

10. A method comprising contacting a cell with an antisense oligonucleotide according to any of claims 1-9.

11. A method of inducing inclusion of exon 7 of SMN2 in a cell comprising contacting the cell with an antisense oligonucleotide according to any of claims 1-9 and thereby inducing inclusion of exon 7 of SMN2 in the cell.

12. The method of claim 11 comprising detecting exon 7 inclusion of SMN2 in the cell.

13. The method of any of claims 10-12 wherein the cell is in an animal.

14. The method of claim 13 wherein the animal is a mammal.

15. The method of claim 13, wherein the animal is a mouse.

16. The method of claim 13, wherein the animal is a human.

17. A pharmaceutical composition comprising at least one antisense oligonucleotide according to any of claims 1-9 and a pharmaceutically acceptable carrier or diluent.

18. The pharmaceutical composition of claim 17, wherein the pharmaceutically acceptable carrier or diluent is sterile, pharmaceutical grade saline.

19. A method of administering a pharmaceutical composition according to any of claims 17-19 to an animal.

20. The method of claim 19, wherein the administering is by injection.

21. The method of claim 20, wherein the administering is by injection into the spinal column.

22. The method of claim 20, wherein the administering is by injection into the brain.

23. The method of any of claims 19-22, wherein the animal is a mouse.

24. The method of any of claims 19-22, wherein the animal is a human.

25. The method of any of claims 19-24, wherein the antisense oligonucleotide is co-administered with at least one other pharmaceutical agent.

26. The method of claim 25, wherein the antisense oligonucleotide and the other pharmaceutical agent are administered separately.

27. Use of an antisense oligonucleotide according to any of claims 1-9 for the preparation of a medicament for modulating splicing of an SMN2 pre-mRNA.

28. The use of claim 27 wherein modulating splicing results in an increase in exon 7 inclusion.

29. Use of an antisense oligonucleotide according to any of claims 1-9 for the preparation of a medicament for the treatment of spinal muscular atrophy.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 10/30940

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - C07H 21/00, C12P 19/34 (2010.01)
USPC - 536/24.5, 514/44A
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)
USPC: 536/24.5, 514/44A

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC: 536/23.1

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Electronic data bases searched: PubWEST (EPAB, PGPB, UPST, JPAB); GenCore Sequence Search (NT)
Search terms used: SMN1, SMN2, exon 7, spinal muscular atrophy, 2'-O-methoxyethyl sugar modification, antisense, pre-mRNA splicing
SEQ ID NO: 117

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2007/002390 A2 (BAKER et al.) 4 January 2007 (04.01.2007). Especially pg 14 para 3, pg 15 para 4, pg 34 claim 1, SEQ ID NO: 82.	1-8
A	KASHIMA et al. A negative element in SMN2 exon 7 inhibits splicing in spinal muscular atrophy. Nature Genetics, August 2003, Vol 34, No 4, Pages 460-463.	1-8

☐ Further documents are listed in the continuation of Box C. ☐

* 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	Date of mailing of the international search report
8 June 2010 (08.06.2010)	13 JUL 2010
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-3201	Authorized officer: Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (July 2009)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 10/30940

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 filed or furnished:

   a. (means)

   ☒ on paper

   ☐ in electronic form

   b. (time)

   ☒ in the international application as filed

   ☐ together with the international application in electronic form

   ☐ subsequently to this Authority for the purposes of search

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 in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

GenCore ver 6.3 SEQ ID NO: 117

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 10/30940

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.: 9-29
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:

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.:

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.