

## Antisense oligonucleotides targeting RAGE

### Field of the invention

**[0001]** The present invention relates to splice-switching antisense oligonucleotides that modulate alternative splicing of pre-mRNA coding for the Receptor for Advanced Glycation End-products (RAGE) or part thereof, and methods of treatment of RAGE-related disorders using said antisense oligonucleotides.

### Related application

**[0002]** This application claims the benefit of priority from Australian provisional application no. 2023903354 filed 20 October 2023, the entire disclosure of which is incorporated herein by reference.

### Background of the invention

**[0003]** The Receptor for Advanced Glycation End-products (RAGE) is a multivalent type I transmembrane glycoprotein belonging to the immunoglobulin (Ig) superfamily. The human RAGE (*Ager*) gene lies within the major histocompatibility complex class III region on chromosome 6. It comprises 11 exons and 10 introns, and a 5' flanking region that regulates its transcription. The transcribed RAGE mRNA is ~1.4 kb, with a short 3'UTR.

**[0004]** The 50-55 kDa glycosylated RAGE protein is constitutively expressed in a limited range of cells (e.g. vascular endothelium, type I pneumocytes, leukocytes), although RAGE expression may be induced in most cell types and tissues following injury, stress, hypoxia or inflammation, providing a conduit for pro-inflammatory and pro-proliferative signalling. RAGE expression is consequently upregulated in inflammatory and metabolic disorders including but not limited to neurodegenerative disease, cancer, cardiovascular disease, diabetes, autoimmune and ischaemic injury in which RAGE is also implicated in the development and progression.

**[0005]** Human RAGE is composed of an immunoglobulin-like ecto-domain, a single transmembrane domain and a short (42 amino acid) cytosolic tail. The ecto-domain (also known as the extracellular domain) of RAGE includes three immunoglobulin-like regions: an N-terminal V-type domain followed by two C-type domains (termed C and C' or alternatively C1 and C2).

**[0006]** Binding of Advanced Glycation End-products (AGEs) and non-AGE ligands to the ecto-domain of RAGE activates intracellular signal transduction cascades implicated in inflammation, injury and cell proliferation and differentiation. RAGE activation also triggers a positive feedback loop in which RAGE ligand-receptor interaction increases expression of RAGE via NFκB activation, thereby augmenting subsequent RAGE-induced cellular activation. In fact, the only means the inventors know to strongly downregulate RAGE expression is to reduce activation of RAGE. This situation contrasts with other receptors, in which increased levels of ligand decrease expression of the receptor.

**[0007]** In humans, the cytosolic tail of RAGE is 43 amino acids long (residue 362 to residue 404). This cytosolic tail contains motifs which are critical for RAGE-dependent cellular activation but not ligand binding. The cytosolic tail of RAGE may be trans-activated following activation of co-located G-protein coupled receptors by their cognate ligands without the binding of AGEs or non-AGE ligands to the RAGE ecto-domain (also known as ligand-independent activation of RAGE) leading to activation of the same pathways (Pickering et al. J Clin Invest 2019; 129: 406-421). RAGE ligand-independent activation of the RAGE cytosolic tail by certain co-located activated G-protein coupled receptors appears to be an important pathway by which RAGE is activated *in vivo*.

**[0008]** In both ligand-dependent activation of RAGE and ligand-independent activation of RAGE, intracellular signalling is mediated by the cytosolic domain of RAGE, which interacts with a range of signalling partners.

**[0009]** The alternative splicing of RAGE is also important for the regulation of RAGE activity, through the generation of RAGE isoforms that have an altered ability to be activated by ligand-dependent and ligand-independent signalling pathways. The alternative splicing of RAGE is altered in disease states including malignancy, diabetes and Alzheimer's disease.

**[0010]** Alternative splicing appears to be important for RAGE regulation/dysregulation, and splice modifying antisense oligonucleotides have been described that can promote alternative splicing.

**[0011]** However, there is a need for new and/or improved antisense oligonucleotides that more potently promote alternative splicing leading to the production of endogenous soluble RAGE and the reduction of membrane bound RAGE.

**[0012]** Reference to any prior art in the specification is not an acknowledgment or suggestion that this prior art forms part of the common general knowledge in any jurisdiction or that this prior art could reasonably be expected to be understood, regarded as relevant, and/or combined with other pieces of prior art by a skilled person in the art.

### Summary of the invention

**[0013]** The present invention is based on the design and production of antisense oligonucleotides that exhibit high potency for promoting alternative splicing of RAGE pre-mRNA (e.g. inclusion of exon 9b and/or the exclusion (e.g. skipping) of exon 10) resulting in increased production endogenous soluble RAGE (also known as “esRAGE”) and/or the reduction of membrane bound RAGE (also known as “mRAGE” or “full length RAGE”). These antisense oligonucleotides surprisingly promote significantly greater alternative splicing of RAGE pre-mRNA (e.g. inclusion of exon 9b and/or the exclusion (e.g. skipping) of exon 10) than existing antisense oligonucleotides.

**[0014]** Accordingly, the present invention provides an antisense oligonucleotide (AON) consisting of a nucleotide sequence of:

TGCCGCCTTTGCCACAAG (5'-3'; SEQ ID NO: 1); or

GCCGCCTTTGCCACAAGA (5'-3'; SEQ ID NO: 2).

**[0015]** Preferably, the antisense oligonucleotide comprises at least one modified nucleotide.

**[0016]** In any embodiment, the modified nucleotide has a modified internucleotide linkage, preferably a phosphorothioate linkage.

**[0017]** In any embodiment, the modified nucleotide has a modified sugar moiety or a sugar mimetic. Preferably the modified sugar moiety or sugar mimetic is selected from the group consisting of: morpholino, 2'-O-methyl (2'-OMe), and 2'-O-methoxyethyl (2'-MOE), 2',4' constrained 2'-O-methoxyethyl (cMOE), 2',4'-constrained 2'-O-Ethyl (cEt)

and locked nucleic acid (LNA). More preferably, the modified sugar moiety is 2'-O-methoxyethyl (2'-MOE).

**[0018]** In any embodiment, the modified nucleotide has both a modified internucleotide linkage and a modified sugar moiety or sugar mimetic. For example, where the modified internucleotide linkage is a phosphorothioate linkage and the modified sugar moiety is 2'-OMe, or the modified internucleotide linkage is phosphorothioate linkage and the modified sugar moiety is 2'-MOE.

**[0019]** In any embodiment, each nucleotide of the antisense oligonucleotide may comprise the same modified internucleotide linkage and/or modified sugar moiety.

**[0020]** In any embodiment, the modified nucleotide has a modified nucleoside base, preferably a C5-methylated cytosine.

**[0021]** In any embodiment, the 5' terminal end of the antisense oligonucleotide has a 5'-hydroxyl.

**[0022]** In any embodiment, the 3' terminal end of the antisense oligonucleotide has a 3'-hydroxyl.

**[0023]** In any embodiment, the antisense oligonucleotide is RNA, or each nucleotide in the antisense oligonucleotide is a ribonucleotide, preferably a modified ribonucleotide (e.g. wherein each ribose sugar and/or internucleotide linkage is modified).

**[0024]** In any embodiment, the Thymine (T) as shown in SEQ ID NO: 1 or 2 may be replaced with Uracil (U) and/or pseudouracil ( $\psi$ ).

**[0025]** In any embodiment, one or more of the Thymines (Ts) as shown in SEQ ID NO: 1 or 2 may be replaced with Uracil (U) and/or pseudouracil ( $\psi$ ).

**[0026]** In any embodiment, all the Ts as shown in SEQ ID NO:1 or 2 may be replaced with uracil (U).

**[0027]** In any embodiment, all Ts as shown in SEQ ID NO:1 or 2 may be replaced with pseudouracil ( $\psi$ ).

**[0028]** In any embodiment, one, two or three of the three contiguous Ts as shown in SEQ ID NO: 1 or 2 may be replaced with pseudouracil ( $\psi$ ).

**[0029]** In any embodiment, the Ts may be C5-methyl-U.

**[0030]** In one embodiment, each nucleotide of the antisense oligonucleotide is modified and comprises:

- a phosphorothioate linkage; and
- a 2'-*O*-methoxyethyl (2'-MOE) sugar moiety.

**[0031]** In one embodiment, each nucleotide of the antisense oligonucleotide is modified and comprises:

- a phosphorothioate linkage; and
- a 2'-*O*-methoxyethyl (2'-MOE) sugar moiety,

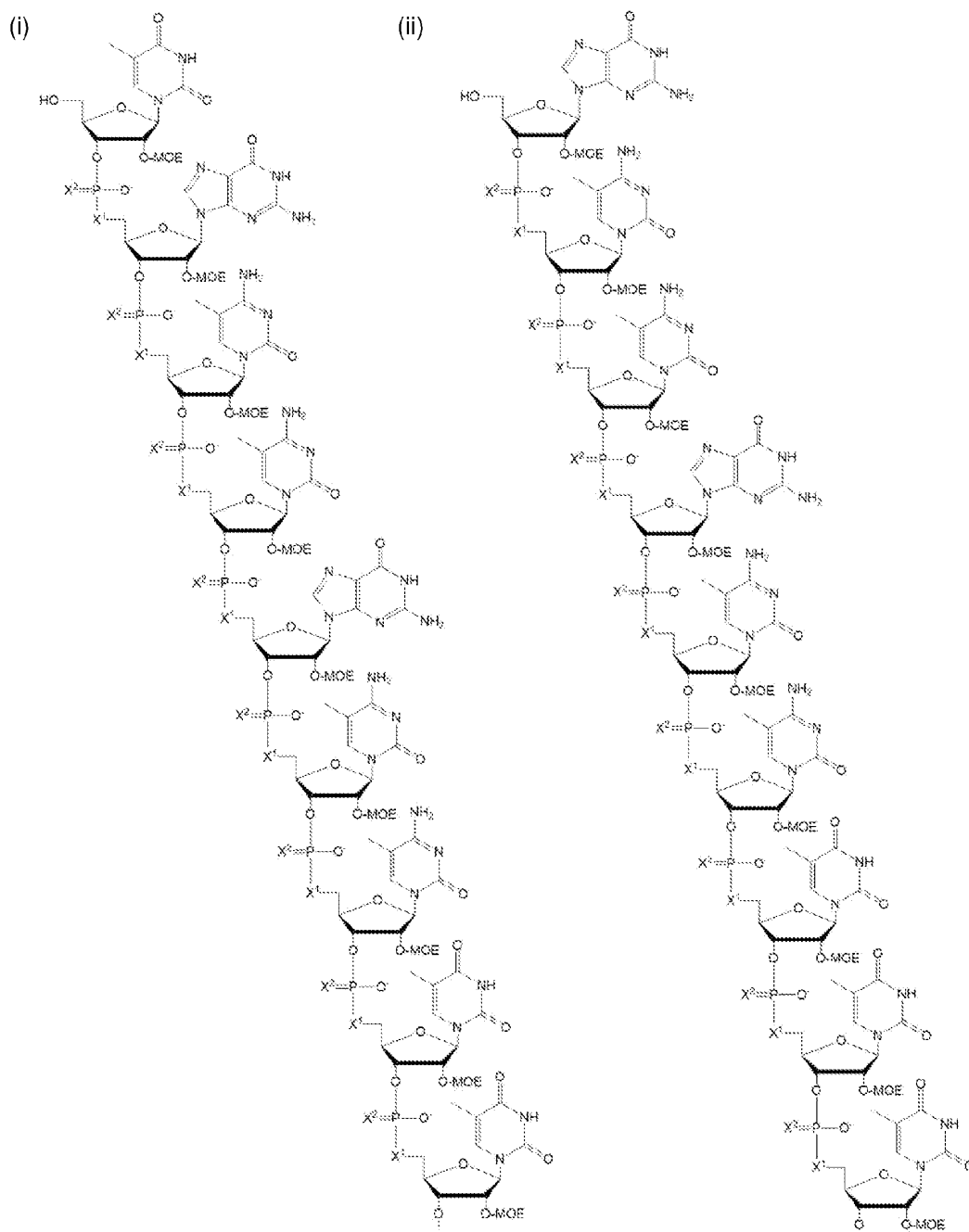
and wherein each cytosine of the antisense oligonucleotide is C5-methylated.

**[0032]** In one embodiment, each nucleotide of the antisense oligonucleotide is modified and comprises:

- a phosphorothioate linkage; and
- a 2'-*O*-methoxyethyl (2'-MOE) sugar moiety,

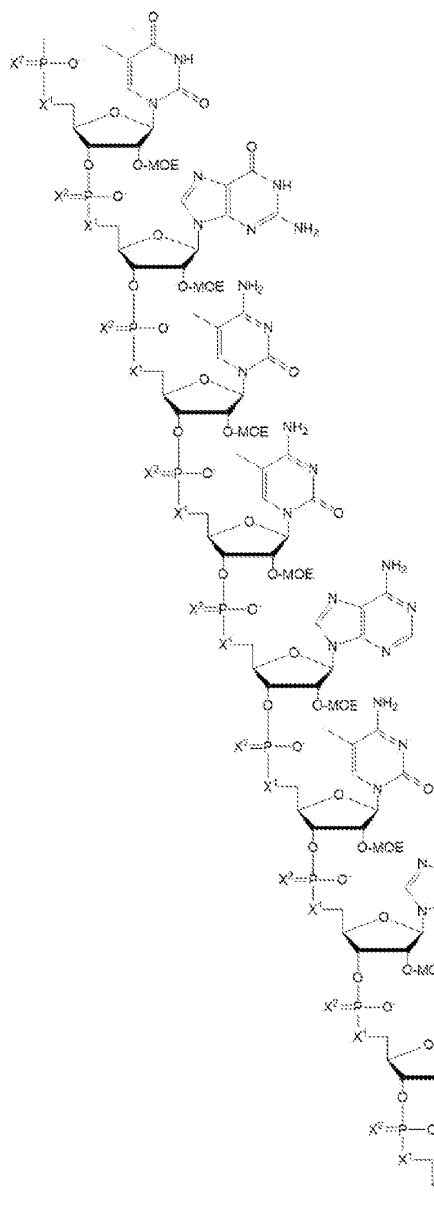
and wherein each cytosine of the antisense oligonucleotide is C5-methylated and each T is C5-methylated uracil.

**[0033]** In one embodiment, the antisense oligonucleotide may consist of one of the following structures:

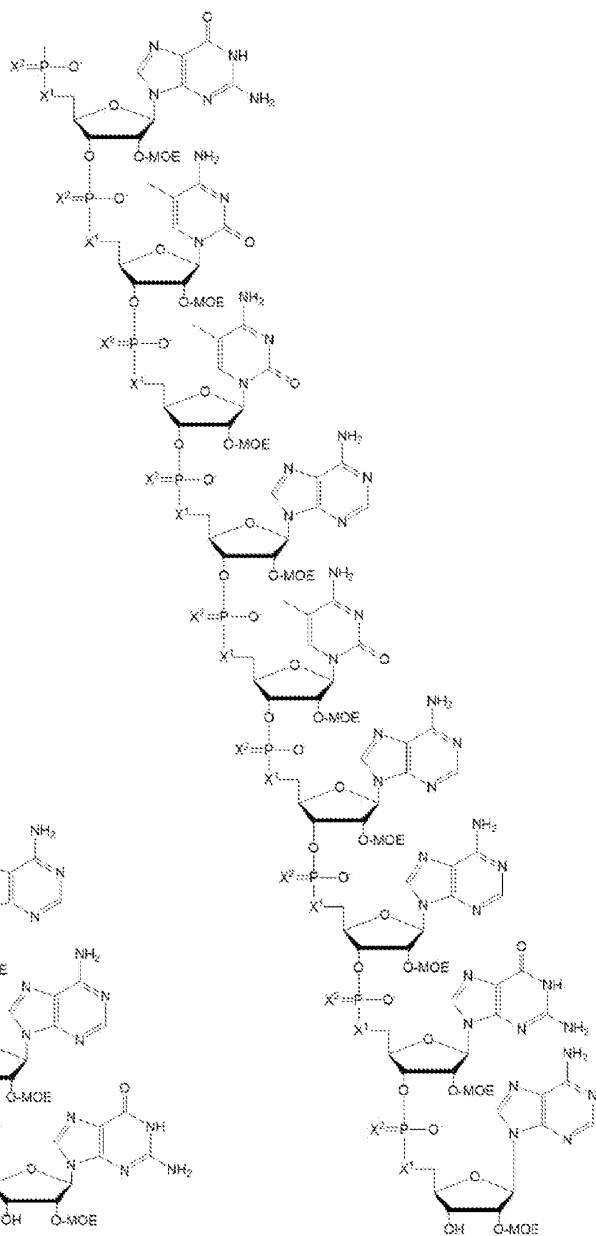


- continued

- (i) continued



- (ii) continued



wherein  $x^1$  is S and  $x^2$  is O, or  $x^1$  is O and  $x^2$  is S;

and MOE is 2-methoxyethyl.

**[0034]** In another aspect, the present invention provides a conjugate comprising an antisense oligonucleotide of the invention conjugated to a molecule. Such molecules

may enhance the delivery of the antisense oligonucleotide and include, for example, cell-penetrating peptides (CPPs). Preferably, the molecule is not an antisense oligonucleotide sufficiently complementary to a RAGE pre-mRNA sequence, such as exon 10 of RAGE pre-mRNA (*e.g* the molecule does not bind to exon 10 of RAGE pre-mRNA).

**[0035]** In one embodiment, the conjugate is a vivo-morpholinos (VMO) or peptide phosphorodiamidate morpholino oligomers (PPMO).

**[0036]** In one embodiment, the antisense oligonucleotide is chemically conjugated to the molecule.

**[0037]** In another aspect, the present invention provides a prodrug comprising an antisense oligonucleotide of the invention.

**[0038]** In one embodiment, the prodrug comprises an antisense oligonucleotide conjugated to a molecule. Preferably, the molecule is cleaved from the antisense oligonucleotide when administered to a subject.

**[0039]** In another aspect, the present invention provides a composition comprising an antisense oligonucleotide, a conjugate, or a prodrug of the invention.

**[0040]** In one embodiment, the composition comprises antisense oligonucleotides consisting of different oligonucleotide stereoisomers, preferably antisense oligonucleotides consisting of different oligonucleotide phosphorothioate stereoisomers.

**[0041]** In another embodiment, the composition comprises antisense oligonucleotides consisting of substantially a single oligonucleotide stereoisomer, preferably a single oligonucleotide phosphorothioate stereoisomer. In one embodiment, the composition comprises antisense oligonucleotides that are essentially a pure stereoisomer. In other words, all antisense oligonucleotides in the composition have the same stereoisomerisation, for example all antisense oligonucleotides have the same phosphorothioate stereoisomerisation.

**[0042]** In another aspect, the present invention provides a pharmaceutical composition comprising an antisense oligonucleotide, a conjugate or a prodrug of the invention, preferably also comprising a pharmaceutically or physiologically acceptable diluent, excipient or carrier.

**[0043]** In another aspect, the present invention provides a method for manipulating splicing in a RAGE pre-mRNA resulting in the skipping of exon 10, the method comprising contacting a cell with an antisense oligonucleotide, a conjugate or a prodrug of the invention, thereby manipulating splicing in a RAGE pre-mRNA resulting in the skipping of exon 10 in the cell.

**[0044]** In another aspect, the present invention provides a method for promoting the production of endogenous soluble RAGE in a subject, the method comprising administering an antisense oligonucleotide, a conjugate or a prodrug of the invention, thereby promoting the production of endogenous soluble RAGE in the subject.

**[0045]** In another aspect, the present invention provides a method for preventing or treating a disease associated with, or caused by, RAGE in a subject, the method comprising administering an antisense oligonucleotide, a conjugate or a prodrug of the invention, thereby preventing or treating a disease associated with, or caused by, RAGE in a subject.

**[0046]** In another aspect, the present invention provides a use of an antisense oligonucleotide, a conjugate or a prodrug of the invention in the manufacture of a medicament for promoting the production of endogenous soluble RAGE in a subject.

**[0047]** In another aspect, the present invention provides a use of an antisense oligonucleotide, a conjugate or a prodrug of the invention in the manufacture of a medicament for preventing or treating a disease associated with, or caused by, RAGE in a subject.

**[0048]** In another aspect, the present invention provides an antisense oligonucleotide, a conjugate or a prodrug of the invention for use in manipulating splicing of RAGE pre-mRNA in a cell resulting in the skipping of exon 10.

**[0049]** In another aspect, the present invention provides an antisense oligonucleotide, a conjugate or a prodrug of the invention for use in promoting the production of endogenous soluble RAGE in a subject.

**[0050]** In another aspect, the present invention provides an antisense oligonucleotide, a conjugate or a prodrug of the invention for use in preventing or treating a disease associated with, or caused by, RAGE in a subject.

**[0051]** In any aspect, an antisense oligonucleotide, a conjugate, a prodrug, method or use of the invention may promote the production of endogenous soluble RAGE in a subject, particularly the respiratory tract of the subject. In any embodiment, a method or use of an AON, a conjugate or a prodrug of the invention may promote the production of endogenous soluble RAGE in one or more tissues of the respiratory tract, for example one or more of the tissues of the respiratory tract described herein.

**[0052]** In any aspect, an antisense oligonucleotide, a conjugate, a prodrug, method or use of the invention may reduce the production of full-length RAGE (*i.e* mRAGE) in a subject, particularly in the respiratory tract of the subject. In any embodiment, a method or use of an AON, a conjugate or a prodrug of the invention may reduce the production of full-length RAGE in one or more tissues of the respiratory tract, for example one or more of the tissues of the respiratory tract described herein.

**[0053]** In any aspect, an antisense oligonucleotide, a conjugate, a prodrug, method or use of the invention may result in the retention of exon 9b.

**[0054]** In any aspect, the antisense oligonucleotide, the conjugate or the prodrug is administered to the total respiratory tract, the upper respiratory tract or the lower respiratory tract.

**[0055]** As used herein, the upper respiratory tract may include any one or more of the following regions: the nose and nasal passages, paranasal sinuses, the pharynx, and the portion of the larynx above the vocal folds (cords). Typically, the lower respiratory tract includes any one or more of the following regions: the portion of the larynx below the vocal folds, trachea, bronchi and bronchioles. The lungs can be included in the lower respiratory tract and include the respiratory bronchioles, alveolar ducts, alveolar sacs, and alveoli.

**[0056]** In any aspect, the antisense oligonucleotide, the conjugate or the prodrug is administered as an aerosol, a dry powder or as nasal drops. In any embodiment, the antisense oligonucleotide, the conjugate or the prodrug may be administered using a nasal spray pump, intranasal installation, intratracheal instillation, metered dose inhaler (MDI), dry powder inhaler (DPI), nebuliser (jet, ultrasonic mesh or vibrating mesh) or soft mist inhaler (SMI).

[0057] As used herein, except where the context requires otherwise, the term "comprise" and variations of the term, such as "comprising", "comprises" and "comprised", are not intended to exclude further additives, components, integers or steps.

[0058] Further aspects of the present invention and further embodiments of the aspects described in the preceding paragraphs will become apparent from the following description, given by way of example and with reference to the accompanying drawings.

#### **Brief description of the drawings**

[0059] **Figure 1. RAGE\_v1 mRNA expression in A549 cells following transfection with an antisense oligonucleotide targeting the ESE in exon 10 or control oligonucleotide.** A549 were transfected with control AON, AON h79 (79), AON 6643 (3), AON 6644 (4), AON 6645 (5), or AON 6646 (6) at various doses and incubated at 37°C for 48 hours. RAGE\_v1 mRNA levels (encoding esRAGE) were measured from cell lysates collected 48 hours following transfection with the AONs. Data shows mean  $\pm$  SEM; \*p<0.05 vs control oligonucleotide.

[0060] **Figure 2. RAGE mRNA expression in A549 cells following transfection with an antisense oligonucleotide targeting the ESE in exon 10 or control oligonucleotide.** A549 were transfected with control AON, AON h79 (79), AON 6643 (3), AON 6644 (4), AON 6645 (5), or AON 6646 (6) at various doses and incubated at 37°C for 48 hours. RAGE mRNA levels (encoding RAGE containing exon 10) were measured from cell lysates collected 48 hours following transfection with the AONs. Data shows mean  $\pm$  SEM; \*p<0.05 vs control oligonucleotide.

[0061] **Figure 3. RAGE\_v1 / exon 10 mRNA ratio in A549 cells following transfection with an antisense oligonucleotide targeting the ESE in exon 10 or control oligonucleotide.** A549 were transfected with control AON, AON h79 (79), AON 6643 (3), AON 6644 (4), AON 6645 (5), or AON 6646 (6) at various doses. RAGE mRNA levels (encoding esRAGE and RAGE containing exon 10) were measured from cell lysates collected 48 hours following transfection with the AONs. Data shows mean  $\pm$  SEM; \*p<0.05 vs control oligonucleotide.

[0062] **Figure 4. AON 6777 and AON 6745 are potent inducers of RAGE\_v1 mRNA expression in primary SAEC cells.** Primary SAEC cells were transfected with

control AON, AON 6776, AON 6777, AON 6645, AON 6778 and AON 6779 at various doses. RAGE mRNA levels (encoding esRAGE) were measured from cell lysates collected 48 hours following transfection with the AONs. Data shows mean  $\pm$  SEM; \* $p < 0.05$  vs control oligonucleotide.

**[0063] Figure 5. AON 6777 and AON 6745 increases RAGE\_v1 mRNA expression in D-SAEC #1, D-SAEC #2384 and D-SAEC #36139 cells.** Primary SAEC cells were transfected with control AON, AON 6776, AON 6777, AON 6645, AON 6778 and AON 6779 at various doses. RAGE mRNA levels (encoding esRAGE) measured from cell lysates collected 48 hours following transfection with the AONs. Data shows mean  $\pm$  SEM; \* $p < 0.05$  vs control oligonucleotide.

#### **Detailed description of the embodiments**

**[0064]** Reference will now be made in detail to certain embodiments of the invention. While the invention will be described in conjunction with the embodiments, it will be understood that the intention is not to limit the invention to those embodiments. On the contrary, the invention is intended to cover all alternatives, modifications, and equivalents, which may be included within the scope of the present invention as defined by the claims.

**[0065]** One skilled in the art will recognise many methods and materials similar or equivalent to those described herein, which could be used in the practice of the present invention. The present invention is in no way limited to the methods and materials described.

**[0066]** It will be understood that the invention disclosed and defined in this specification extends to all alternative combinations of two or more of the individual features mentioned or evident from the text or drawings. All of these different combinations constitute various alternative aspects of the invention.

**[0067]** All of the patents and publications referred to herein are incorporated by reference in their entirety.

**[0068]** Unless defined otherwise, technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs.

**[0069]** The oligonucleotides of the invention may also exist as tautomeric isomers or stereoisomers (such as enantiomers or diastereomers) in certain cases. Although only one resonance structure or stereoisomer may be depicted, all such forms are contemplated within the scope of the invention. For example, olo/ono tautomers or different stereoisomers can exist for each independent phosphorothioate group in the nucleotides of the invention and all their possible tautomeric forms or stereoisomers are within the scope of the invention. For example, all phosphorothioate tautomers/stereoisomers comprising either P=S or P=O bonds are contemplated.

**[0070]** For purposes of interpreting this specification, the following definitions will generally apply and whenever appropriate, terms used in the singular will also include the plural and vice versa.

**[0071]** As used herein, the term “and/or”, e.g., “X and/or Y” will be understood to mean either “X and Y” or “X or Y” and shall be taken to provide explicit support for both meanings or for either meaning.

**[0072]** The articles “a” and “an” are used herein to refer to one or to more than one (i.e. to at least one) of the grammatical object of the article.

**[0073]** Stereochemical definitions and conventions used herein generally follow S. P. Parker, Ed., *McGraw-Hill Dictionary of Chemical Terms* (1984) McGraw-Hill Book Company, New York; and Eliel, E. and Wilen, S., “Stereochemistry of Organic Compounds”, John Wiley & Sons, Inc., New York, 1994. The oligonucleotides of the invention may contain asymmetric or chiral centers, and therefore exist in different stereoisomeric forms. The term “stereoisomers” refers to oligonucleotides which have identical chemical constitution, but differ with regard to the arrangement of the atoms or groups in space. As used herein, the term “stereoisomer” includes but is not limited to diastereomers, enantiomers and atropisomers, as well as mixtures thereof such as racemic mixtures.

**[0074]** The inventors have surprisingly identified that oligonucleotides having specific sequences, and in some embodiments specific chemistry, display significantly greater potency in promoting alternative splicing resulting in an increase in RAGE\_v1 mRNA and esRAGE production, as well as a reduction in RAGE mRNA containing exon 10.

These two oligonucleotides drive the greatest shift in the ratio of RAGE\_v1 mRNA / RAGE mRNA containing exon 10 compared to all oligonucleotides tested.

**[0075]** The oligonucleotides of the invention, unexpectedly, are more potent than any other oligonucleotides tested including those that have very similar sequences. It was therefore surprising that oligonucleotides with these 2 sequences would exhibit the observed level of potency.

**[0076]** Without being bound by theory or mode of action, the inventors believe that the oligonucleotides of the invention have reduced self-annealing properties compared to other oligonucleotides targeting exon 10 of RAGE pre-mRNA. Furthermore, the inventors believe that these lead oligonucleotides specifically target and disrupt the duplex stem of the stem-loop of RAGE pre-mRNA, thereby disrupting the SRSF binding site on pre-mRNA that regulates exon 10 splicing. In addition, the inventors believe the positioning of the lead oligonucleotides on RAGE pre-mRNA not only disrupts the duplex of the stem-loop but also directly competes with SRSF or splicing factors for binding to the same site of the pre-mRNA.

#### **RAGE pre-mRNA alternate splicing**

**[0077]** Alternative splicing is recognized as an important layer of post-transcriptional gene regulation for the Receptor for Advanced Glycation End-products (RAGE). Although most RAGE is expressed in its full length isoform, a number of different coding isoforms are generated through alternative splicing (also known as splicing), including splicing forms with N-terminal truncations, C-terminal truncations, and splicing forms retaining intronic sequences. These different splicing forms may act as possible regulators of the full-length RAGE receptor either by competitive ligand binding or by displacing the full-length protein from binding partners. Over twenty splicing forms have been identified in different tissues such as lung, liver, kidney, smooth muscle, endothelial cells and brain.

**[0078]** The different RAGE gene splice variants have been named RAGE, RAGE\_v1 to RAGE\_v19 according to the Human Gene Nomenclature Committee and are described in Hudson et al., (2008) The FASEB Journal, 22: 1572-1580, the contents of which are incorporated in its entirety. For example, (run on) retention of intron 9 (exon 9b) results in a premature stop and the complete loss of the trans-membrane and

cytoplasmic domains generating a C-terminus truncated splicing form. For example, RAGE\_v1 is C-terminus truncated splicing form generated by skipping of exon 10 and retention of intron 9 (exon 9b). Alternatively, in another example, an antisense oligonucleotide as described herein may increase RAGE\_v10 mRNA levels in a tissue or sample by promoting splicing in the RAGE pre-mRNA that results in the skipping of exon 10 and exon 11.

**[0079]** The C-terminus truncated splicing forms as described herein lack any signalling elements or the transmembrane domain and translate a C-terminus truncated RAGE, such as endogenous secretory RAGE (esRAGE), that is able to act as a decoy receptor, competing with full length RAGE for ligands or increasing ligand clearance. In particular, esRAGE constitutes ~5% of circulating RAGE in humans.

**[0080]** Higher circulating levels of esRAGE are associated with improved health outcomes and longevity while lower esRAGE is associated with many disease states including but not limited to atherosclerosis, diabetes, the metabolic syndrome, cardiovascular mortality, anaemia, autism and various tumorigenic states. Treatment of diabetic mice with recombinant esRAGE reduces atherosclerosis, vascular inflammation, renal and retinal damage.

**[0081]** Aberrant splicing of RAGE (and therefore dysfunctional RAGE signalling) has been reported in diabetes, some cancers and Alzheimer's disease.

**[0082]** The skipping of exon 10 in esRAGE-type splicing is ascribed to the limitation of intron length in higher eukaryotes. Roughly 45 nucleotides must separate the 5' splice site and branch point, and the minimum distance between the branch point and 3' splice site appears to be approximately 18 nucleotides, respectively. Therefore, introns shorter than 70 nucleotides are extremely rare in mammals and cannot be spliced out efficiently. When the esRAGE 5' splice site in intron 9 is selected, the distance between this site and the 3' splice site that borders exon 10 is 46 nucleotides, which is considerably shorter than the lower limit of the intron length. Therefore, the use of the downstream, esRAGE 5' splice site of intron 9 and the inclusion of exon 10 would be mutually exclusive. Among the known splice variants analyzed, all variants that used the downstream esRAGE 5' splice site in intron 9 skipped exon 10; in contrast, all variants that used the upstream RAGE 5' splice site in intron 9 included exon 10. Thus, the

available evidence indicates that the selection of either one of the two alternative 5' splice sites in intron 9 couples with inclusion or exclusion of exon 10.

**[0083]** RAGE pre-mRNA comprises an exon splicing enhancer (ESE) at residues 89 to 94. Without being bound by theory, the inventors believe that the nucleotide sequences 5'-adjacent to the ESE form a stem-loop in the secondary structure of the RAGE pre-mRNA.

#### **Antisense Oligonucleotides (AONs)**

**[0084]** Antisense oligonucleotide (AON) are short, synthetic, antisense, modified strands of DNA or RNA that can selectively hybridise to pre-RNA/mRNA through Watson–Crick base pairing to form an oligonucleotide:RNA heteroduplex within the target sequence and selectively modulate the function of the target RNA.

**[0085]** The terms “AON”, “ASO”, “oligomer” and “antisense compound” may be used interchangeably to refer to an antisense oligonucleotide.

**[0086]** When AONs are used to modulate alternative splicing of mRNA, they are often referred to as splice-switching oligonucleotides (SSO). In the present invention, the terms AON and SSO may be used interchangeably. SSOs base-pair with a pre-mRNA and disrupt the normal splicing repertoire of the transcript by blocking the RNA–RNA base-pairing or protein–RNA binding interactions that occur between components of the splicing machinery and the pre-mRNA. SSOs can induce “skipping” of selected exons and/or retention of intronic sequences to modulate the product of translation. This can be achieved by targeting splice sites directly or by targeting cis-acting sequences involved in enhancing or silencing splicing by modulating binding of specific proteins or altering secondary structure of the pre mRNA.

**[0087]** Therapeutic SSOs may be used for the treatment of genetic disorders, to skip faulty or misaligned sections allowing for the generation of internally deleted, but now functional protein as a therapy.

**[0088]** The AONs of the present invention selectively manipulate the alternative splicing pattern of RAGE pre-mRNA, resulting in the generation of either natural RAGE mRNA spliciforms that are either non-functional or that act as a decoy receptor to

antagonise ligand dependent activation and ligand-independent transactivation of full length RAGE.

**[0089]** Notably, there are no common RAGE polymorphisms at these splice sites. The RAGE sequence is highly conserved. Therefore, personalisation or individualised sequence modification is not required, unlike the management of genetic disorders with exon skipping technologies.

**[0090]** According to any aspect of the invention, the AONs as described herein are capable of binding to a selected target on a RAGE gene transcript to modulate pre-mRNA splicing in a RAGE gene transcript or part thereof. Broadly, the AON may be an isolated or purified AON. By “isolated” is meant material that is substantially or essentially free from components that normally accompany it in its native state. For example, an “isolated polynucleotide” or “isolated oligonucleotide,” as used herein, may refer to a polynucleotide that has been purified or removed from the sequences that flank it in a naturally-occurring state, e.g., a DNA fragment that is removed from the sequences that are adjacent to the fragment in the genome. The term “isolating” as it relates to cells, refers to the purification of cells (e.g., fibroblasts, lymphoblasts) from a source subject (e.g., a subject with a polynucleotide repeat disease). In the context of DNA, mRNA or protein, “isolating” refers to the recovery of the DNA, mRNA or protein from a source, e.g., cells.

**[0091]** According to any aspect of the invention, the AONs as described herein promote splicing in the RAGE pre-mRNA resulting in the inclusion of exon 9b and/or exclusion (eg skipping) of exon 10, resulting in a premature stop and the complete loss of the trans-membrane and cytoplasmic domains. Therefore, the AONs as described herein may increase the level of RAGE\_v1, RAGE\_v6, RAGE\_v8, RAGE\_v9, RAGE\_v10, RAGE\_v15, RAGE\_v18, and RAGE\_v19 mRNA, preferably an increase in the level of RAGE\_v1, RAGE\_v6, RAGE\_v8, RAGE\_v9, RAGE\_v10, RAGE\_v15, RAGE\_v18, and RAGE\_v19 mRNA in a tissue or sample. For example, an AON as described herein may increase RAGE\_v1 mRNA levels in a tissue or sample by promoting splicing in the RAGE pre-mRNA that results in the skipping of exon 10 and retention of intron 9 (exon 9b). In another example, an AON as described herein may increase RAGE\_v10 mRNA levels in a tissue or sample by promoting splicing in the RAGE pre-mRNA that results in the skipping of exon 10 and exon 11.

**[0092]** An AON can be said to be “directed to” or “targeted against” a target sequence with which it hybridizes.

**[0093]** The term “modulate” or “modulates” includes to “increase” or “decrease” one or more quantifiable parameters, optionally by a defined and/or statistically significant amount. The terms “increase” or “increasing,” “enhance” or “enhancing,” or “stimulate” or “stimulating” refer generally to the ability of an AON or compositions to produce or cause a greater physiological response (i.e., downstream effects) in a cell or a subject relative to the response caused by either no AON or a control compound. The terms “decreasing” or “decrease” refer generally to the ability of an AON or compositions to produce or cause a reduced physiological response (i.e., downstream effects) in a cell or a subject relative to the response caused by either no AON or a control compound.

**[0094]** Relevant physiological or cellular responses (*in vivo* or *in vitro*) will be apparent to persons skilled in the art, and may include increases in the expression of specific exons in a RAGE-coding pre-mRNA, decreases in the amount of RAGE-coding pre-mRNA or decreases in the expression of functional RAGE protein in a cell, tissue, or subject in need thereof. An “increased” or “enhanced” amount is typically a statistically significant amount compared to the amount produced by no AON (the absence of an agent) or a control compound. The term “reduce” or “inhibit” may relate generally to the ability of one or more AONs or compositions to “decrease” a relevant physiological or cellular response, such as a symptom of a disease or condition described herein. Relevant physiological or cellular responses (*in vivo* or *in vitro*) will be apparent to persons skilled in the art, and may include reductions in the symptoms or pathology of a disease such as cancer, neurodegenerative diseases, lung disorders, and other inflammatory diseases. A “decrease” in a response may be statistically significant as compared to the response produced by no AON or a control composition.

**[0095]** The cyclic subunits may be based on ribose or another pentose sugar or, in certain embodiments, a morpholino group (see description of morpholino oligonucleotides below). Also contemplated are peptide nucleic acids (PNAs), locked nucleic acids (LNAs), and 2'-O-Methyl oligonucleotides, 2'-O-Methoxyethyl oligonucleotides, among other antisense agents known in the art.

**[0096]** Included are non-naturally-occurring AONs, or “oligonucleotide analogs”, including AONs or oligonucleotides having (i) a modified backbone structure, e.g., a

backbone other than the standard phosphodiester linkage found in naturally-occurring oligo- and polynucleotides, and/or (ii) modified sugar moieties or sugar mimetics, e.g., morpholino moieties rather than ribose or deoxyribose moieties. Oligonucleotide analogs support bases capable of hydrogen bonding by Watson-Crick base pairing to standard polynucleotide bases, where the analog backbone presents the bases in a manner to permit such hydrogen bonding in a sequence-specific fashion between the oligonucleotide analog molecule and bases in a standard polynucleotide (e.g., single-stranded RNA or single-stranded DNA). Preferred analogs are those having a substantially uncharged, phosphorus containing backbone.

**[0097]** One method for producing AONs is the methylation of the 2' hydroxyribose position and the incorporation of a phosphorothioate backbone produces molecules that superficially resemble RNA but that are much more resistant to nuclease degradation, although persons skilled in the art of the invention will be aware of other forms of suitable backbones that may be useable in the objectives of the methods of the invention.

**[0098]** Increased splice-switching may also be achieved with alternative oligonucleotide chemistry. For example, the AON may be chosen from the list comprising: phosphoramidate or phosphorodiamidate morpholino oligomer (PMO); PMO-X; PPMO; peptide nucleic acid (PNA); a locked nucleic acid (LNA) and derivatives including alpha-L-LNA, 2'-amino LNA, 4'-methyl LNA and 4'-O-methyl LNA; ethylene bridged nucleic acids (ENA) and their derivatives; phosphorothioate oligomer; tricyclo-DNA oligomer (tcDNA); tricyclophosphorothioate oligomer; 2'-O-Methyl-modified oligomer (2'-OMe); 2',4' constrained 2'-O-methoxyethyl (cMOE); 2',4' constrained 2'-O-Ethyl (cEt); 2'-O-methoxy ethyl (2'-MOE); 2'-fluoro, 2'-fluroarabino (FANA); unlocked nucleic acid (UNA); thermostable twisted intercalating nucleic acid (TINA), hexitol nucleic acid (HNA); cyclohexenyl nucleic acid (CeNA); 2'-amino (2'-NH<sub>2</sub>); 2'-O-ethyleneamine or any combination of the foregoing as mixmers or as gapmers.

**[0099]** To further improve the delivery efficacy, the above mentioned modified nucleotides are often conjugated with fatty acids/lipid/cholesterol/amino acids/carbohydrates/polysaccharides/nanoparticles etc. to the sugar or nucleobase moieties. These conjugated nucleotide derivatives can also be used to construct exon skipping AONs.

**[0100]** Conjugate groups may be added to the antisense oligonucleotide to modify one or more properties of the attached oligonucleotide, including but not limited to pharmacodynamics, pharmacokinetics, stability, binding, absorption, tissue distribution, cellular distribution, cellular uptake, charge and clearance. In certain embodiments, conjugate groups impart a new property on the attached oligonucleotide, e.g., fluorophores or reporter groups that enable detection of the oligonucleotide.

**[0101]** Antisense oligonucleotide-induced splice modification of the human RAGE gene transcripts have generally used either oligoribonucleotides, PNAs, 2'-OMe or MOE modified bases on a phosphorothioate backbone. When alternative chemistries are used to generate the AONs for use in the methods of the invention, the thymine (T) of the sequences provided herein may be replaced by a uracil (U) and/or pseudouracil ( $\psi$ ).

**[0102]** Preferred modifications for the AONs described herein and as exemplified by the oligonucleotides described in the Examples described herein, include phosphorothioate internucleotides linkages, 2'-O-methoxyethyl (2'-MOE; also known as 2'-O-MOE and 2'-O-(2-methoxyethyl)) sugar moieties, C5-methylation of cytosine residues (also referred to as 5-methylcytosine, C5-methyl cytosine or C5-methyl-C), and T that is C5-methylated uracil (also referred to as 5-methyluracil, C5-methyl uracil or C5-methyl-U).

**[0103]** Each internucleotide linkage may contain asymmetric or chiral centres and therefore exist in different stereoisomeric forms. Compositions of the invention may comprise a mixture of different oligonucleotide stereoisomers. For example, an oligonucleotide of the invention comprising phosphorothioate internucleotide linkages may comprise different oligonucleotide phosphorothioate stereoisomers, due to the chirality introduced by the two sulfur atoms in the PS internucleotide linkages. The compositions of the invention may comprise oligonucleotides as described herein consisting of substantially a single phosphorothioate stereoisomer. In other words, each oligonucleotide of the composition may comprise the same phosphorothioate stereochemistry. These compositions may be free, or essentially free, of any other oligonucleotides consisting of a different phosphorothioate stereoisomer, ie the oligonucleotides are in pure stereoisomer composition. The oligonucleotide may comprise additional chiral centres, for example on one or more ribose sugars, and therefore may comprise mixtures of, or single, stereoisomers at the additional chiral centres.

**[0104]** Antisense oligonucleotides that do not activate RNase H can be made in accordance with known techniques (see, e.g., U.S. Pat. 5,149,797). Such AONs, which may be deoxyribonucleotide or ribonucleotide sequences, simply contain any structural modification which sterically hinders or prevents binding of RNase H to a duplex molecule containing the oligomer as one member thereof, which structural modification does not substantially hinder or disrupt duplex formation. Because the portions of the oligomer involved in duplex formation are substantially different from those portions involved in RNase H binding thereto, numerous AONs that do not activate RNase H are available. For example, such AONs may be oligomers wherein at least one, or all, of the inter-nucleotide bridging phosphate residues are modified phosphates, such as methyl phosphonates, methyl phosphorothioates, phosphoromorpholidates, phosphoropiperazidates boranophosphates, amide linkages and phosphoramidates. For example, every other one of the internucleotide bridging phosphate residues may be modified as described. In another non-limiting example, such AONs are molecules wherein at least one, or all, of the nucleotides contain a 2' lower alkyl moiety (such as, for example, C<sub>1</sub>-C<sub>4</sub>, linear or branched, saturated or unsaturated alkyl, such as methyl, ethyl, ethenyl, propyl, 1-propenyl, 2-propenyl, and isopropyl). For example, every other one of the nucleotides may be modified as described.

**[0105]** Specific examples of alternate AONs of the present invention include oligomers containing modified backbones or non-natural inter-nucleoside linkages. As defined in this specification, oligomers having modified backbones include those that retain a phosphorus atom in the backbone and those that do not have a phosphorus atom in the backbone. For the purposes of this specification, and as sometimes referenced in the art, modified oligomers that do not have a phosphorus atom in their inter-nucleoside backbone can also be considered to be AONs.

**[0106]** In other alternate oligomer mimetics, both the sugar and the inter-nucleoside linkage, i.e., the backbone, of the nucleotide units are replaced with novel groups. The base units are maintained for hybridization with an appropriate nucleic acid target compound. One such oligomeric compound, an oligomer mimetic that has been shown to have excellent hybridization properties, is referred to as a peptide nucleic acid (PNA). In PNA compounds, the sugar-backbone of an oligomer is replaced with an amide containing backbone, in particular an aminoethylglycine backbone. The nucleo-bases

are retained and are bound directly or indirectly to aza nitrogen atoms of the amide portion of the backbone.

**[0107]** Another alternate chemistry is the phosphorodiamidate morpholino oligomer (PMO) oligomeric compounds, which are not degraded by any known nuclease or protease. These compounds are uncharged, do not activate RNase H activity when bound to a RNA strand and have been shown to exert sustained splice modulation after *in vivo* administration (Summerton and Weller, *Antisense Nucleic Acid Drug Development*, 7, 187-197).

**[0108]** Modified oligomers may also contain one or more substituted sugar moieties. Oligomers may also include nucleobase (often referred to in the art simply as "base") modifications or substitutions. Certain nucleobases are particularly useful for increasing the binding affinity of the oligomeric compounds as described herein. These include 5-substituted pyrimidines, 6-azapyrimidines, and N-2, N-6 and O-6 substituted purines, including 2-aminopropyladenine, 5-propynyluracil and 5-propynylcytosine. 5-methylcytosine substitutions have been shown to increase nucleic acid duplex stability by 0.6-1.2°C, even more particularly when combined with 2'-O-methoxyethyl sugar modifications.

**[0109]** The activity of AONs and variants thereof can be assayed according to routine techniques in the art. For example, splice forms and expression levels of surveyed RNAs and proteins may be assessed by any of a wide variety of well-known methods for detecting splice forms and/or expression of a transcribed nucleic acid or protein. Non-limiting examples of such methods include RT-PCR of spliced forms of RNA followed by size separation of PCR products, nucleic acid hybridization methods e.g., Northern blots and/or use of nucleic acid arrays; nucleic acid amplification methods; immunological methods for detection of proteins; protein purification methods; and protein function or activity assays.

**[0110]** RNA expression levels can be assessed by preparing mRNA/cDNA (i.e., a transcribed polynucleotide) from a cell, tissue or organism, and by hybridizing the mRNA/cDNA with a reference polynucleotide, which is a complement of the assayed nucleic acid, or a fragment thereof. cDNA can, optionally, be amplified using any of a variety of polymerase chain reaction or *in vitro* transcription methods prior to hybridization with the complementary polynucleotide; preferably, it is not amplified.

Expression of one or more transcripts can also be detected using quantitative PCR to assess the level of expression of the transcript(s).

**[0111]** The AONs of the present invention may be conveniently 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, Calif.). One method for synthesising oligomers on a modified solid support is described in U.S. Pat. No. 4,458,066.

**[0112]** 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 oligomers such as the phosphorothioates and alkylated derivatives. In one such automated embodiment, diethyl-phosphoramidites are used as starting materials and may be synthesized as described by Beaucage, et al., (1981) Tetrahedron Letters, 22:1859-1862.

**[0113]** In one embodiment, the AONs of the present invention are synthesised *in vitro* and do not include antisense compositions of biological origin, or genetic vector constructs designed to direct the *in vivo* synthesis of AONs.

#### **Administration, dosage and formulation**

**[0114]** In any aspect, the methods of the present invention may be used to prevent or treat a disease associated with, or caused by, RAGE in a subject. Preferably, the disease associated with, or caused by, RAGE is a respiratory (pulmonary) disorder or disease and is selected from the group: Acute upper respiratory infections, rhinitis, nasopharyngitis, sinusitis, laryngitis, influenza and pneumonia, acute bronchitis, acute bronchiolitis, asthma, chronic obstructive pulmonary disease (COPD), bronchiectasis, emphysema, chronic lung diseases due to external agents, Acute Respiratory Distress Syndrome (ARDS), pulmonary eosinophilia, and pleuritic, lung trauma and recovery from lung injury, trauma and/or surgery.

**[0115]** The term 'respiratory' refers to the process by which oxygen is taken into the body and carbon dioxide is discharged, through the bodily system including the nose, throat, larynx, trachea, bronchi and lungs.

**[0116]** As used herein, the upper respiratory tract may include the following regions: nose and nasal passages, paranasal sinuses, the pharynx, and the portion of the larynx above the vocal folds (cords). Typically, the lower respiratory tract includes any one of more of the following regions: portion of the larynx below the vocal folds, trachea, bronchi and bronchioles. The lungs can be included in the lower respiratory tract and include the respiratory bronchioles, alveolar ducts, alveolar sacs, and alveoli.

**[0117]** The term 'respiratory disease' or 'respiratory condition' refers to any one of several ailments that involve inflammation and affect a component of the respiratory system including the upper (including the nasal cavity, pharynx and larynx) and lower respiratory tract (including trachea, bronchi and lungs).

**[0118]** A symptom of respiratory disease may include cough, excess sputum production, a sense of breathlessness or chest tightness with audible wheeze. Exercise capacity may be quite limited. In asthma the FEV1.0 (forced expiratory volume in one second) as a percentage of that predicted nomographically based on weight, height and age, may be decreased as may the peak expiratory flow rate in a forced expiration. In COPD the FEV1.0 as a ratio of the FVC is typically reduced to less than 0.7. The impact of each of these conditions may also be measured by days of lost work/school, disturbed sleep, requirement for bronchodilator drugs, requirement for glucocorticoids including oral glucocorticoids.

**[0119]** The existence of, improvement in, treatment of or prevention of a respiratory disease may be determined by any clinically or biochemically relevant method of the subject or a biopsy therefrom. For example, a parameter measured may be the presence or degree of lung function, signs and symptoms of obstruction; exercise tolerance; night time awakenings; days lost to school or work; bronchodilator usage; inhaled corticosteroid (ICS) dose; oral (glucocorticoid) GC usage; need for other medications; need for medical treatment; hospital admission.

**[0120]** The terms "treatment" or "treating" of a subject includes the application or administration of an antisense oligonucleotide, a conjugate or a prodrug as described herein with the purpose of delaying, slowing, stabilizing, curing, healing, alleviating, relieving, altering, remedying, less worsening, ameliorating, improving, or affecting the disease or condition, the symptom of the disease or condition, or the risk of (or susceptibility to) the disease or condition. The term "treating" refers to any indication of

success in the treatment or amelioration of an injury, pathology or condition, including any objective or subjective parameter such as abatement; remission; lessening of the rate of worsening; lessening severity of the disease; stabilization, diminishing of symptoms or making the injury, pathology or condition more tolerable to the subject; slowing in the rate of degeneration or decline; making the final point of degeneration less debilitating; or improving a subject's physical or mental well-being.

**[0121]** The antisense oligonucleotides, the conjugates or the prodrugs as described herein may be adapted to aid in the prophylactic or therapeutic treatment, prevention or amelioration of symptoms of a disease such as a RAGE expression related disease or pathology in a form suitable for delivery to a patient.

**[0122]** A positive response to therapy may also be prevention or attenuation of worsening of respiratory symptoms, e.g. asthma symptoms (exacerbation), following a respiratory virus infection. This could be assessed by comparison of the mean change in disease score from baseline to end of study period based on Juniper Asthma Control Questionnaire (ACQ-6), and could also assess lower respiratory symptom score (LRSS – symptoms of chest tightness, wheeze, shortness of breath and cough) daily following infection/onset of cold symptoms. Change from baseline lung function (peak expiratory flow PEF) could also be assessed and a positive response to therapy could be a significant attenuation in reduced PEF. For example, a placebo treated group would show a significant reduction in morning PEF of 15% at the peak of exacerbation whilst the treatment group would show a non-significant reduction in PEF less than 15% change from baseline.

**[0123]** Although the methods of the present invention finds application in humans, the methods of the invention is also useful for therapeutic veterinary purposes. The invention is useful for domestic or farm animals such as cattle, sheep, horses and poultry; for companion animals such as cats and dogs; and for zoo animals.

**[0124]** In certain embodiments, the antisense oligonucleotides, the conjugates, or the prodrugs as described herein can be delivered by pulmonary or nasal routes (e.g., via nebulised saline incorporating the AONs). The highest endogenous expression of RAGE mRNA in the healthy human tissues is found in the lung and is accessible via the airways. Inhaled oligonucleotides are an emerging therapeutic modality for respiratory diseases. The airways are uniquely lined with pulmonary surfactants, which are

primarily composed of zwitterionic lipids. These surfactant lipids possess cationic properties at the pH of the respiratory tract. When anionic oligonucleotides are inhaled, they tend to be adsorbed by the surfactants, resulting in reformulated particles that have been hypothesised to be efficiently taken up by bronchial and alveolar epithelial cells into the pulmonary cells. Of note, AONs have been shown to be able to withstand the nebulization process.

**[0125]** The antisense oligonucleotides, the conjugates, or the prodrugs as described herein may be in compositions formulated for administration to the lower respiratory tract only. Limitation to the lower respiratory tract may be achieved by an amount, particularly volume and composition of form ie. particle size, physical form whether dry powder or solution droplet, of composition that would otherwise be administered to the upper respiratory tract. Alternatively, the antisense oligonucleotides, the conjugates, or the prodrugs as described herein may be administered via a device that ensures retention in the lower respiratory tract only.

**[0126]** The antisense oligonucleotides, the conjugates, or the prodrugs as described herein may be formulated for intranasal administration, including dry powder, sprays, mists, or aerosols. This may be particularly preferred for treatment of a respiratory infection.

**[0127]** Suitable formulations, wherein the carrier is a liquid, for administration, as for example, a nasal spray or as nasal drops, include aqueous or oily solutions of the active ingredient. Alternatively, the antisense oligonucleotides, the conjugates, or the prodrugs as described herein may be provided as a dry powder and administered to the upper respiratory tract only as defined herein.

**[0128]** The selection of appropriate carriers depends upon the particular type of administration that is contemplated. For administration via the upper respiratory tract, e.g., the nasal mucosal surfaces, the antisense oligonucleotide, the conjugate or the prodrug can be formulated into a solution, e.g., water or isotonic saline, buffered or unbuffered, or as a suspension, for intranasal administration as drops or as a spray. Preferably, such solutions or suspensions are isotonic relative to nasal secretions and of about the same pH, ranging e.g., from about pH 4.0 to about pH 7.4 or, from pH 6.0 to pH 7.0. Buffers should be physiologically compatible and include, simply by way of example, phosphate buffers. For example, a representative nasal decongestant is

described as being buffered to a pH of about 6.2 (Remington's, Id. at page 1445). Of course, the ordinary artisan can readily determine a suitable saline content and pH for an innocuous aqueous carrier for nasal and/or upper respiratory administration.

**[0129]** Other ingredients, such as art known preservatives, colorants, lubricating or viscous mineral or vegetable oils, perfumes, natural or synthetic plant extracts such as aromatic oils, and humectants and viscosity enhancers such as, e.g., glycerol, can also be included to provide additional viscosity, moisture retention and a pleasant texture and odour for the formulation. For nasal administration of solutions or suspensions according to the methods of the invention, various devices are available in the art for the generation of drops, droplets and sprays. For example, an AON as described herein can be administered into the nasal passages by means of a simple dropper (or pipet) that includes a glass, plastic or metal dispensing tube from which the contents are expelled drop by drop by means of air pressure provided by a manually powered pump, e.g., a flexible rubber bulb, attached to one end.

**[0130]** The delivery of a therapeutically useful amount of the antisense oligonucleotides, the conjugates, or the prodrugs may be achieved by methods previously published. For example, intracellular delivery of the AON may be via a composition comprising an admixture of the AON and an effective amount of a block copolymer. An example of this method is described in US patent application US20040248833. Other methods of delivery of AONs to the nucleus are described in Mann CJ et al. (2001) Proc. Natl. Acad. Science, 98(1) 42-47, and in Gebiski et al. (2003) Human Molecular Genetics, 12(15): 1801-1811. A method for introducing a nucleic acid molecule into a cell by way of an expression vector either as naked DNA or complexed to lipid carriers, is described in US 6,806,084.

**[0131]** The antisense oligonucleotides, the conjugates, or the prodrugs for use in the methods or uses of the present invention is to be administered in an effective amount. The phrase 'therapeutically effective amount' or 'effective amount' generally refers to an amount of an AON, a conjugate, a pharmaceutically acceptable salt, polymorph or prodrug thereof as described herein that (i) treats the particular disease, condition, or disorder, (ii) attenuates, ameliorates, or eliminates one or more symptoms of the particular disease, condition, or disorder, or (iii) delays the onset of one or more symptoms of the particular disease, condition, or disorder described herein. Undesirable effects, e.g. side effects, are sometimes manifested along with the desired therapeutic

effect; hence, a practitioner balances the potential benefits against the potential risks in determining what is an appropriate "effective amount".

**[0132]** The exact amount required will vary from subject to subject, depending on the species, age and general condition of the subject, mode of administration and the like. Thus, it may not be possible to specify an exact "effective amount". However, an appropriate "effective amount" in any individual case may be determined by one of ordinary skill in the art using only routine experimentation. In one aspect, the dose administered to a subject is any dose that reduces viral load.

**[0133]** The phrase "pharmaceutically acceptable" refers to molecular entities and compositions that are physiologically tolerable and do not typically produce an allergic or similarly untoward reaction, such as gastric upset and the like, when administered to a patient. The term "carrier" refers to a diluent, adjuvant, excipient, or vehicle with which the compound is administered. Such pharmaceutical carriers can be sterile liquids, such as water and oils, including those of petroleum, animal, vegetable or synthetic origin, such as peanut oil, soybean oil, mineral oil, sesame oil and the like. Water or saline solutions and aqueous dextrose and glycerol solutions are preferably employed as carriers, particularly for injectable solutions. Suitable pharmaceutical carriers are described in Martin, *Remington's Pharmaceutical Sciences*, 18th Ed., Mack Publishing Co., Easton, PA, (1990).

**[0134]** In any embodiment, an antisense oligonucleotide, a conjugates, or a prodrug, as described herein, is administered in an amount and manner effective to result in a peak blood concentration of at least 200-400 nM of the antisense oligonucleotide.

**[0135]** In any embodiment an antisense oligonucleotide, a conjugates, or a prodrug is administered in an amount of a human equivalent dose of those doses described in the preclinical studies in the Examples.

**[0136]** Treatment may be monitored, e.g., by general indicators of disease known in the art. The efficacy of an *in vivo* administered the antisense oligonucleotides, the conjugates, or a prodrugs for use in the methods of the present invention may be determined from biological samples (tissue, blood, urine etc.) taken from a subject prior to, during and subsequent to administration of the antisense oligonucleotides, the conjugates, or the prodrugs. Assays of such samples include (1) monitoring the

presence or absence of heteroduplex formation with target and non-target sequences, using procedures known to those skilled in the art, e.g., an electrophoretic gel mobility assay; (2) monitoring the amount of a mutant mRNA in relation to a reference normal mRNA or protein as determined by standard techniques such as RT-PCR, Northern blotting, ELISA or Western blotting.

**[0137]** Kits are also provided for use in the methods of the invention, in particular to treat, prevent or ameliorate a disease or condition associated with RAGE expression in a patient, which kit comprises at least an antisense oligonucleotide, a conjugates, or a prodrug as described herein together with instructions for its use, wherein the instructions for use describe a method of the invention.

**[0138]** The contents of the kit can be lyophilized and the kit can additionally contain a suitable solvent for reconstitution of the lyophilized components. Individual components of the kit would be packaged in separate containers and, associated with such containers, can be a notice in the form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals or biological products, which notice reflects approval by the agency of manufacture, use or sale for human administration.

**[0139]** As used herein the term “derived” and “derived from” shall be taken to indicate that a specific integer may be obtained from a particular source albeit not necessarily directly from that source.

**[0140]** Other than in the operating example, or where otherwise indicated, all numbers expressing quantities of ingredients, reaction conditions, and so forth used in the specification and claims are to be understood as being modified in all instances by the term “about”. Accordingly, unless indicated to the contrary, the numerical parameters set forth in the specification and claims are approximations that may vary depending upon the desired properties sought to be obtained by the methods of the present invention. Hence “about 80 %” means “about 80 %” and also “80 %”. At the very least, each numerical parameter should be construed in light of the number of significant digits and ordinary rounding approaches.

**[0141]** Notwithstanding that the numerical ranges and parameters setting forth the broad scope of the methods of the present invention are approximations, the numerical values set forth in the specific examples are reported as precisely as possible. Any

numerical value; however, inherently contains certain errors necessarily resulting from the standard deviation found in their respective testing measurements

**[0142]** Other definitions for selected terms used herein may be found within the detailed description of the invention and apply throughout. Unless otherwise defined, all other scientific and technical terms used herein have the same meaning as commonly understood to one of ordinary skill in the art to which the invention belongs.

**[0143]** It will be understood that the invention disclosed and defined in this specification extends to all alternative combinations of two or more of the individual features mentioned or evident from the text or drawings. All of these different combinations constitute various alternative aspects of the invention.

### Examples

**[0144]** The inventors have found antisense oligonucleotides that promote significantly greater alternative splicing of RAGE pre-mRNA (e.g. inclusion of exon 9b and/or the exclusion (e.g. skipping) of exon 10) than existing antisense oligonucleotides. This is surprisingly as it has been shown that usually only a small proportion of ASOs are functional and even fewer ASOs are potent. Without being bound by theory, the inventors believe that potency is determined not only by Watson-Crick complementarity to splice regulatory elements within target pre-mRNA, but that the likelihood of successful hybridization, and therein pharmacodynamic activity, is also influenced by other features, including secondary structural features of the ASO and/or the target pre-mRNA, which influence nucleation at an accessible (single-stranded, unstructured) site and subsequent elongation by a 'zippering' process.

#### *Example 1 – Materials and Methods*

##### Synthesis of ASOs.

**[0145]** All ASOs were synthesized by Syngenis using a GE ÄKTA oligopilot plus 10 (GE Healthcare Life Sciences) oligonucleotide synthesizer using standard phosphoramidite chemistry in 1 µmol scale. All synthesis reagents were purchased from Merck Millipore. Synthesised oligonucleotides were deprotected and cleaved from the solid support by treatment with NH<sub>4</sub>OH (Merck Millipore) at 55°C overnight, and the crude oligonucleotides were then purified by desalting through illustra NAP-10 columns (GE Healthcare Life Sciences).

**[0146]** The two ASOs synthesised were designated 6777 and 6645. ASO 6777 has the sequence shown in SEQ ID NO: 1. ASO 6645 has the sequence shown in SEQ ID NO: 2. For both ASO all links are phosphorothioate, all ribose are 2'-O-methoxyethyl; all Cs are C5-methylated; T is thymidine, i.e. C5-methyl-U; 5' terminal ribose is 5'-hydroxyl.

#### Transfection of with ASOs.

**[0147]** Cells were seeded into 48-well plates for 48 hours and then transfected using lipofectamine 3000 reagent (0.15ul/well of lipofectamine 3000; 0.4ul/well P3000/well) with ASOs (0.1-100nM). Cells were then incubated with ASO/cationic lipoplexes at 37°C for 48 hours.

#### Cell lines

**[0148]** Human lung carcinoma cells (A549) were cultured in F12K medium (GIBCO) supplemented with 10% FBS (Scientifix Life). Validation studies were undertaken in primary human diseased small airways epithelial cells (D-SAEC) isolated from the distal portion of the lung in the 1mm bronchiole area from three different patients with chronic lung disease (clones 23484,36139; Lonza). D-SAEC were cultured in small airways growth medium (Lonza).

#### Real time quantitative PCR

**[0149]** To measure the effect of interventions on the alternative splicing of RAGE pre-mRNA, the expression of RAGE mRNA splice variants was determined using real-time RT-qPCR, performed using the TaqMan system based on real-time detection of accumulated fluorescence (ABI Prism 7700, Perkin-Elmer Inc, PE Biosystems, Foster City, CA, USA). After 48 hours of exposure to each ASO, cells were lysed, RNA was extracted and cDNA was synthesized using the Cells to CT method. RAGE mRNA expression was estimated by RT-qPCR, performed using either the Taqman or SYBR Green (Sigma) system on the basis of real-time detection of accumulation of fluorescence (Applied Biosystems Q3 and Q5). To define the expression of RAGE mRNA splice variants retaining exon 9b a probe was designed to span exon 9b. To define the expression of RAGE mRNA splice variants retaining exon 10, a PCR primer/probes spanning exon 8-10 were designed. To define the expression of all RAGE mRNA, PCR probe/primers spanning exon 8 and 11 were designed. A reduction in the signal of exon 8-10 of the cytosolic tail relative to exon 8-11 indicates that less

full-length RAGE transcript (signalling capable) is produced by the cell. Gene Expression was normalized to 18S mRNA and reported as fold change compared to the level of expression in control RNA treated cells, which were given an arbitrary value of 1.

#### Statistical Analysis

**[0150]** Data shown are means  $\pm$  SEM. All datasets were determined to be normally distributed based on previous experiments and were also tested using the Kolmogorov–Smirnov test ( $\alpha = 0.05$ ). Data were analysed using one-way ANOVA with Tukey's multiple comparison test using PRISM software (V6.0d, GraphPad, La Jolla, CA, USA), with  $P < 0.05$  considered statistically significant.

#### *Example 2 – In vitro pharmacodynamic activity of 18-mer ASOs targeting the critical ESE in exon 10 of RAGE in A549 cells*

**[0151]** The pharmacodynamic activity of ASO 6643, 6644, 6645 and 6646 were compared with ASO h79 across a range of different doses (0.3-30nM). Notably at low doses ( $\leq 1$ nM), the pharmacodynamic activity of 6645 and 6644 exceeded that of ASO h79 (**Figures 1, 2 & 3**). Equally, the maximal response to ASO 6644, 6645 and 6646 at highest dose (30nM) also exceeded that of ASO h79 following transfection of A548 cells. Notably, ASO 6644, 6645 and 6646 also share identity across the region predicted to contain an ESE that regulates the alternative splicing of RAGE pre-mRNA.

#### *Example 3 – In vitro pharmacodynamic activity of 18-mer ASOs targeting the critical ESE in exon 10 of RAGE in primary SAEC cells*

**[0152]** Having identified the additional pharmacodynamic activity of 6644, 6645 and 6646 over ASO h79, the inventors next investigated the activity of ASOs targeting the adjacent sequences across the same area as 6644, 6645 and 6646.

**[0153]** In this assay, the activity of ASO these 18mer ASOs and others targeting the sequences directly adjacent to the were compared to ASO 6780, a 25-mer 2'MOE-PS ASO targeting exactly the same sequence as h79. Notably, all these 18mers were at least as potent as ASO 6780, while the activity of ASO 6777 significantly exceeded that of ASO 6780 at low dose 1nM and was numerically greater across all other doses (**Figure 4**).

**[0154]** Finally, the pharmacodynamic activity of these 18mer ASO was validated in three different D-SAEC lines obtained from humans with chronic lung disease. In these experiments ASO 6777 and 6645 had similar pharmacodynamic activity following transfection of D-SAEC (**Figure 5**).

**CLAIMS**

1. An antisense oligonucleotide (AON) consisting of a nucleotide sequence of:  
  
TGCCGCCTTTGCCACAAG (5'-3'; SEQ ID NO: 1); or  
  
GCCGCCTTTGCCACAAGA (5'-3'; SEQ ID NO: 2),
2. The antisense oligonucleotide of claim 1, wherein the antisense oligonucleotide comprises at least one modified nucleotide.
3. The antisense oligonucleotide of claim 2, wherein the modified nucleotide has a modified internucleotide linkage.
4. The antisense oligonucleotide of claim 3, wherein the modified internucleotide linkage has a phosphorothioate linkage.
5. The antisense oligonucleotide of any one of claims 2 to 4, wherein the modified nucleotide has a modified sugar moiety or a sugar mimetic.
6. The antisense oligonucleotide of claim 5, wherein the modified sugar moiety or sugar mimetic is selected from the group consisting of: morpholino, 2' O-methyl (2'-OMe), and 2'-O-methoxyethyl (2'-MOE), 2',4'-constrained 2'-O-methoxyethyl (cMOE), 2',4' constrained 2'-O-Ethyl (cEt) and locked nucleic acid (LNA), preferably, the modified sugar moiety is 2'-O-methoxyethyl (2'MOE) sugar moiety.
7. The antisense oligonucleotide of any one of claims 2 to 6, wherein the modified nucleotide has a modified nucleoside base.
8. The antisense oligonucleotide of claim 7, wherein the modified nucleoside base is a C5-methylated cytosine.
9. The antisense oligonucleotide of any one of claims 1 to 8, wherein the 5' terminal end of the antisense oligonucleotide has a 5'-hydroxyl.
10. The antisense oligonucleotide of any one of claims 1 to 5 and 7 to 9, wherein the antisense oligonucleotide is RNA, or each nucleotide in the antisense oligonucleotide is a ribonucleotide.

11. The antisense oligonucleotide of any one of claims 1 to 10, wherein one or more of the Thymines (Ts) as shown in SEQ ID NO: 1 or 2 is replaced with Uracil (U) and/or pseudouracil ( $\psi$ ).

12. The antisense oligonucleotide of claim 11, wherein one, two or three of the three contiguous Ts as shown in SEQ ID NO: 1 or 2 are replaced with pseudouracil ( $\psi$ ).

13. The antisense oligonucleotide of any one of claims 1 to 10, wherein the Ts are C5-methyl-U.

14. The antisense oligonucleotide of any one of claims 1 to 13, wherein each nucleotide of the antisense oligonucleotide is modified and comprises:

- a phosphorothioate linkage; and
- a 2'-O-methoxyethyl (2'-MOE) sugar moiety.

15. The antisense oligonucleotide of any one of claims 1 to 13, wherein each nucleotide of the antisense oligonucleotide is modified and comprises:

- a phosphorothioate linkage; and
- a 2'-O-methoxyethyl (2'-MOE) sugar moiety,

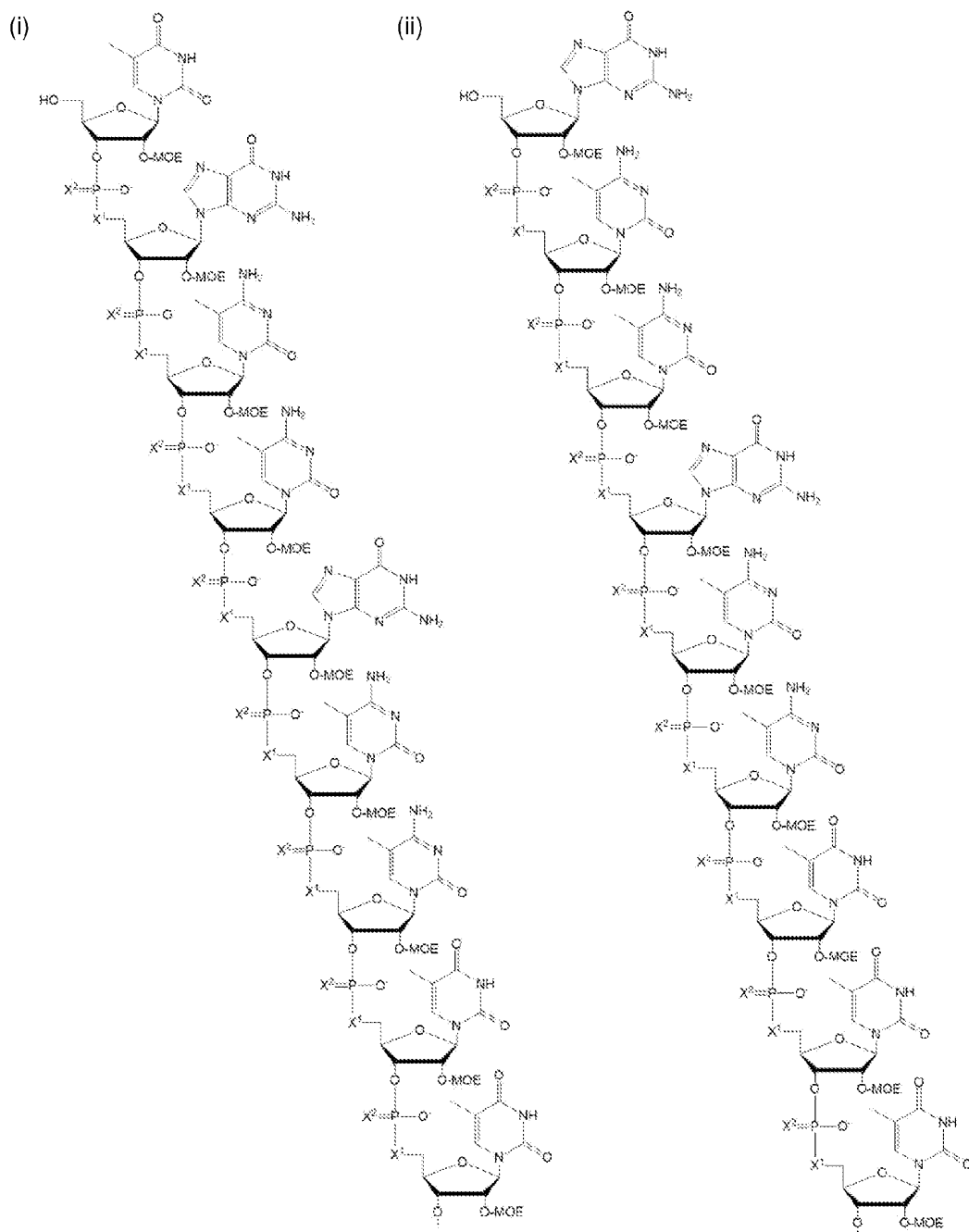
and wherein each cytosine of the antisense oligonucleotide is C5-methylated.

16. The antisense oligonucleotide of any one of claims 1 to 13, wherein each nucleotide of the antisense oligonucleotide is modified and comprises:

- a phosphorothioate linkage; and
- a 2'-O-methoxyethyl (2'-MOE) sugar moiety,

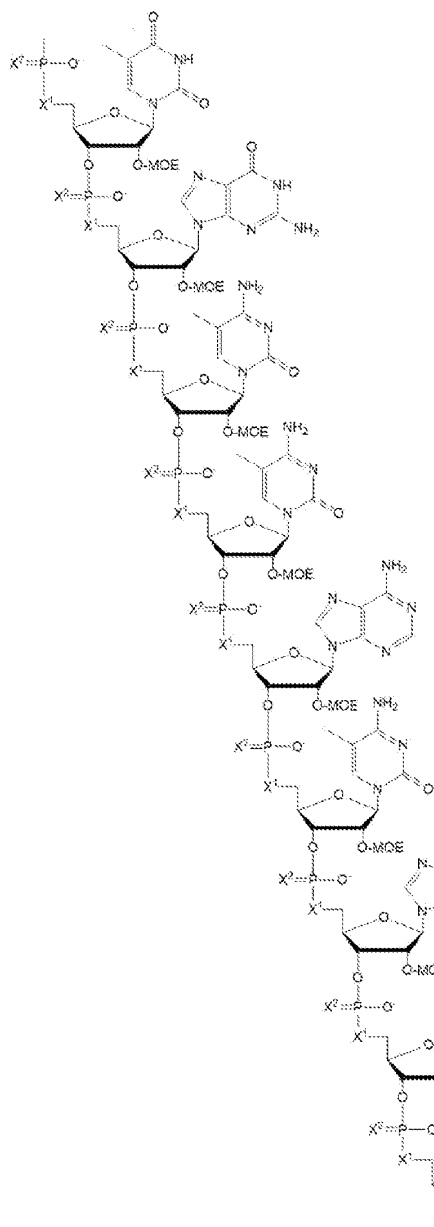
and wherein each cytosine of the antisense oligonucleotide is C5-methylated and each T is C5-methylated uracil.

17. The antisense oligonucleotide of any one of claim 1 to 16, wherein the antisense oligonucleotide consists of one of the following structures:

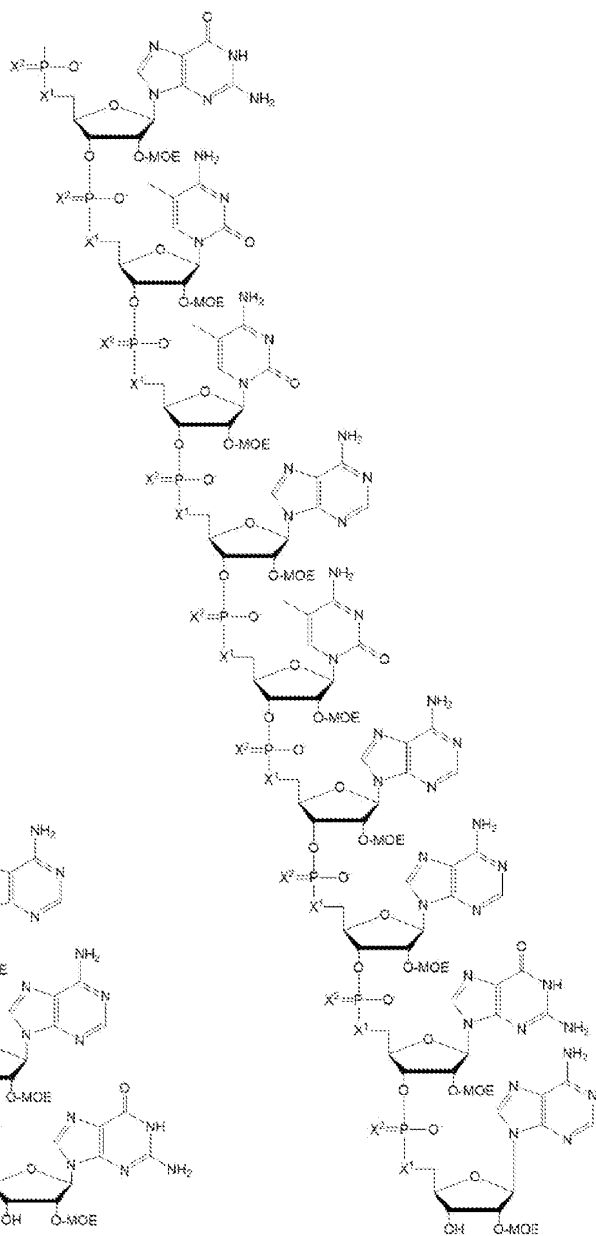


- continued

- (i) continued



- (ii) continued



wherein  $x^1$  is S and  $x^2$  is O, or  $x^1$  is O and  $x^2$  is S;

and MOE is 2-methoxyethyl.

18. A conjugate comprising an antisense oligonucleotide according to claims 1 to 17 conjugated to a molecule.

19. The conjugate according to claim 18, wherein the molecule is a cell-penetrating peptide (CPP).
20. The conjugate according to claim 18, wherein the conjugate is a vivo-morpholinos (VMO) or peptide phosphorodiamidate morpholino oligomers (PPMO).
21. The conjugate according to any one of claims 18 to 20, wherein the antisense oligonucleotide is chemically conjugated to the moiety.
22. A prodrug comprising an antisense oligonucleotide according to claims 1 to 17, preferably the antisense oligonucleotide is conjugated to a molecule.
23. The prodrug of claim 22, wherein the molecule is cleaved from the antisense oligonucleotide when administered to a subject.
24. A composition comprising antisense oligonucleotide of any one of claims 1 to 17, the conjugate of any one of claims 18 to 21, or the prodrug of claim 22 or 23.
25. A pharmaceutical composition comprising antisense oligonucleotide of any one of claims 1 to 16, the conjugate of any one of claims 18 to 21, or the prodrug of claim 22 or 23, optionally wherein the composition further comprises a pharmaceutically or physiologically acceptable diluent, excipient or carrier.
26. A method for manipulating splicing in a RAGE pre-mRNA resulting in the skipping of exon 10, the method comprising contacting a cell with an antisense oligonucleotide of any one of claims 1 to 17, the conjugate of any one of claims 18 to 21, the prodrug of claim 22 or 23, the composition of claim 24, or the pharmaceutical composition of claim 25, thereby manipulating splicing in a RAGE pre-mRNA resulting in the skipping of exon 10 in the cell.
27. A method for promoting the production of endogenous soluble RAGE in a subject, the method comprising administering an antisense oligonucleotide of any one of claims 1 to 17, the conjugate of any one of claims 18 to 21, the prodrug of claim 22 or 23, the composition of claim 24, or the pharmaceutical composition of claim 25, thereby promoting the production of endogenous soluble RAGE in the subject.
28. A method for preventing or treating a disease associated with, or caused by, RAGE in a subject, the method comprising administering an antisense oligonucleotide

of any one of claims 1 to 17, the conjugate of any one of claims 18 to 21, the prodrug of claim 22 or 23, the composition of claim 24, or the pharmaceutical composition of claim 25, thereby preventing or treating a disease associated with, or caused by, RAGE in a subject.

29. Use of an antisense oligonucleotide of any one of claims 1 to 17, the conjugate of any one of claims 18 to 21, the prodrug of claim 22 or 23, the composition of claim 24, or the pharmaceutical composition of claim 25, in the manufacture of a medicament for promoting the production of endogenous soluble RAGE in a subject.

30. Use of an antisense oligonucleotide of any one of claims 1 to 17, the conjugate of any one of claims 18 to 21, the prodrug of claim 22 or 23, the composition of claim 24, or the pharmaceutical composition of claim 25, in the manufacture of a medicament for preventing or treating a disease associated with, or caused by, RAGE in a subject.

31. An antisense oligonucleotide of any one of claims 1 to 17, the conjugate of any one of claims 18 to 21, the prodrug of claim 22 or 23, the composition of claim 24, or the pharmaceutical composition of claim 25, for use in manipulating splicing of RAGE pre-mRNA in a cell resulting in the skipping of exon 10.

32. An antisense oligonucleotide of any one of claims 1 to 17, the conjugate of any one of claims 18 to 21, the prodrug of claim 22 or 23, the composition of claim 24, or the pharmaceutical composition of claim 25, for use in promoting the production of endogenous soluble RAGE in a subject.

33. An antisense oligonucleotide of any one of claims 1 to 17, the conjugate of any one of claims 18 to 21, the prodrug of claim 22 or 23, the composition of claim 24, or the pharmaceutical composition of claim 25, for use in preventing or treating a disease associated with, or caused by, RAGE in a subject.

34. The method, use or antisense oligonucleotide of any one of claims 26 to 33, wherein administration of the antisense oligonucleotide, the conjugate, the prodrug or the compound promotes the production of endogenous soluble RAGE in one or more tissues of the respiratory tract.

35. The method, use or antisense oligonucleotide of any one of claims 26 to 33, wherein the antisense oligonucleotide, the conjugate, the prodrug or the compound is

administered to the total respiratory tract, the upper respiratory tract or the lower respiratory tract.

36. The method, use or antisense oligonucleotide of any one of claims 26 to 33, wherein the antisense oligonucleotide, the conjugate, the prodrug or the compound is administered as an aerosol, a dry powder or as nasal drops.

37. The method, use or antisense oligonucleotide of any one of claims 26 to 33, wherein the antisense oligonucleotide, the conjugate, the prodrug or the compound is administered using a nasal spray pump, intranasal installation, intratracheal instillation, metered dose inhaler (MDI), dry powder inhaler (DPI), nebuliser (jet, ultrasonic mesh or vibrating mesh) or soft mist inhaler (SMI).