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(54) Title: COMPOUNDS AND METHODS FOR MODULATING TMPRSS6 EXPRESSION

(57) Abstract: Disclosed herein are compositions and compounds comprising modified oligonucleotides for modulating TMPRSS6 and modulating an iron accumulation disease, disorder and/or condition in an individual in need thereof. Iron accumulation diseases in an individual such as polycythemia, hemochromatosis or β-thalassemia can be treated, ameliorated, delayed or prevented with the administration of antisense compounds targeted to TMPRSS6.

COMPOUNDS AND METHODS FOR MODULATING TMPRSS6 EXPRESSION

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 BIOL0271WOSEQ_ST25.txt created March 23, 2016, which is 148 kb in size. The information in the electronic format of the sequence listing is incorporated herein by reference in its entirety.

FIELD OF THE INVENTION

The present invention provides methods, compounds, and compositions for modulating TMPRSS6 expression for the purpose of reducing iron accumulation in an animal.

BACKGROUND OF THE INVENTION

Maintenance of iron balance in human beings is delicate because of the limited capacity of the human physiology for iron absorption and excretion (Finch, C.A. and Huebers, H. N. Engl. J. Med. 1982. 306: 1520-1528). Iron deficiency is a widespread disorder and results from any condition in which dietary iron intake does not meet the body's demands. Often, pathological blood loss contributes to negative iron balance. Iron overload is also a common condition, and may result from a genetic cause, for example, mutations of different genes of iron metabolism (Camaschella, C. Blood. 2005. 106: 3710-3717). The hepatic peptide hormone, hepcidin plays a key role in body iron metabolism as it controls iron absorption and recycling (Ganz, T. Am. Soc. Hematol. Educ. Program 2006. 507: 29-35; Kemna, E. H. et al., Clin. Chem. 2007. 53: 620-628). Several proteins, including HFE (hemochromatosis protein) (Ahmad, K.A. et al., Blood Cells Mol Dis. 2002. 29: 361), transferrin receptor 2 (Kawabata, H. et al., Blood 2005. 105: 376), and hemojuvelin (Papanikolaou, G. et al., Nat. Genet. 2004. 36: 77) also regulate the body's iron levels.

Transmembrane protease, serine 6 (TMPRSS6) is a type II transmembrane serine protease and is expressed primarily in the liver (Velasco, G. et al., J. Biol. Chem. 2002. 277: 37637-37646). Mutations in TMPRSS6 have been implicated in iron deficiency anemia (Finberg, K. E. et al., Nat. Genet. 2008. 40: 569-571), where the level of hepcidin was found to be unusually elevated. A study of a human population with microcytic anemia found that loss-of-function mutations in the TMPRSS6 gene lead to overproduction of hepcidin, which, in turn, lead to defective iron absorption and utilization (Melis, M.A. et al., Hematologica 2008. 93: 1473-1479). TMPRSS6 participates in a transmembrane signaling pathway triggered by iron deficiency and suppresses diverse pathways of *Hamp* activation, the gene that encodes hepcidin (Du, X. et al., Science 2008. 320: 1088-1092). Heterozygous loss of TMPRSS6 in $HFE^{-/-}$ mice reduces systemic iron overload, while homozygous loss of TMPRSS6 in $HFE^{-/-}$ mice causes systemic iron deficiency and elevated hepatic expression of hepcidin (Finberg, K.E. et al., Blood 2011. 117: 4590-4599).

An example of an iron overload disorder is Hemochromatosis. Hemochromatosis (e.g. hemochromatosis type 1 or hereditary hemochromatosis) is a disorder that results in excess intestinal absorption of dietary iron from the gastrointestinal tract (Allen, K.J. et al., N. Engl. J. Med. 2008. 358: 221-230). This results in a pathological increase in total body iron stores. Excess iron accumulates in tissues and organs, particularly the liver, adrenal glands, heart, skin, gonads, joints and pancreas, and disrupt their normal function. Secondary complications, such as cirrhosis (Ramm, G.A. and Ruddell, R.G. Semin. Liver Dis. 2010. 30: 271-287), polyarthropathy (Carroll, G.J. et al., Arthritis Rheum. 2011. 63: 286-294), adrenal insufficiency, heart failure and diabetes (Huang, J. et al., Diabetes 2011. 60: 80-87) are common. Another example of an iron overload disorder is β-thalassemia, where patients can develop iron overload caused by ineffective erythropoiesis or transfusions to treat β-thalassemia.

To date, therapeutic strategies to treat iron overload disorders have been limited. Nucleic acid inhibitors such as siRNA and antisense oligonucleotides have been suggested or developed, but none of the compounds directly targeting TMPRSS6 (PCT Publications WO2014/076195, WO2012/135246, WO2014/190157, WO2005/0032733, WO 2013/070786 and WO2013/173635; U.S. Patent Number 8,090,542; Schmidt et al. Blood. 2013, 121(7):1200-8) have been approved for treating iron overload disorders. Accordingly, there is an unmet need for highly potent and tolerable compounds to inhibit TMPRSS6. The invention disclosed herein relates to the discovery of novel, highly potent inhibitors of TMPRSS6 expression and their use in treatment.

All documents, or portions of documents, cited in this application, including, but not limited to, patents, patent applications, articles, books, and treatises, are hereby expressly incorporated-by-reference for the portions of the document discussed herein, as well as in their entirety.

SUMMARY OF THE INVENTION

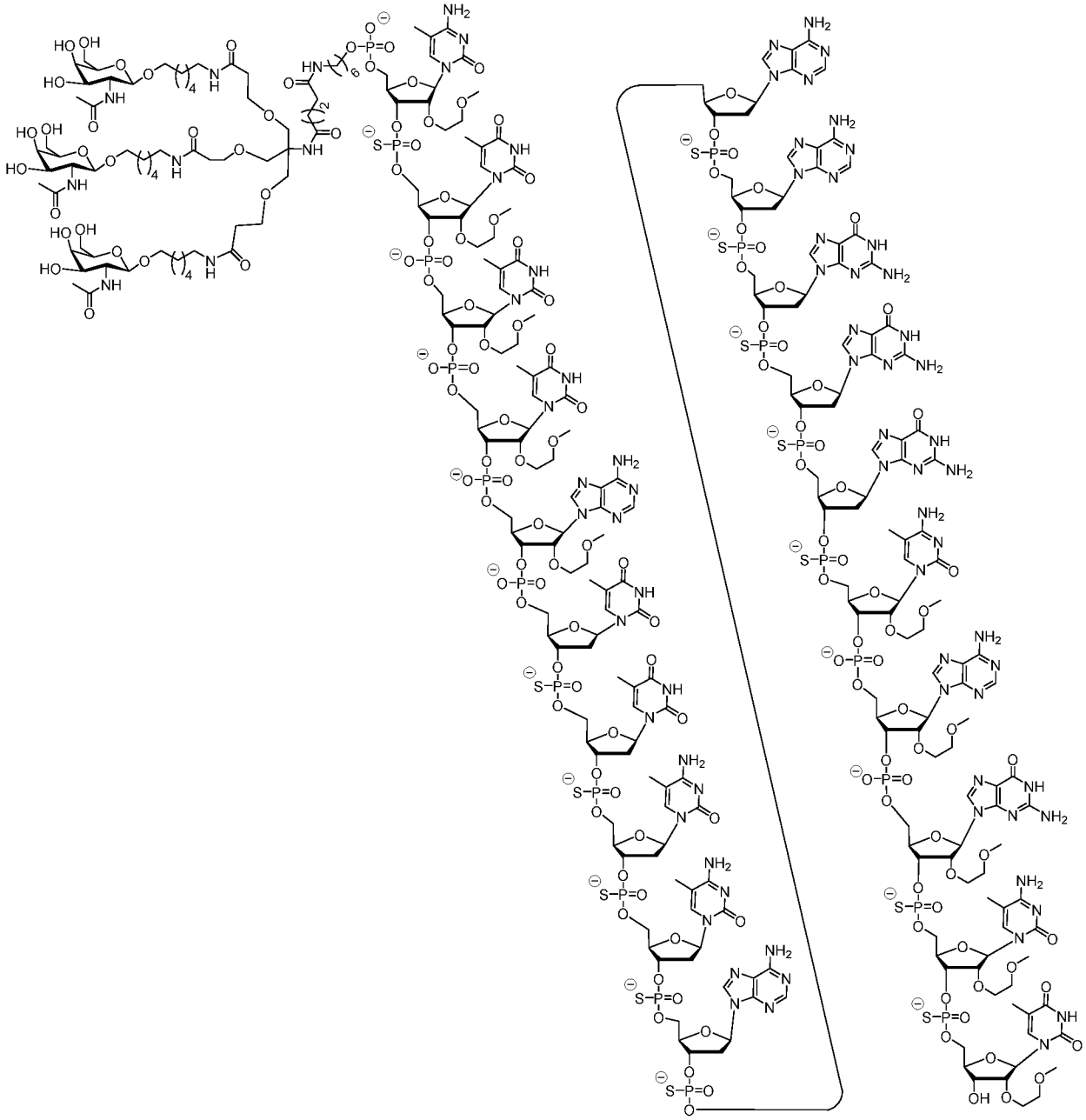
Provided herein are compositions, compounds and methods for modulating the levels of TMPRSS6 mRNA and/or protein in an animal. Provided herein are compositions, compounds and methods for lowering TMPRSS6 levels.

Certain embodiments disclosed herein provide a compound comprising a modified oligonucleotide targeting a nucleic acid sequence encoding TMPRSS6. In certain embodiments, the compound targets a TMPRSS6 sequence as shown in the nucleobase sequences of any of SEQ ID NOs: 1-6.

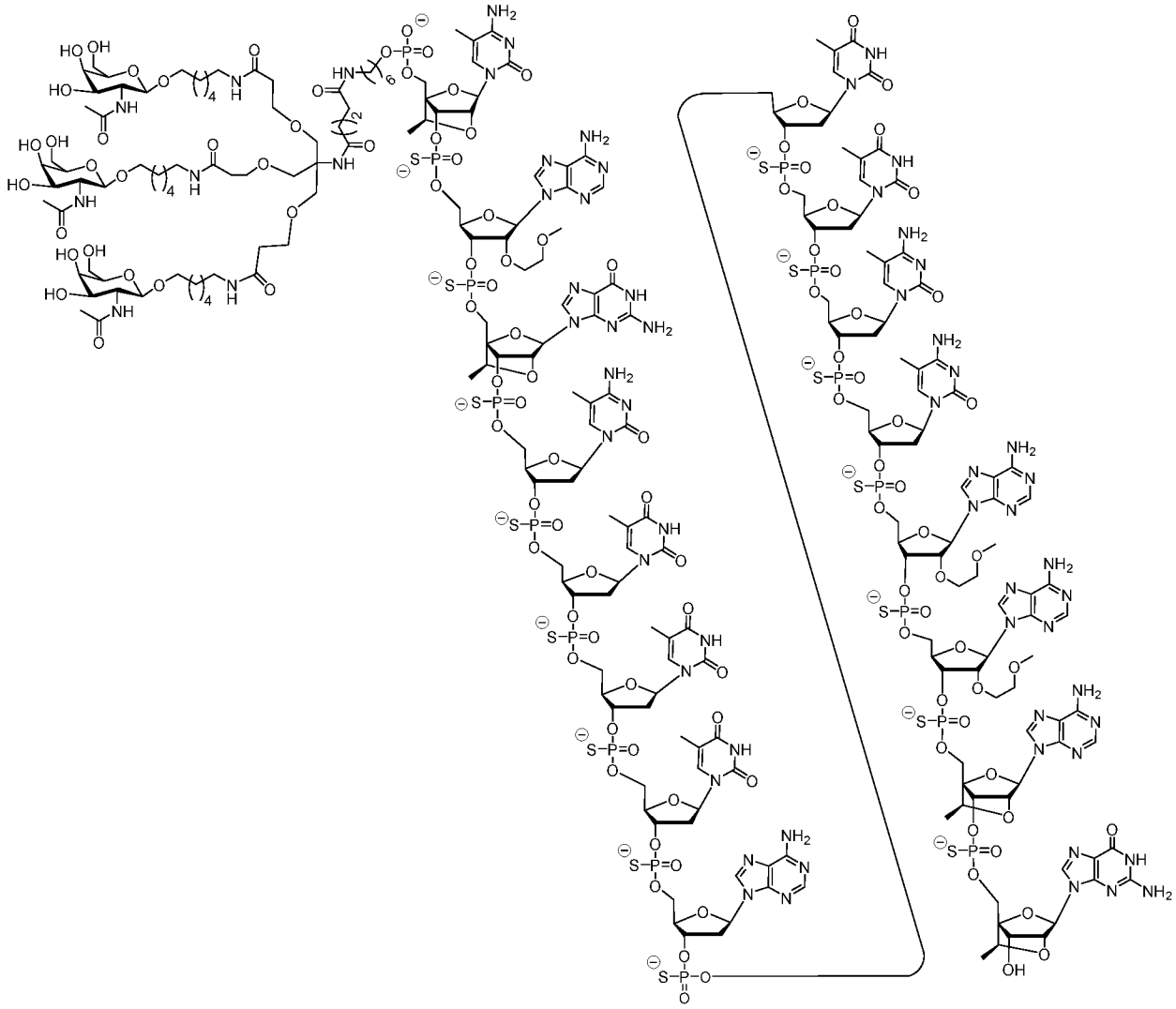
Certain embodiments disclosed herein provide a compound comprising a modified oligonucleotide consisting of 12 to 30 linked nucleosides and comprising a nucleobase sequence comprising a portion of at least 8 contiguous nucleobases complementary to an equal length portion of nucleobases 3162 to 3184 of SEQ ID NO: 1, wherein the nucleobase sequence of the modified oligonucleotide is at least 80% complementary to SEQ ID NO: 1.

Certain embodiments disclosed herein provide a compound comprising a modified oligonucleotide consisting of 12 to 30 linked nucleosides and having a nucleobase sequence comprising at least 8 contiguous nucleobases of any of the nucleobase sequences of SEQ ID NOs: 23, 36, 37, 63, 77.

Certain embodiments disclosed herein provide a compound comprising a modified oligonucleotide with the following formula:



Certain embodiments disclosed herein provide a compound comprising a modified oligonucleotide with the following formula:



DETAILED DESCRIPTION OF THE INVENTION

It is to be understood that both the foregoing general description and the following detailed description are exemplary and explanatory only and are not restrictive of the invention, as claimed. Herein, the use of the singular includes the plural unless specifically stated otherwise. As used herein, the use of "or" means "and/or" unless stated otherwise. Furthermore, the use of the term "including" as well as other forms, such as "includes" and "included", is not limiting. Also, terms such as "element" or "component" encompass both elements and components comprising one unit and elements and components that comprise more than one subunit, unless specifically stated otherwise.

The section headings used herein are for organizational purposes only and are not to be construed as limiting the subject matter described. All documents, or portions of documents, cited in this application, including, but not limited to, patents, patent applications, articles, books, and treatises, are hereby expressly incorporated by reference for the portions of the document discussed herein, as well as in their entirety.

Definitions

Unless specific definitions are provided, the nomenclature utilized in connection with, and the procedures and techniques of, analytical chemistry, synthetic organic chemistry, and medicinal and pharmaceutical chemistry described herein are those well known and commonly used in the art. Standard techniques may be used for chemical synthesis, and chemical analysis. Where permitted, all patents, applications, published applications and other publications, GENBANK Accession Numbers and associated sequence information obtainable through databases such as National Center for Biotechnology Information (NCBI) and other data referred to throughout the disclosure herein are incorporated by reference for the portions of the document discussed herein, as well as in their entirety.

Unless otherwise indicated, the following terms have the following meanings:

"2'-O-methoxyethyl" (also 2'-MOE and 2'-$O(CH_2)_2$-OCH_3) refers to an O-methoxy-ethyl modification of the 2' position of a furosyl ring. A 2'-O-methoxyethyl modified sugar is a modified sugar.

"2'-O-methoxyethyl nucleotide" means a nucleotide comprising a 2'-O-methoxyethyl modified sugar moiety.

"5-methylcytosine" means a cytosine modified with a methyl group attached to the 5' position. A 5-methylcytosine is a modified nucleobase.

"About" means within ±10 % of a value. For example, if it is stated, "a marker may be increased by about 50%", it is implied that the marker may be increased between 45%-55%.

"Active pharmaceutical agent" or "Pharmaceutical agent" means the substance or substances in a pharmaceutical composition that provide a therapeutic benefit when administered to an individual. For example, in certain embodiments, an antisense oligonucleotide targeted to TMPRSS6 is an active pharmaceutical agent.

"Active target region" or "target region" means a region to which one or more active antisense compounds is targeted.

"Active antisense compounds" means antisense compounds that reduce target nucleic acid levels or protein levels.

"Administered concomitantly" refers to the co-administration of two agents in any manner in which the pharmacological effects of both are manifest in the patient time. Concomitant administration does not require that both agents be administered in a single pharmaceutical composition, in the same dosage form, or by the same route of administration. The effects of both agents need not manifest themselves at the same time. The effects need only be overlapping for a period of time and need not be coextensive.

"Administering" means providing a pharmaceutical agent to an individual, and includes, but is not limited to administering by a medical professional and self-administering.

"Agent" means an active substance that can provide a therapeutic benefit when administered to an animal. "First Agent" means a therapeutic compound provided herein. For example, a first agent is an

antisense oligonucleotide targeting TMPRSS6. "Second agent" means a second therapeutic compound described herein. For example, a second agent can be a second antisense oligonucleotide targeting TMPRSS6 or a non-TMPRSS6 target. Alternatively, a second agent can be a compound other than an antisense oligonucleotide.

"Amelioration" or "ameliorate" refers to a lessening of at least one indicator, marker, sign, or symptom of an associated disease, disorder and/or condition. In certain embodiments, amelioration includes a delay or slowing in the progression of one or more indicators of a condition, disorder and/or disease. The severity of indicators may be determined by subjective or objective measures, which are known to those skilled in the art.

"Anemia" is a disease characterized by a lower than normal number of red blood cells (erythrocytes) in the blood, usually measured by a decrease in the amount of hemoglobin. The cause of anemia can include chronic inflammation, chronic kidney disease, kidney dialysis treatment, genetic (hereditary) disorders, chronic infection, acute infection, cancer and cancer treatments. Altered iron homeostasis and/or erythropoiesis in these diseases, disorders and/or conditions can also result in decreased erythrocyte production. Clinical signs of anemia include low serum iron (hypoferremia), low hemoglobin levels, low hematocrit levels, decreased red blood cells, decreased reticulocytes, increased soluble transferrin receptor and iron restricted erythropoiesis. Examples of anemia include thalassemias (i.e. α-thalassemia, β-thalassemia (minor, intermedia and major) and δ-thalassemia), sickle cell anemia, aplastic anemia, Fanconi anemia, Diamond Blackfan anemia, Shwachman Diamond syndrome, red cell membrane disorders, glucose-6-phosphate dehydrogenase deficiency, hereditary hemorrhagic telangiectasia, hemolytic anemia, anemia of chronic disease and the like.

"Animal" refers to a human or non-human animal, including, but not limited to, mice, rats, rabbits, dogs, cats, pigs, and non-human primates, including, but not limited to, monkeys and chimpanzees.

"Antibody" refers to a molecule characterized by reacting specifically with an antigen in some way, where the antibody and the antigen are each defined in terms of the other. Antibody may refer to a complete antibody molecule or any fragment or region thereof, such as the heavy chain, the light chain, Fab region, and Fc region.

"Antisense activity" means any detectable or measurable activity attributable to the hybridization of an antisense compound to its target nucleic acid. In certain embodiments, antisense activity is a decrease in the amount or expression of a target nucleic acid or protein encoded by such target nucleic acid.

"Antisense compound" means an oligomeric compound that is capable of undergoing hybridization to a target nucleic acid through hydrogen bonding.

"Antisense inhibition" means reduction of target nucleic acid levels or target protein levels in the presence of an antisense compound complementary to a target nucleic acid compared to target nucleic acid levels or target protein levels in the absence of the antisense compound.

"Antisense oligonucleotide" means a single-stranded oligonucleotide having a nucleobase sequence that permits hybridization to a corresponding region or segment of a target nucleic acid.

"Bicyclic sugar" means a furosyl ring modified by the bridging of two non-geminal ring atoms. A bicyclic sugar is a modified sugar.

"Bicyclic nucleic acid" or "BNA" refers to a nucleoside or nucleotide wherein the furanose portion of the nucleoside or nucleotide includes a bridge connecting two carbon atoms on the furanose ring, thereby forming a bicyclic ring system.

"Blood transfusion" refers to the process of receiving blood products into one's circulation intravenously. Transfusions are used in a variety of medical disease, disorder and/or conditions to replace lost blood components.

"Cap structure" or "terminal cap moiety" means chemical modifications, which have been incorporated at either terminus of an antisense compound.

"cEt" or "constrained ethyl" means a bicyclic sugar moiety comprising a bridge connecting the 4'-carbon and the 2'-carbon, wherein the bridge has the formula: 4'-$CH(CH_3)$-O-2'.

"Constrained ethyl nucleoside" (also cEt nucleoside) means a nucleoside comprising a bicyclic sugar moiety comprising a 4'-$CH(CH_3)$-O-2' bridge.

"Chemically distinct region" refers to a region of an antisense compound that is in some way chemically different than another region of the same antisense compound. For example, a region having 2'-O-methoxyethyl nucleotides is chemically distinct from a region having nucleotides without 2'-O-methoxyethyl modifications.

"Chimeric antisense compound" means an antisense compound that has at least two chemically distinct regions.

"Co-administration" means administration of two or more pharmaceutical agents to an individual. The two or more pharmaceutical agents may be in a single pharmaceutical composition, or may be in separate pharmaceutical compositions. Each of the two or more pharmaceutical agents may be administered through the same or different routes of administration. Co-administration encompasses concomitant, parallel or sequential administration.

"Complementarity" means the capacity for pairing between nucleobases of a first nucleic acid and a second nucleic acid. In certain embodiments, the first nucleic acid is an antisense compound and the second nucleic acid is a target nucleic acid.

"Contiguous nucleobases" means nucleobases immediately adjacent to each other.

"Deoxyribonucleotide" means a nucleotide having a hydrogen at the 2' position of the sugar portion of the nucleotide. Deoxyribonucleotides may be modified with any of a variety of substituents.

"Diluent" means an ingredient in a composition that lacks pharmacological activity, but is pharmaceutically necessary or desirable. For example, the diluent in an injected composition may be a liquid, e.g. phosphate buffered saline (PBS).

"Dosage unit" means a form in which a pharmaceutical agent is provided, e.g. pill, tablet, or other dosage unit known in the art. In certain embodiments, a dosage unit is a vial containing lyophilized antisense oligonucleotide. In certain embodiments, a dosage unit is a vial containing reconstituted antisense oligonucleotide.

"Dose" means a specified quantity of a pharmaceutical agent provided in a single administration, or in a specified time period. In certain embodiments, a dose may be administered in one, two, or more boluses, tablets, or injections. For example, in certain embodiments where subcutaneous administration is desired, the desired dose requires a volume not easily accommodated by a single injection, therefore, two or more injections may be used to achieve the desired dose. In certain embodiments, the pharmaceutical agent is administered by infusion over an extended period of time or continuously. Doses may be stated as the amount of pharmaceutical agent per hour, day, week, or month.

"Effective amount" or "therapeutically effective amount" means the amount of active pharmaceutical agent sufficient to effectuate a desired physiological outcome in an individual in need of the agent. The effective amount can vary among individuals depending on the health and physical condition of the individual to be treated, the taxonomic group of the individuals to be treated, the formulation of the composition, assessment of the individual's medical condition, and other relevant factors.

"Fully complementary" or "100% complementary" means that each nucleobase of a nucleobase sequence of a first nucleic acid has a complementary nucleobase in a second nucleobase sequence of a second nucleic acid. In certain embodiments, the first nucleic acid is an antisense compound and the second nucleic acid is a target nucleic acid.

"Gapmer" means a chimeric antisense compound in which an internal region having a plurality of nucleosides that support RNase H cleavage is positioned between external regions having one or more nucleosides, wherein the nucleosides comprising the internal region are chemically distinct from the nucleoside or nucleosides comprising the external regions. The internal region may be referred to as a "gap segment" and the external regions may be referred to as "wing segments."

"Gap-widened" means a chimeric antisense compound having a gap segment of 12 or more contiguous 2'-deoxynucleosides positioned between and immediately adjacent to 5' and 3' wing segments having from one to six nucleosides.

"Hemochromatosis" is a disorder of iron metabolism that results in excess iron being absorbed from the gastrointestinal tract, leading to excess iron accumulation and deposition in various tissues of the body. Primary or hereditary or classic hemochromatosis is caused by a genetic mutation, for example, in the HFE gene. Subjects with this disease have excess amounts of iron, which is absorbed in the gastrointestinal tract and builds up in the body tissues, particularly in the liver. Secondary or acquired hemochromatosis can be caused by frequent blood transfusions, high oral or parenteral intake of iron supplements, or a secondary effect of other diseases.

"Hematopoiesis" refers to the formation of cellular components of the blood, derived from hematopoietic stem cells. These stem cells reside in the medulla of the bone marrow and have the unique ability to give rise to all the different mature blood cell types.

"Hemolysis" refers to the rupturing of erythrocytes or red blood cells and the release of their contents into surrounding fluid. Hemolysis in an animal may occur due to a large number of medical conditions, including bacterial infection, parasitic infection, autoimmune disorders and genetic disorders.

"Hepcidin" refers to both an mRNA as well as a protein encoded by the mRNA that is produced by hepatocytes in response to inflammation or to rising levels of iron in the blood. The primary role of hepcidin is to regulate blood iron levels by facilitating a decrease in these blood iron levels. Hepcidin expression is increased in conditions of acute and chronic inflammation resulting in decreased iron availability for erythropoiesis. "Hepcidin" is also referred to as hepcidin antimicrobial peptide; HAMP; HAMP1; HEPC; HFE2; LEAP-1; LEAP1; and liver-expressed antimicrobial peptide.

"Hereditary anemia" refers to anemia which is caused by a hereditary condition that causes red blood cells in the body to die faster than normal, be ineffective in transporting oxygen from the lungs to the different parts of the body, or not be created at all. Examples include, but are not limited to, sickle cell anemia, thalassemia, Fanconi anemia, Diamond Blackfan anemia, Shwachman Diamond syndrome, red cell membrane disorders, glucose-6-phosphate dehydrogenase deficiency, or hereditary hemorrhagic telangiectasia.

"HFE" refers to the human hemochromatosis gene or protein.

"HFE gene mutation" refers to mutations in the HFE gene, which may result in hereditary hemochromatosis.

"Hybridization" means the annealing of complementary nucleic acid molecules. In certain embodiments, complementary nucleic acid molecules include an antisense compound and a target nucleic acid.

"Identifying an animal at risk for or having a disease, disorder and/or condition associated with excess accumulation of iron" means identifying an animal having been diagnosed with a disease, disorder and/or condition or identifying an animal predisposed to develop a disease, disorder and/or condition associated with excess accumulation of iron. For example, an animal can be predisposed to develop a disease, disorder and/or condition associated with excess accumulation of iron if the animal has a family history of hemochromatosis. Such identification may be accomplished by any method including evaluating an animal's medical history and standard clinical tests or assessments.

"Immediately adjacent" means that there are no intervening elements between the immediately adjacent elements.

"Individual" or "subject" or "animal" means a human or non-human animal selected for treatment or therapy.

"Inhibiting the expression or activity" refers to a reduction or blockade of the expression or activity of a RNA or protein and does not necessarily indicate a total elimination of expression or activity.

"Internucleoside linkage" refers to the chemical bond between nucleosides.

"Intravenous administration" means administration into a vein.

"Iron accumulation" or "iron overload" indicates accumulation and deposition of iron in the body from any cause. The most common causes are hereditary causes, transfusional iron overload, which can result from repeated blood transfusions, or excessive dietary iron intake.

"Iron supplements" refer to supplements prescribed for a medical reason to treat iron deficiency in a patient. Iron can be supplemented by the oral route or given parenterally.

"Linked nucleosides" means adjacent nucleosides which are bonded together.

"Marker" or "biomarker" is any measurable and quantifiable biological parameter that serves as an index for health- or physiology-related assessments. For example, an increase in the percentage saturation of transferrin, an increase in iron levels, or a decrease in hepcidin levels can be considered markers of an iron overload disease, disorder and/or condition.

"MCH" refers to "mean corpuscular hemoglobin" or "mean cell hemoglobin", a value to express the average mass of hemoglobin (Hb) per red blood cell in a sample of blood.

"MCV" refers to "mean corpuscular volume" or "mean cell volume", a value to express the average red blood cell size.

"Mismatch" or "non-complementary nucleobase" or "MM" refers to the case when a nucleobase of a first nucleic acid is not capable of pairing with the corresponding nucleobase of a second or target nucleic acid.

"Modified internucleoside linkage" refers to a substitution or any change from a naturally occurring internucleoside bond (i.e. a phosphodiester internucleoside bond).

"Modified nucleobase" refers to any nucleobase other than adenine, cytosine, guanine, thymidine, or uracil. For example, a modified nucleobase can be 5-methylcytosine. An "unmodified nucleobase" means the purine bases adenine (A) and guanine (G), and the pyrimidine bases thymine (T), cytosine (C), and uracil (U).

"Modified nucleoside" means a nucleoside having, independently, a modified sugar moiety and/or modified nucleobase.

"Modified nucleotide" means a nucleotide having, independently, a modified sugar moiety, modified internucleoside linkage, and/or modified nucleobase.

"Modified oligonucleotide" means an oligonucleotide comprising a modified internucleoside linkage, a modified sugar, and/or a modified nucleobase.

"Modified sugar" refers to a substitution or change from a natural sugar. For example, a modified sugar can be 2'-MOE.

"Modulating" refers to changing or adjusting a feature in a cell, tissue, organ or organism. For example, modulating TMPRSS6 level can mean to increase or decrease the level of TMPRSS6 mRNA or

TMPRSS6 protein in a cell, tissue, organ or organism. A "modulator" effects the change in the cell, tissue, organ or organism. For example, a TMPRSS6 antisense oligonucleotide can be a modulator that increases or decreases the amount of TMPRSS6 mRNA or TMPRSS6 protein in a cell, tissue, organ or organism.

"Monomer" refers to a single unit of an oligomer. Monomers include, but are not limited to, nucleosides and nucleotides, whether naturally occuring or modified.

"Motif" means the pattern of chemically distinct regions in an antisense compound.

"Mutations" refer to changes in a nucleic acid sequence. Mutations can be caused in a variety of ways including, but not limited to, radiation, viruses, transposons and mutagenic chemicals, as well as errors that occur during meiosis, DNA replication, RNA transcription and post-transcriptional processing. Mutations can result in several different changes in sequence; they can have either no effect, alter the product of a gene, or prevent the gene from functioning properly or completely. For example, HFE mutation can lead to the improper functioning of the gene product, leading to excess iron absorption in the intestines.

"Myelodysplastic syndrome" refers to a diverse collection of hematological medical disease, disorder and/or conditions that involve ineffective production of the myeloid class of blood cells. The syndrome is caused by disorders of the stem cells in the bone marrow. In myelodysplastic syndrome, hematopoiesis is ineffective and the number and quality of blood cells decline irreversibly, further impairing blood production. As a result, patients with myelodysplastic syndrome develop severe anemia and require frequent blood transfusions.

"Naturally occurring internucleoside linkage" means a 3' to 5' phosphodiester linkage.

"Natural sugar moiety" means a sugar found in DNA (2'-H) or RNA (2'-OH).

"Nucleic acid" refers to molecules composed of monomeric nucleotides. A nucleic acid includes ribonucleic acids (RNA), deoxyribonucleic acids (DNA), single-stranded nucleic acids, double-stranded nucleic acids, small interfering ribonucleic acids (siRNA), and microRNAs (miRNA).

"Nucleobase" means a heterocyclic moiety capable of pairing with a base of another nucleic acid.

"Nucleobase sequence" means the order of contiguous nucleobases independent of any sugar, linkage, or nucleobase modification.

"Nucleoside" means a nucleobase linked to a sugar.

"Nucleoside mimetic" includes those structures used to replace the sugar or the sugar and the base and not necessarily the linkage at one or more positions of an oligomeric compound; such as, for example, nucleoside mimetics having morpholino, cyclohexenyl, cyclohexyl, tetrahydropyranyl, bicyclo or tricyclo sugar mimetics e.g. non furanose sugar units.

"Nucleotide" means a nucleoside having a phosphate group covalently linked to the sugar portion of the nucleoside.

"Nucleotide mimetic" includes those structures used to replace the nucleoside and the linkage at one or more positions of an oligomeric compound; such as, for example, peptide nucleic acids or morpholinos (morpholinos linked by -N(H)-C(=O)-O- or other non-phosphodiester linkage).

"Oligomeric compound" or "oligomer" refers to a polymeric structure comprising two or more sub-structures (monomers) and capable of hybridizing to a region of a nucleic acid molecule. In certain embodiments, oligomeric compounds are oligonucleosides. In certain embodiments, oligomeric compounds are oligonucleotides. In certain embodiments, oligomeric compounds are antisense compounds. In certain embodiments, oligomeric compounds are antisense oligonucleotides. In certain embodiments, oligomeric compounds are chimeric oligonucleotides.

"Oligonucleotide" means a polymer of linked nucleosides each of which can be modified or unmodified, independent one from another.

"Parenteral administration" means administration through injection or infusion. Parenteral administration includes subcutaneous administration, intravenous administration, intramuscular administration, intra-arterial administration, intraperitoneal administration, or intracranial administration, e.g., intrathecal or intracerebroventricular administration. Administration can be continuous, or chronic, or short or intermittent.

"Peptide" refers to a molecule formed by linking at least two amino acids by amide bonds. Peptide refers to polypeptides and proteins.

"Percentage saturation of transferrin" refers to the ratio of serum iron to total iron binding capacity multiplied by 100. Of the transferrin molecules that are available to bind iron, this value tells a clinician how much serum iron are actually bound.

"Pharmaceutical composition" means a mixture of substances suitable for administering to an individual. For example, a pharmaceutical composition may comprise one or more active pharmaceutical agents and a sterile aqueous solution.

"Pharmaceutically acceptable carrier" means a medium or diluent that does not interfere with the structure of the oligonucleotide. Certain of such carriers enable pharmaceutical compositions to be formulated as, for example, tablets, pills, dragees, capsules, liquids, gels, syrups, slurries, suspension and lozenges for the oral ingestion by a subject. For example, a pharmaceutically acceptable carrier can be a sterile aqueous solution, such as PBS.

"Pharmaceutically acceptable derivative" encompasses pharmaceutically acceptable salts, conjugates, prodrugs or isomers of the compounds described herein.

"Pharmaceutically acceptable salts" means physiologically and pharmaceutically acceptable salts of antisense compounds, i.e., salts that retain the desired biological activity of the parent oligonucleotide and do not impart undesired toxicological effects thereto.

"Phosphorothioate linkage" means a linkage between nucleosides where the phosphodiester bond is modified by replacing one of the non-bridging oxygen atoms with a sulfur atom. A phosphorothioate linkage is a modified internucleoside linkage.

"Polycythemia" refers to a condition of increased red blood cells (RBCs) in a specified volume due to either an increase in red blood cell numbers (absolute polycythemia) or a decrease in plasma volume

(relative polycythemia). Blood volume to red blood cell proportions can be measured as Hematocrit (Hct) levels. The increased proportion of RBCs can make the blood viscous which can lead to slower blood flow through the circulatory system and potential formation of blood clots. Slower blood flow can decrease oxygen transport to cells, tissue and/or organs leading to diseases, disorders or conditions such as angina or heart failure. Formation of blood clots in the circulatory system can lead to cell, tissue and/or organ damage leading to diseases, disorders or conditions such as myocardial infarction or stroke. Treatment for polycythemia includes phlebotomy or drugs to decrease RBC production (e.g., INF-α, hydroxyurea, anagrelide). Examples of polycythemia include, but is not limited to, polycythemia vera (PCV), polycythemia rubra vera (PRV) and erythremia. In certain instances, polycythemia can progress into erythroid leukemia in a subject.

"Portion" means a defined number of contiguous (i.e. linked) nucleobases of a nucleic acid. In certain embodiments, a portion is a defined number of contiguous nucleobases of a target nucleic acid. In certain embodiments, a portion is a defined number of contiguous nucleobases of an antisense compound.

"Prevent" refers to delaying or forestalling the onset, development, or progression of a disease, disorder, or condition for a period of time from minutes to indefinitely. Prevent also means reducing risk of developing a disease, disorder, or condition.

"Prodrug" means a therapeutic agent that is prepared in an inactive form that is converted to an active form within the body or cells thereof by the action of endogenous enzymes or other chemicals or conditions.

"Side effects" means physiological responses attributable to a treatment other than the desired effects. In certain embodiments, side effects include injection site reactions, liver function test abnormalities, renal function abnormalities, liver toxicity, renal toxicity, central nervous system abnormalities, myopathies, and malaise. For example, increased aminotransferase levels in serum may indicate liver toxicity or liver function abnormality.

"Single-stranded oligonucleotide" means an oligonucleotide which is not hybridized to a complementary strand.

"Specifically hybridizable" refers to an antisense compound having a sufficient degree of complementarity with a target nucleic acid to induce a desired effect, while exhibiting minimal or no effects on non-target nucleic acids under conditions in which specific binding is desired, i.e. under physiological conditions in the case of *in vivo* assays and therapeutic treatments.

"Subcutaneous administration" means administration just below the skin.

"Targeting" or "targeted" means the process of design and selection of an antisense compound that will specifically hybridize to a target nucleic acid and induce a desired effect.

"Target nucleic acid," "target RNA," and "target RNA transcript" all refer to a nucleic acid capable of being targeted by antisense compounds.

"Target segment" means the sequence of nucleotides of a target nucleic acid to which an antisense compound is targeted. "5' target site" refers to the 5'-most nucleotide of a target segment. "3' target site" refers to the 3'-most nucleotide of a target segment.

"Thalassemia" refers to a subgroup of anemias (e.g., α-thalassemia, β-thalassemia, δ-thalassemia, non-transfusion dependent thalassemia (NTDT)) caused by the formation of abnormal hemoglobin molecules leading to the destruction or degradation of red blood cells. Complications of thalassemia include excess iron (i.e. iron overload in the blood either from the thalassemia itself or from frequent transfusions to treat the thalassemia), increased risk of infection, bone deformities, enlarged spleens (i.e. splenomegaly), slowed growth rates and heart problems (e.g., congestive heart failure and arrhythmias).

"Therapeutically effective amount" means an amount of a pharmaceutical agent that provides a therapeutic benefit to an animal.

"TMPRSS6" (also known as "matriptase-2") refers to any nucleic acid or protein of TMPRSS6.

"TMPRSS6 nucleic acid" means any nucleic acid encoding TMPRSS6. For example, in certain embodiments, a TMPRSS6 nucleic acid includes a DNA sequence encoding TMPRSS6, a RNA sequence transcribed from DNA encoding TMPRSS6 (including genomic DNA comprising introns and exons), and a mRNA sequence encoding TMPRSS6. "TMPRSS6 mRNA" means a mRNA encoding a TMPRSS6 protein.

"TMPRSS6 specific inhibitor" refers to any agent capable of specifically inhibiting the expression of TMPRSS6 gene, TMPRSS6 RNA and/or TMPRSS6 protein at the molecular level. For example, TMPRSS6 specific inhibitors include nucleic acids (including antisense compounds), peptides, antibodies, small molecules, and other agents capable of inhibiting the level of TMPRSS6. In certain embodiments, by specifically modulating TMPRSS6, TMPRSS6 specific inhibitors may affect components of the iron accumulation pathway.

"Treat" refers to administering a pharmaceutical composition to an animal in order to effect an alteration or improvement of a disease, disorder, or condition in the animal. In certain embodiments, one or more pharmaceutical compositions can be administered to the animal.

"Unmodified nucleotide" means a nucleotide composed of naturally occuring nucleobases, sugar moieties, and internucleoside linkages. In certain embodiments, an unmodified nucleotide is an RNA nucleotide (i.e. β-D-ribonucleotide) or a DNA nucleotide (i.e. β-D-deoxyribonucleotide).

Certain Embodiments

In certain embodiments disclosed herein, TMPRSS6 has the sequence as set forth in: GenBank Accession No. NM_153609.2 (incorporated herein as SEQ ID NO: 1); the complement of GENBANK Accession NT_011520.12 truncated from 16850000 to 16897000 (incorporated herein as SEQ ID NO: 2); GENBANK Accession CR456446.1 (incorporated herein as SEQ ID NO: 3); GENBANK Accession No. BC039082.1 (incorporated herein as SEQ ID NO: 4); GENBANK Accession No. AY358398.1 (incorporated

herein as SEQ ID NO: 5); and GENBANK Accession No. DB081153.1 (incorporated herein as SEQ ID NO: 6).

Certain embodiments disclosed herein provide a compound comprising a modified oligonucleotide targeting a nucleic acid sequence encoding TMPRSS6. In certain embodiments, the compound targets a TMPRSS6 sequence as shown in the nucleobase sequences of any of SEQ ID NOs: 1-6.

Certain embodiments disclosed herein provide a compound comprising a modified oligonucleotide consisting of 12 to 30 linked nucleosides having a nucleobase sequence comprising at least 8, least 9, least 10, least 11, at least 12, least 13, at least 14, at least 15, at least 16, least 17, least 18, least 19, or 20 contiguous nucleobases complementary to an equal length portion of SEQ ID NOs: 1-6.

Certain embodiments disclosed herein provide a compound comprising a modified oligonucleotide consisting of 12 to 30 linked nucleosides having a nucleobase sequence comprising a portion of at least 8 contiguous nucleobases complementary to an equal length portion of nucleobases 3162 to 3184 of SEQ ID NO: 1, wherein the nucleobase sequence of the modified oligonucleotide is at least 80% complementary to SEQ ID NO: 1.

Certain embodiments disclosed herein provide a compound comprising a modified oligonucleotide consisting of 12 to 30 linked nucleosides having a nucleobase sequence comprising a portion of at least 8 contiguous nucleobases complementary to an equal length portion of nucleobases 1286 to 1305 of SEQ ID NO: 1, wherein the nucleobase sequence of the modified oligonucleotide is at least 80% complementary to SEQ ID NO: 1.

Certain embodiments disclosed herein provide a compound comprising a modified oligonucleotide consisting of 12 to 30 linked nucleosides having a nucleobase sequence comprising a portion of at least 8, at least 9, at least 10, at least 11, at least 12, at least 13, at least 14, at least 15, at least 16, at least 17, at least 18, at least 19, or at least 20 contiguous nucleobases complementary to an equal length portion of nucleobases 3162 to 3184 of SEQ ID NO: 1, wherein the nucleobase sequence of the modified oligonucleotide is at least 80% complementary to SEQ ID NO: 1.

Certain embodiments disclosed herein provide a compound comprising a modified oligonucleotide consisting of 12 to 30 linked nucleosides having a nucleobase sequence comprising a portion of at least 8, at least 9, at least 10, at least 11, at least 12, at least 13, at least 14, at least 15, at least 16, at least 17, at least 18, at least 19, or at least 20 contiguous nucleobases complementary to an equal length portion of nucleobases 1286 to 1305 of SEQ ID NO: 1, wherein the nucleobase sequence of the modified oligonucleotide is at least 80% complementary to SEQ ID NO: 1.

Certain embodiments disclosed herein provide a compound comprising a modified oligonucleotide consisting of 12 to 30 linked nucleosides having a nucleobase sequence comprising at least 8, at least 9, at least 10, at least 11, at least 12, at least 13, at least 14, at least 15, at least 16, at least 17, at least 18, at least 19, or 20 contiguous nucleobases of any of the nucleobase sequences of SEQ ID NOs: 7-85.

Certain embodiments disclosed herein provide a compound comprising a modified oligonucleotide consisting of 12 to 30 linked nucleosides having a nucleobase sequence comprising at least 8, at least 9, at least 10, at least 11, at least 12, at least 13, at least 14, at least 15, at least 16, at least 17, at least 18, at least 19, or 20 contiguous nucleobases of any of the nucleobase sequences of SEQ ID NOs: 23, 36, 37, 63, 77.

Certain embodiments disclosed herein provide a compound comprising a modified oligonucleotide consisting of 12 to 30 linked nucleosides having a nucleobase sequence comprising at least 8, at least 9, at least 10, at least 11, at least 12, at least 13, at least 14, at least 15, at least 16, at least 17, at least 18, at least 19, or 20 contiguous nucleobases of SEQ ID NO: 36.

Certain embodiments disclosed herein provide a compound comprising a modified oligonucleotide consisting of 12 to 30 linked nucleosides having a nucleobase sequence comprising at least 8, at least 9, at least 10, at least 11, at least 12, at least 13, at least 14, at least 15, at least 16, at least 17, at least 18, at least 19, or 20 contiguous nucleobases of SEQ ID NO: 77.

In certain embodiments, the nucleobase sequence of the modified oligonucleotide is at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% complementary to an equal length portion of any of SEQ ID NOs: 1-6. In certain embodiments, the modified oligonucleotide comprises a nucleobase sequence 100% complementary to an equal length portion of any of SEQ ID NOs: 1-6.

In certain embodiments, the compound comprises a modified oligonucleotide consisting of 8 to 80, 20 to 80, 10 to 50, 20 to 35, 10 to 30, 12 to 30, 15 to 30, 16 to 30, 20 to 30, 20 to 29, 20 to 28, 20 to 27, 20 to 26, 20 to 25, 20 to 24, 20 to 23, 20 to 22, 20 to 21, 15 to 25, 16 to 25, 15 to 24, 16 to 24, 17 to 24, 18 to 24, 19 to 24, 19 to 22, 16 to 21, 18 to 21 or 16 to 20 linked nucleobases. In certain embodiments, the compound comprises a modified oligonucleotide consisting of 16 linked nucleosides. In certain embodiments, the compound comprises a modified oligonucleotide consisting of 20 linked nucleosides.

In certain embodiments, the compound comprises a modified oligonucleotide consisting of 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, or 80 linked nucleobases in length, or a range defined by any two of the above values.

In certain embodiments, the modified oligonucleotide is single-stranded.

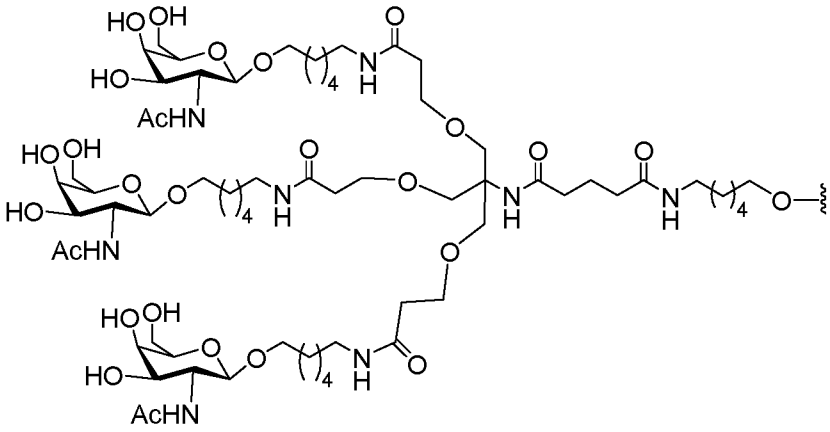
In certain embodiments, the modified oligonucleotide comprises at least one modified internucleoside linkage. In certain embodiments, the modified internucleoside linkage is a phosphorothioate internucleoside linkage. In certain embodiments, at least one modified internucleoside linkage is a phosphorothioate internucleoside linkage. In certain embodiments, each modified internucleoside linkage is a phosphorothioate internucleoside linkage.

In certain embodiments, the modified oligonucleotide comprises at least one nucleoside comprising a modified sugar. In certain embodiments, at least one modified sugar comprises a bicyclic sugar. In certain

embodiments, at least one modified sugar comprises a 2'-O-methoxyethyl, a constrained ethyl, a 3'-fluoro-HNA or a 4'- $(CH_2)_n$-O-2' bridge, wherein n is 1 or 2.

In certain embodiments, the modified oligonucleotide comprises at least one nucleoside comprising a modified nucleobase. In certain embodiments, the modified nucleobase is a 5-methylcytosine.

In certain embodiments, the modified oligonucleotide comprises a conjugate group. In certain embodiments, the conjugate is a carbohydrate moiety. In certain embodiments, the conjugate is a GalNAc moiety. In certain embodiments, the GalNAc is 5'-Trishexylamino-(THA)-C6 $GalNAc_3$. In certain embodiments, the conjugate has the formula



In certain embodiments, the compound comprises a modified oligonucleotide consisting of 12 to 30 linked nucleosides and targeted to or complementary to an equal length portion ofregion 3162 to 3184 of SEQ ID NO: 1, wherein the modified oligonucleotide comprises: (a) a gap segment consisting of linked deoxynucleosides; (b) a 5' wing segment consisting of linked nucleosides; and (c) a 3' wing segment consisting of linked nucleosides; wherein the gap segment is positioned immediately adjacent to and between the 5' wing segment and the 3' wing segment and wherein each nucleoside of each wing segment comprises a modified sugar. In certain embodiments, the modified oligonucleotide further comprises at least one phosphorothioate internucleoside linkage. In certain embodiments, the modified oligonucleotide further comprises a GalNAc conjugate. In certain embodiments, the conjugate is a 5'-Trishexylamino-(THA)-C6 $GalNAc_3$ conjugate.

In certain embodiments, the compound comprises a modified oligonucleotide consisting of 12 to 30 linked nucleosides and targeted to or complementary to an equal length portion of region 1286 to 1305 of SEQ ID NO: 1, wherein the modified oligonucleotide comprises: (a) a gap segment consisting of linked deoxynucleosides; (b) a 5' wing segment consisting of linked nucleosides; and (c) a 3' wing segment consisting of linked nucleosides; wherein the gap segment is positioned immediately adjacent to and between the 5' wing segment and the 3' wing segment and wherein each nucleoside of each wing segment comprises a modified sugar. In certain embodiments, the modified oligonucleotide further comprises at least one

phosphorothioate internucleoside linkage. In certain embodiments, the modified oligonucleotide further comprises a GalNAc conjugate. In certain embodiments, the conjugate is a 5'-Trishexylamino-(THA)-C6 $GalNAc_3$ conjugate.

In certain embodiments, the compound comprises a modified oligonucleotide consisting of 20 linked nucleosides and targeted to or complementary to an equal length portion of region 3162 to 3181 of SEQ ID NO: 1, wherein the modified oligonucleotide comprises: (a) a gap segment consisting of ten linked deoxynucleosides; (b) a 5' wing segment consisting of five linked nucleosides; and (c) a 3' wing segment consisting of five linked nucleosides; wherein the gap segment is positioned immediately adjacent to and between the 5' wing segment and the 3' wing segment, wherein each nucleoside of each wing segment comprises a 2'-O-methoxyethyl sugar, wherein at least one internucleoside linkage is a phosphorothioate linkage and wherein each cytosine residue is a 5-methylcytosine. In certain embodiments, the modified oligonucleotide further comprises a GalNAc conjugate. In certain embodiments, the conjugate is a 5'-Trishexylamino-(THA)-C6 $GalNAc_3$ conjugate.

In certain embodiments, the compound comprises a modified oligonucleotide consisting of 16 linked nucleosides and targeted to or complementary to an equal length portion of region 3169 to 3184 of SEQ ID NO: 1, wherein the modified oligonucleotide comprises: (a) a gap segment consisting of nine linked deoxynucleosides; (b) a 5' wing segment consisting of three linked nucleosides; and (c) a 3' wing segment consisting of four linked nucleosides; wherein the gap segment is positioned immediately adjacent to and between the 5' wing segment and the 3' wing segment, wherein each nucleoside of each wing segment comprises a modified sugar, wherein each internucleoside linkage is a phosphorothioate linkage and wherein each cytosine residue is a 5-methylcytosine. In certain embodiments, the modified oligonucleotide further comprises a GalNAc conjugate. In certain embodiments, the conjugate is a 5'-Trishexylamino-(THA)-C6 $GalNAc_3$ conjugate.

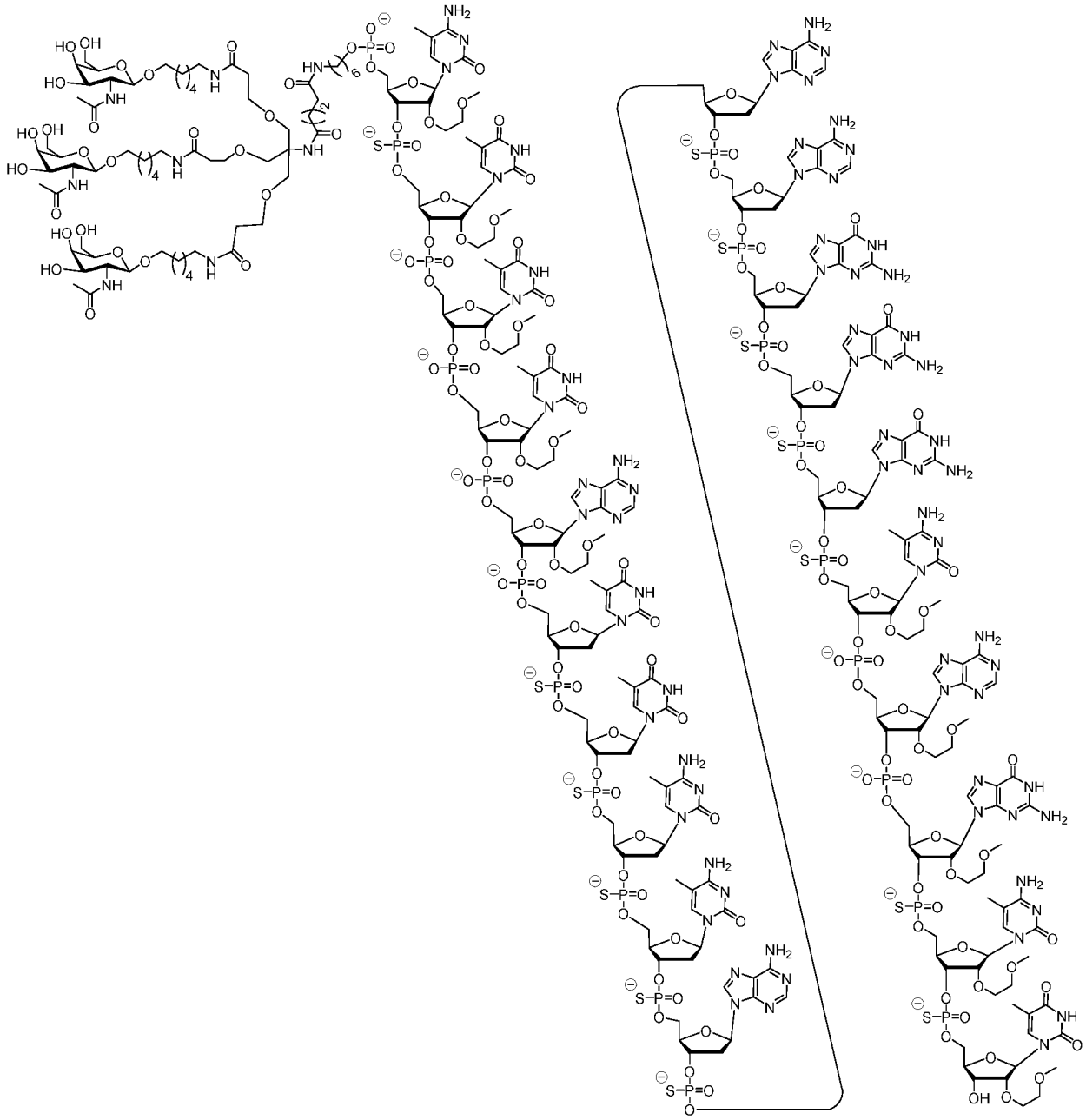
In certain embodiments, the compound comprising a modified oligonucleotide consisting of 20 linked nucleosides and having a nucleobase sequence comprising at least 8 contiguous nucleobases of SEQ ID NO: 36, wherein the modified oligonucleotide comprises: (a) a gap segment consisting of ten linked deoxynucleosides; (b) a 5' wing segment consisting of five linked nucleosides; and (c) a 3' wing segment consisting of five linked nucleosides; wherein the gap segment is positioned immediately adjacent to and between the 5' wing segment and the 3' wing segment, wherein each nucleoside of each wing segment comprises a 2'-O-methoxyethyl sugar, wherein at least one internucleoside linkage is a phosphorothioate linkage and wherein each cytosine residue is a 5-methylcytosine. In certain embodiments, the modified oligonucleotide further comprises a GalNAc conjugate. In certain embodiments, the conjugate is a 5'-Trishexylamino-(THA)-C6 $GalNAc_3$ conjugate.

In certain embodiments, the compound comprising a modified oligonucleotide consisting of 16 linked nucleosides and having a nucleobase sequence comprising at least 8 contiguous nucleobases of SEQ ID NO: 77, wherein the modified oligonucleotide comprises: (a) a gap segment consisting of nine linked

deoxynucleosides; (b) a 5' wing segment consisting of three linked nucleosides; and (c) a 3' wing segment consisting of four linked nucleosides; wherein the gap segment is positioned immediately adjacent to and between the 5' wing segment and the 3' wing segment, wherein each nucleoside of each wing segment comprises a modified sugar, wherein each internucleoside linkage is a phosphorothioate linkage and wherein each cytosine residue is a 5-methylcytosine. In certain embodiments, the modified oligonucleotide further comprises a GalNAc conjugate. In certain embodiments, the conjugate is a 5'-Trishexylamino-(THA)-C6 $GalNAc_3$ conjugate.

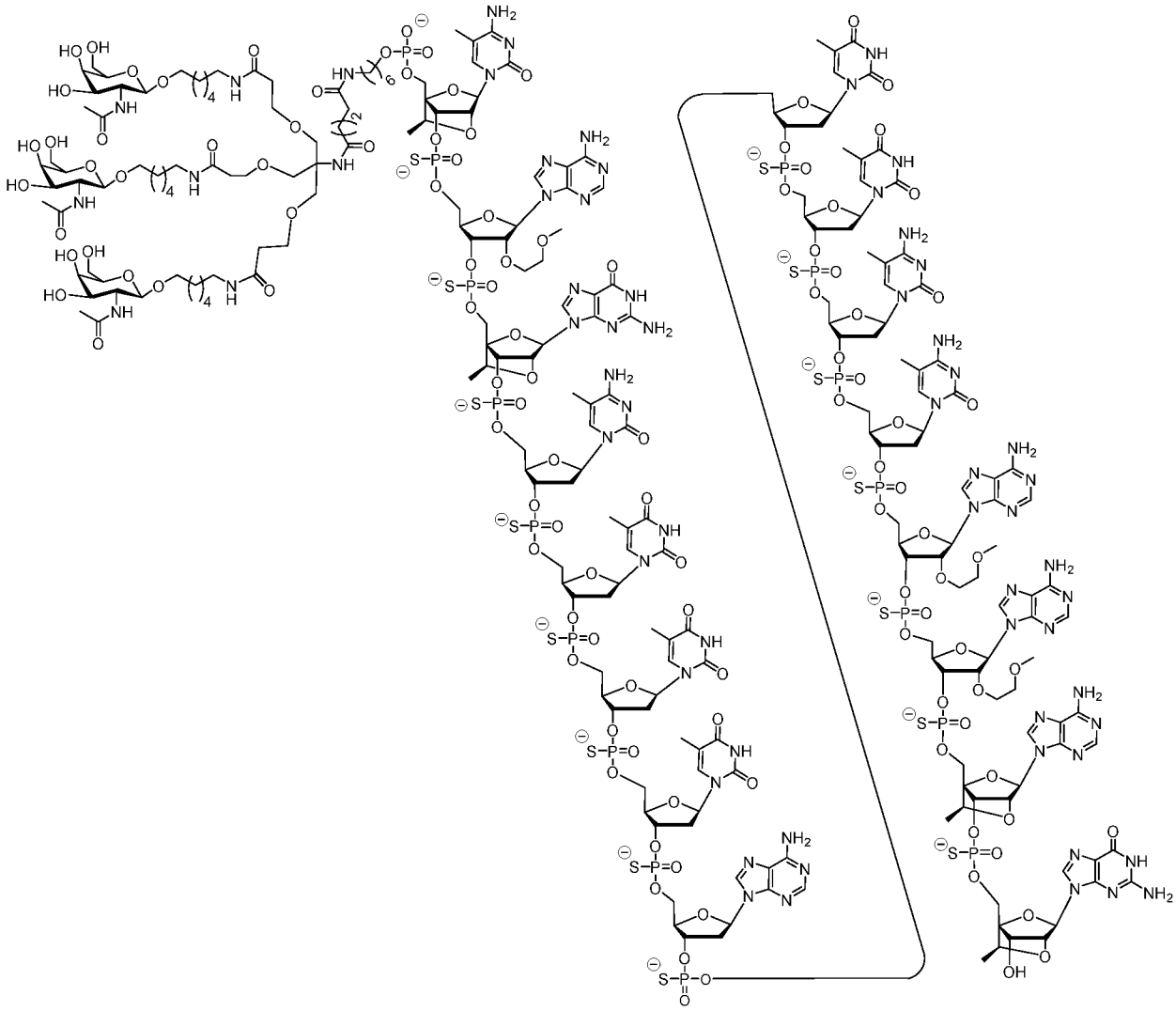
Certain embodiments disclosed herein provide a compound comprising a modified oligonucleotide according to the following formula: mCes Teo Teo Teo Aeo Tds Tds mCds mCds Ads Ads Ads Gds Gds Gds mCeo Aeo Ges mCes Te (SEQ ID NO: 36); wherein, A is an adenine, mC is a 5-methylcytosine, G is a guanine, T is a thymine, e is a 2'-O-methoxyethyl modified nucleoside, d is a 2'-deoxynucleoside, and s is a phosphorothioate internucleoside linkage. In certain embodiments, the modified oligonucleotide further comprises a GalNAc conjugate. In certain embodiments, the conjugate is a 5'-Trishexylamino-(THA)-C6 $GalNAc_3$ conjugate.

Certain embodiments disclosed herein provide a compound comprising a modified oligonucleotide with the following formula:



Certain embodiments disclosed herein provide a compound comprising a modified oligonucleotide according to the following formula: mCks Aes Gks mCds Tds Tds Tds Ads Tds Tds mCds mCds Aes Aes Aks Gk (SEQ ID NO: 77); wherein, A is an adenine, mC is a 5-methylcytosine, G is a guanine, T is a thymine, e is a 2'-O-methoxyethyl modified nucleoside, d is a 2'-deoxynucleoside, s is a phosphorothioate internucleoside linkage, and k is a cEt. In certain embodiments, the modified oligonucleotide further comprises a GalNAc conjugate. In certain embodiments, the conjugate is a 5'-Trishexylamino-(THA)-C6 $GalNAc_3$ conjugate.

Certain embodiments disclosed herein provide a compound comprising a modified oligonucleotide with the following formula:



In certain embodiments, the compounds or compositions disclosed herein comprise a salt of the modified oligonucleotide.

In certain embodiments, the compounds or compositions disclosed herein further comprise a pharmaceutically acceptable carrier or diluent.

In certain embodiments, the animal is a human.

Certain embodiments provide a composition or compound comprising a modified oligonucleotide as described herein, wherein the viscosity level is less than 40 cP. In certain embodiments, the composition has a viscosity level less than 15 cP. In certain embodiments, the composition has a viscosity level less than 12 cP. In certain embodiments, the composition has a viscosity level less than 10 cP.

Certain embodiments disclosed herein provide compounds and compositions comprising a modified oligonucleotide targeting TMPRSS6 for use in reducing TMPRSS6 in a cell, tissue, organ or animal.

Certain embodiments disclosed herein provide compounds and compositions comprising a modified oligonucleotide targeting TMPRSS6 for use in reducing iron levels in a cell, tissue, organ or animal. In certain embodiments, the compounds and compositions reduce serum iron levels. In certain embodiments, the compounds and compositions reduce liver iron levels. In certain embodiments, the compounds and compositions reduce iron absorption. In certain embodiments, the compounds and compositions reduce iron overload or accumulation. In certain embodiments, reducing iron overload/accumulation ameliorates, treats, prevents or delays a disease, disorder or condition related to iron overload.

Certain embodiments disclosed herein provide compounds and compositions comprising a modified oligonucleotide targeting TMPRSS6 for use in increasing hepcidin levels, such as mRNA or protein expression levels, in an animal.

Certain embodiments disclosed herein provide compounds and compositions comprising a modified oligonucleotide targeting TMPRSS6 for use in decreasing the percentage saturation of transferrin in an animal. In certain embodiments, decreasing transferrin saturation leads to a decrease in iron supply for erythropoiesis. In certain embodiments, the decrease in erythropoiesis treats, prevents, delays the onset of, ameliorates, and/or reduces polycythemia, or symptom thereof, in the animal. In certain embodiments, the polycythemia is polycythemia vera. In certain embodiments, treatment with the modified oligonucleotide targeting TMPRSS6 prevents or delays the polycythemia from progressing into erythroid leukemia.

Certain embodiments disclosed herein provide compounds and compositions comprising a modified oligonucleotide targeting TMPRSS6 for reducing iron accumulation in an animal. In certain embodiments, compounds and compositions comprising a modified oligonucleotide targeting TMPRSS6 are used for treating, preventing, slowing the progression, delaying the onset of, ameliorating and/or reducing a disease, disorder and/or condition, or symptom thereof, associated with the excess accumulation of iron in an animal.

In certain embodiments, the iron accumulation is the result of, or cause of, a disease, disorder or condition in the animal. In certain embodiments, the disease, disorder or condition is ineffective erythropoiesis, polycythemia, hemochromatosis or anemia. In certain embodiments, the hemochromatosis is hereditary hemochromatosis. In certain embodiments, the anemia is hereditary anemia, myelodysplastic syndrome or severe chronic hemolysis. In certain embodiments, the hereditary anemia is sickle cell anemia, thalassemia, Fanconi anemia, Diamond Blackfan anemia, Shwachman Diamond syndrome, red cell membrane disorders, glucose-6-phosphate dehydrogenase deficiency, or hereditary hemorrhagic telangiectasia. In certain embodiments, the thalassemia is β-thalassemia. In certain embodiments, the β-thalassemia is β-thalassemia major, β-thalassemia intermedia or β-thalassemia minor. In certain embodiments, the the disease, disorder or condition is associated with mutations in the HFE gene. In other embodiments, the disease is associated with mutations in the hemojuvelin gene. In other embodiments, the disease is associated with mutations in the hepcidin gene.

In certain embodiments, the iron accumulation is the result of a therapy to treat a disease, disorder or condition in the animal. In certain embodiments, the therapy is phlebotomy or transfusion therapy. In certain

embodiments, the disease, disorder and/or condition may be due to multiple blood transfusions. In certain embodiments, multiple transfusions may lead to polycythemia. In certain embodiments, multiple blood transfusions are associated with the animal having anemia. Examples of anemia requiring multiple blood transfusions are hereditary anemia, myelodysplastic syndrome and severe chronic hemolysis.

In certain embodiments, the disease, disorder and/or condition is associated with excess parenteral iron supplement intake or excess dietary iron intake.

In certain embodiments, provided are compounds and compositions comprising a modified oligonucleotide targeting TMPRSS6 for use in therapy. In certain embodiments, the compounds and compositions comprising a modified oligonucleotide targeting TMPRSS6 are administered to an animal in a therapeutically effective amount.

In certain embodiments, provided are compounds and compositions comprising a modified oligonucleotide targeting TMPRSS6 for use in the preparation of a medicament. In certain embodiments, the medicament is used for treating, preventing, slowing the progression, delaying the onset of, and/or reducing a disease, disorder and/or condition, or symptom thereof, associated with excess accumulation of iron in an animal.

In certain embodiments, the composition or compound comprising a modified oligonucleotide targeting TMPRSS6 is co-administered with one or more second agent(s). In certain embodiments the second agent is an iron chelator or a hepcidin agonist. In further embodiments, the iron chelator includes FBS0701 (FerroKin), Exjade, Desferal or Deferiprone (DFP). In certain embodiments, the second agent is a second antisense compound. In further embodiments, the second antisense compound targets TMPRSS6. In other embodiments, the second antisense compound targets a non-TMPRSS6 compound. In other embodiments, the composition or compound comprising a modified oligonucleotide targeting TMPRSS6 is administered before, during or after phlebotomy or transfusion therapy.

Antisense Compounds

Oligomeric compounds include, but are not limited to, oligonucleotides, oligonucleosides, oligonucleotide analogs, oligonucleotide mimetics, antisense compounds, antisense oligonucleotides, and siRNAs. An oligomeric compound can be "antisense" to a target nucleic acid, meaning that it is capable of undergoing hybridization to a target nucleic acid through hydrogen bonding.

In certain embodiments, an antisense compound has a nucleobase sequence that, when written in the 5' to 3' direction, comprises the reverse complement of the target segment of a target nucleic acid to which it is targeted. In certain such embodiments, an antisense oligonucleotide has a nucleobase sequence that, when written in the 5' to 3' direction, comprises the reverse complement of the target segment of a target nucleic acid to which it is targeted.

In certain embodiments, an antisense compound targeted to TMPRSS6 nucleic acid is 10 to 30 nucleotides in length. In other words, antisense compounds are from 10 to 30 linked nucleobases. In other

embodiments, the antisense compound comprises a modified oligonucleotide consisting of 8 to 80, 10 to 80, 12 to 50, 15 to 30, 18 to 24, 19 to 22, or 20 linked nucleobases. In certain such embodiments, the antisense compound comprises a modified oligonucleotide consisting of 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, or 80 linked nucleobases in length, or a range defined by any two of the above values. In some embodiments, the antisense compound is an antisense oligonucleotide.

In certain embodiments, the antisense compound comprises a shortened or truncated modified oligonucleotide. The shortened or truncated modified oligonucleotide can have a single nucleoside deleted from the 5' end (5' truncation), the central portion or alternatively from the 3' end (3' truncation). A shortened or truncated oligonucleotide can have two or more nucleosides deleted from the 5' end, two or more nucleosides deleted from the central portion or alternatively can have two or more nucleosides deleted from the 3' end. Alternatively, the deleted nucleosides can be dispersed throughout the modified oligonucleotide, for example, in an antisense compound having one or more nucleoside deleted from the 5' end, one or more nucleoside deleted from the central portion and/or one or more nucleoside deleted from the 3' end.

When a single additional nucleoside is present in a lengthened oligonucleotide, the additional nucleoside can be located at the 5' end, 3' end or central portion of the oligonucleotide. When two or more additional nucleosides are present, the added nucleosides can be adjacent to each other, for example, in an oligonucleotide having two nucleosides added to the 5' end (5' addition), to the 3' end (3' addition) or the central portion, of the oligonucleotide. Alternatively, the added nucleoside can be dispersed throughout the antisense compound, for example, in an oligonucleotide having one or more nucleoside added to the 5' end, one or more nucleoside added to the 3' end, and/or one or more nucleoside added to the central portion.

It is possible to increase or decrease the length of an antisense compound, such as an antisense oligonucleotide, and/or introduce mismatch bases without eliminating activity. For example, in Woolf et al. (Proc. Natl. Acad. Sci. USA 89:7305-7309, 1992), a series of antisense oligonucleotides 13-25 nucleobases in length were tested for their ability to induce cleavage of a target RNA in an oocyte injection model. Antisense oligonucleotides 25 nucleobases in length with 8 or 11 mismatch bases near the ends of the antisense oligonucleotides were able to direct specific cleavage of the target mRNA, albeit to a lesser extent than the antisense oligonucleotides that contained no mismatches. Similarly, target specific cleavage was achieved using 13 nucleobase antisense oligonucleotides, including those with 1 or 3 mismatches.

Gautschi et al (J. Natl. Cancer Inst. 93:463-471, March 2001) demonstrated the ability of an oligonucleotide having 100% complementarity to the bcl-2 mRNA and having 3 mismatches to the bcl-xL mRNA to reduce the expression of both bcl-2 and bcl-xL in vitro and in vivo. Furthermore, this oligonucleotide demonstrated potent anti-tumor activity in vivo.

Maher and Dolnick (Nuc. Acid. Res. 16:3341-3358, 1988) tested a series of tandem 14 nucleobase antisense oligonucleotides, and a 28 and 42 nucleobase antisense oligonucleotides comprised of the sequence of two or three of the tandem antisense oligonucleotides, respectively, for their ability to arrest translation of human DHFR in a rabbit reticulocyte assay. Each of the three 14 nucleobase antisense oligonucleotides alone was able to inhibit translation, albeit at a more modest level than the 28 or 42 nucleobase antisense oligonucleotides.

Certain Antisense Compound Motifs and Mechanisms

In certain embodiments, antisense compounds have chemically modified subunits arranged in patterns, or motifs, to confer to the antisense compounds properties such as enhanced inhibitory activity, increased binding affinity for a target nucleic acid, or resistance to degradation by *in vivo* nucleases.

Chimeric antisense compounds typically contain at least one region modified so as to confer increased resistance to nuclease degradation, increased cellular uptake, increased binding affinity for the target nucleic acid, and/or increased inhibitory activity. A second region of a chimeric antisense compound may confer another desired property e.g., serve as a substrate for the cellular endonuclease RNase H, which cleaves the RNA strand of an RNA:DNA duplex.

Antisense activity may result from any mechanism involving the hybridization of the antisense compound (e.g., oligonucleotide) with a target nucleic acid, wherein the hybridization ultimately results in a biological effect. In certain embodiments, the amount and/or activity of the target nucleic acid is modulated. In certain embodiments, the amount and/or activity of the target nucleic acid is reduced. In certain embodiments, hybridization of the antisense compound to the target nucleic acid ultimately results in target nucleic acid degradation. In certain embodiments, hybridization of the antisense compound to the target nucleic acid does not result in target nucleic acid degradation. In certain such embodiments, the presence of the antisense compound hybridized with the target nucleic acid (occupancy) results in a modulation of antisense activity. In certain embodiments, antisense compounds having a particular chemical motif or pattern of chemical modifications are particularly suited to exploit one or more mechanisms. In certain embodiments, antisense compounds function through more than one mechanism and/or through mechanisms that have not been elucidated. Accordingly, the antisense compounds described herein are not limited by particular mechanism.

Antisense mechanisms include, without limitation, RNase H mediated antisense; RNAi mechanisms, which utilize the RISC pathway and include, without limitation, siRNA, ssRNA and microRNA mechanisms; and occupancy based mechanisms. Certain antisense compounds may act through more than one such mechanism and/or through additional mechanisms.

RNase H-Mediated Antisense

In certain embodiments, antisense activity results at least in part from degradation of target RNA by RNase H. RNase H is a cellular endonuclease that cleaves the RNA strand of an RNA:DNA duplex. It is known in the art that single-stranded antisense compounds which are "DNA-like" elicit RNase H activity in mammalian cells. Accordingly, antisense compounds comprising at least a portion of DNA or DNA-like nucleosides may activate RNase H, resulting in cleavage of the target nucleic acid. In certain embodiments, antisense compounds that utilize RNase H comprise one or more modified nucleosides. In certain embodiments, such antisense compounds comprise at least one block of 1-8 modified nucleosides. In certain such embodiments, the modified nucleosides do not support RNase H activity. In certain embodiments, such antisense compounds are gapmers, as described herein. In certain such embodiments, the gap of the gapmer comprises DNA nucleosides. In certain such embodiments, the gap of the gapmer comprises DNA-like nucleosides. In certain such embodiments, the gap of the gapmer comprises DNA nucleosides and DNA-like nucleosides.

Certain antisense compounds having a gapmer motif are considered chimeric antisense compounds. In a gapmer an internal region having a plurality of nucleotides that supports RNaseH cleavage is positioned between external regions having a plurality of nucleotides that are chemically distinct from the nucleosides of the internal region. In the case of an antisense oligonucleotide having a gapmer motif, the gap segment generally serves as the substrate for endonuclease cleavage, while the wing segments comprise modified nucleosides. In certain embodiments, the regions of a gapmer are differentiated by the types of sugar moieties comprising each distinct region. The types of sugar moieties that are used to differentiate the regions of a gapmer may in some embodiments include β-D-ribonucleosides, β-D-deoxyribonucleosides, 2'-modified nucleosides (such 2'-modified nucleosides may include 2'-MOE and 2'-O-CH_3, among others), and bicyclic sugar modified nucleosides (such bicyclic sugar modified nucleosides may include those having a constrained ethyl). In certain embodiments, nucleosides in the wings may include several modified sugar moieties, including, for example 2'-MOE and bicyclic sugar moieties such as constrained ethyl (cEt) or LNA. In certain embodiments, wings may include several modified and unmodified sugar moieties. In certain embodiments, wings may include various combinations of 2'-MOE nucleosides, bicyclic sugar moieties such as constrained ethyl nucleosides or LNA nucleosides, and 2'-deoxynucleosides.

Each distinct region may comprise uniform sugar moieties, variant, or alternating sugar moieties. The wing-gap-wing motif is frequently described as "X-Y-Z", where "X" represents the length of the 5'-wing, "Y" represents the length of the gap, and "Z" represents the length of the 3'-wing. "X" and "Z" may comprise uniform, variant, or alternating sugar moieties. In certain embodiments, "X" and "Y" may include one or more 2'-deoxynucleosides. "Y" may comprise 2'-deoxynucleosides. As used herein, a gapmer described as "X-Y-Z" has a configuration such that the gap is positioned immediately adjacent to each of the 5'-wing and the 3' wing. Thus, no intervening nucleotides exist between the 5'-wing and gap, or the gap and the 3'-wing. Any of the antisense compounds described herein can have a gapmer motif. In certain

embodiments, "X" and "Z" are the same; in other embodiments they are different. In certain embodiments, "Y" is between 8 and 15 nucleosides. X, Y, or Z can be any of 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30 or more nucleosides.

In certain embodiments, the antisense compound targeted to a TMPRSS6 nucleic acid has a gapmer motif in which the gap consists of 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, or 16 linked nucleosides.

In certain embodiments, the antisense oligonucleotide has a sugar motif described by Formula A as follows: $(J)_m$-$(B)_n$-$(J)_p$-$(B)_r$-$(A)_t$-$(D)_g$-$(A)_v$-$(B)_w$-$(J)_x$-$(B)_y$-$(J)_z$

wherein:

each A is independently a 2'-substituted nucleoside;

each B is independently a bicyclic nucleoside;

each J is independently either a 2'-substituted nucleoside or a 2'-deoxynucleoside;

each D is a 2'-deoxynucleoside;

m is 0-4; n is 0-2; p is 0-2; r is 0-2; t is 0-2; v is 0-2; w is 0-4; x is 0-2; y is 0-2; z is 0-4; g is 6-14; provided that:

at least one of m, n, and r is other than 0;

at least one of w and y is other than 0;

the sum of m, n, p, r, and t is from 2 to 5; and

the sum of v, w, x, y, and z is from 2 to 5.

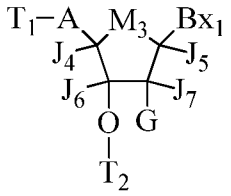
RNAi Compounds

In certain embodiments, antisense compounds are interfering RNA compounds (RNAi), which include double-stranded RNA compounds (also referred to as short-interfering RNA or siRNA) and single-stranded RNAi compounds (or ssRNA). Such compounds work at least in part through the RISC pathway to degrade and/or sequester a target nucleic acid (thus, include microRNA/microRNA-mimic compounds). In certain embodiments, antisense compounds comprise modifications that make them particularly suited for such mechanisms.

i. ssRNA compounds

In certain embodiments, antisense compounds including those particularly suited for use as single-stranded RNAi compounds (ssRNA) comprise a modified 5'-terminal end. In certain such embodiments, the 5'-terminal end comprises a modified phosphate moiety. In certain embodiments, such modified phosphate is stabilized (e.g., resistant to degradation/cleavage compared to unmodified 5'-phosphate). In certain embodiments, such 5'-terminal nucleosides stabilize the 5'-phosphorous moiety. Certain modified 5'-terminal nucleosides may be found in the art, for example in WO 2011/139702.

In certain embodiments, the 5'-nucleoside of an ssRNA compound has Formula IIc:



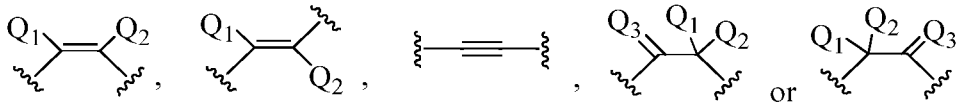
IIc

wherein:

T_1 is an optionally protected phosphorus moiety;

T_2 is an internucleoside linking group linking the compound of Formula IIc to the oligomeric compound;

A has one of the formulas:



Q_1 and Q_2 are each, independently, H, halogen, C_1-C_6 alkyl, substituted C_1-C_6 alkyl, C_1-C_6 alkoxy, substituted C_1-C_6 alkoxy, C_2-C_6 alkenyl, substituted C_2-C_6 alkenyl, C_2-C_6 alkynyl, substituted C_2-C_6 alkynyl or $N(R_3)(R_4)$;

Q_3 is O, S, $N(R_5)$ or $C(R_6)(R_7)$;

each R_3, R_4 R_5, R_6 and R_7 is, independently, H, C_1-C_6 alkyl, substituted C_1-C_6 alkyl or C_1-C_6 alkoxy;

M_3 is O, S, NR_{14}, $C(R_{15})(R_{16})$, $C(R_{15})(R_{16})C(R_{17})(R_{18})$, $C(R_{15})=C(R_{17})$, $OC(R_{15})(R_{16})$ or $OC(R_{15})(Bx_2)$;

R_{14} is H, C_1-C_6 alkyl, substituted C_1-C_6 alkyl, C_1-C_6 alkoxy, substituted C_1-C_6 alkoxy, C_2-C_6 alkenyl, substituted C_2-C_6 alkenyl, C_2-C_6 alkynyl or substituted C_2-C_6 alkynyl;

R_{15}, R_{16}, R_{17} and R_{18} are each, independently, H, halogen, C_1-C_6 alkyl, substituted C_1-C_6 alkyl, C_1-C_6 alkoxy, substituted C_1-C_6 alkoxy, C_2-C_6 alkenyl, substituted C_2-C_6 alkenyl, C_2-C_6 alkynyl or substituted C_2-C_6 alkynyl;

Bx_1 is a heterocyclic base moiety;

or if Bx_2 is present then Bx_2 is a heterocyclic base moiety and Bx_1 is H, halogen, C_1-C_6 alkyl, substituted C_1-C_6 alkyl, C_1-C_6 alkoxy, substituted C_1-C_6 alkoxy, C_2-C_6 alkenyl, substituted C_2-C_6 alkenyl, C_2-C_6 alkynyl or substituted C_2-C_6 alkynyl;

J_4, J_5, J_6 and J_7 are each, independently, H, halogen, C_1-C_6 alkyl, substituted C_1-C_6 alkyl, C_1-C_6 alkoxy, substituted C_1-C_6 alkoxy, C_2-C_6 alkenyl, substituted C_2-C_6 alkenyl, C_2-C_6 alkynyl or substituted C_2-C_6 alkynyl;

or J_4 forms a bridge with one of J_5 or J_7 wherein said bridge comprises from 1 to 3 linked biradical groups selected from O, S, NR_{19}, $C(R_{20})(R_{21})$, $C(R_{20})=C(R_{21})$, $C[=C(R_{20})(R_{21})]$ and C(=O) and the other two of J_5, J_6 and J_7 are each, independently, H, halogen, C_1-C_6 alkyl, substituted C_1-C_6 alkyl, C_1-C_6 alkoxy,

substituted C_1-C_6 alkoxy, C_2-C_6 alkenyl, substituted C_2-C_6 alkenyl, C_2-C_6 alkynyl or substituted C_2-C_6 alkynyl;

each R_{19}, R_{20} and R_{21} is, independently, H, C_1-C_6 alkyl, substituted C_1-C_6 alkyl, C_1-C_6 alkoxy, substituted C_1-C_6 alkoxy, C_2-C_6 alkenyl, substituted C_2-C_6 alkenyl, C_2-C_6 alkynyl or substituted C_2-C_6 alkynyl;

G is H, OH, halogen or O-$[C(R_8)(R_9)]_n$-$[(C=O)_m$-$X_1]_j$-Z;

each R_8 and R_9 is, independently, H, halogen, C_1-C_6 alkyl or substituted C_1-C_6 alkyl;

X_1 is O, S or $N(E_1)$;

Z is H, halogen, C_1-C_6 alkyl, substituted C_1-C_6 alkyl, C_2-C_6 alkenyl, substituted C_2-C_6 alkenyl, C_2-C_6 alkynyl, substituted C_2-C_6 alkynyl or $N(E_2)(E_3)$;

E_1, E_2 and E_3 are each, independently, H, C_1-C_6 alkyl or substituted C_1-C_6 alkyl;

n is from 1 to about 6;

m is 0 or 1;

j is 0 or 1;

each substituted group comprises one or more optionally protected substituent groups independently selected from halogen, OJ_1, $N(J_1)(J_2)$, $=NJ_1$, SJ_1, N_3, CN, $OC(=X_2)J_1$, $OC(=X_2)N(J_1)(J_2)$ and $C(=X_2)N(J_1)(J_2)$;

X_2 is O, S or NJ_3;

each J_1, J_2 and J_3 is, independently, H or C_1-C_6 alkyl;

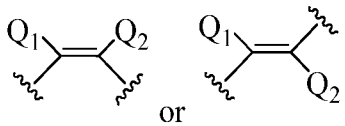
when j is 1 then Z is other than halogen or $N(E_2)(E_3)$; and

wherein said oligomeric compound comprises from 8 to 40 monomeric subunits and is hybridizable to at least a portion of a target nucleic acid.

In certain embodiments, M_3 is O, CH=CH, OCH_2 or $OC(H)(Bx_2)$. In certain embodiments, M_3 is O.

In certain embodiments, J_4, J_5, J_6 and J_7 are each H. In certain embodiments, J_4 forms a bridge with one of J_5 or J_7.

In certain embodiments, A has one of the formulas:

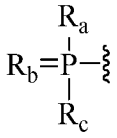


or

wherein:

Q_1 and Q_2 are each, independently, H, halogen, C_1-C_6 alkyl, substituted C_1-C_6 alkyl, C_1-C_6 alkoxy or substituted C_1-C_6 alkoxy. In certain embodiments, Q_1 and Q_2 are each H. In certain embodiments, Q_1 and Q_2 are each, independently, H or halogen. In certain embodiments, Q_1 and Q_2 is H and the other of Q_1 and Q_2 is F, CH_3 or OCH_3.

In certain embodiments, T_1 has the formula:



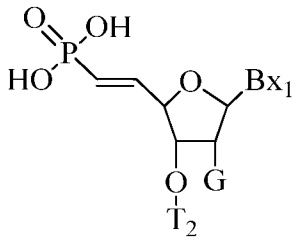
wherein:

R_a and R_c are each, independently, protected hydroxyl, protected thiol, C_1-C_6 alkyl, substituted C_1-C_6 alkyl, C_1-C_6 alkoxy, substituted C_1-C_6 alkoxy, protected amino or substituted amino; and

R_b is O or S. In certain embodiments, R_b is O and R_a and R_c are each, independently, OCH_3, OCH_2CH_3 or $CH(CH_3)_2$.

In certain embodiments, G is halogen, OCH_3, OCH_2F, $OCHF_2$, OCF_3, OCH_2CH_3, $O(CH_2)_2F$, OCH_2CHF_2, OCH_2CF_3, OCH_2-CH=CH_2, $O(CH_2)_2$-OCH_3, $O(CH_2)_2$-SCH_3, $O(CH_2)_2$-OCF_3, $O(CH_2)_3$-$N(R_{10})(R_{11})$, $O(CH_2)_2$-$ON(R_{10})(R_{11})$, $O(CH_2)_2$-$O(CH_2)_2$-$N(R_{10})(R_{11})$, $OCH_2C(=O)$-$N(R_{10})(R_{11})$, $OCH_2C(=O)$-$N(R_{12})$-$(CH_2)_2$-$N(R_{10})(R_{11})$ or $O(CH_2)_2$-$N(R_{12})$-$C(=NR_{13})[N(R_{10})(R_{11})]$ wherein R_{10}, R_{11}, R_{12} and R_{13} are each, independently, H or C_1-C_6 alkyl. In certain embodiments, G is halogen, OCH_3, OCF_3, OCH_2CH_3, OCH_2CF_3, OCH_2-CH=CH_2, $O(CH_2)_2$-OCH_3, $O(CH_2)_2$-$O(CH_2)_2$-$N(CH_3)_2$, $OCH_2C(=O)$-$N(H)CH_3$, $OCH_2C(=O)$-$N(H)$-$(CH_2)_2$-$N(CH_3)_2$ or OCH_2-$N(H)$-$C(=NH)NH_2$. In certain embodiments, G is F, OCH_3 or $O(CH_2)_2$-OCH_3. In certain embodiments, G is $O(CH_2)_2$-OCH_3.

In certain embodiments, the 5'-terminal nucleoside has Formula IIe:



IIe

In certain embodiments, antisense compounds, including those particularly suitable for ssRNA comprise one or more type of modified sugar moieties and/or naturally occurring sugar moieties arranged along an oligonucleotide or region thereof in a defined pattern or sugar modification motif. Such motifs may include any of the sugar modifications discussed herein and/or other known sugar modifications.

In certain embodiments, the oligonucleotides comprise or consist of a region having uniform sugar modifications. In certain such embodiments, each nucleoside of the region comprises the same RNA-like sugar modification. In certain embodiments, each nucleoside of the region is a 2'-F nucleoside. In certain embodiments, each nucleoside of the region is a 2'-OMe nucleoside. In certain embodiments, each nucleoside of the region is a 2'-MOE nucleoside. In certain embodiments, each nucleoside of the region is a cEt nucleoside. In certain embodiments, each nucleoside of the region is an LNA nucleoside. In certain embodiments, the uniform region constitutes all or essentially all of the oligonucleotide. In certain embodiments, the region constitutes the entire oligonucleotide except for 1-4 terminal nucleosides.

In certain embodiments, oligonucleotides comprise one or more regions of alternating sugar modifications, wherein the nucleosides alternate between nucleotides having a sugar modification of a first type and nucleotides having a sugar modification of a second type. In certain embodiments, nucleosides of both types are RNA-like nucleosides. In certain embodiments the alternating nucleosides are selected from: 2'-OMe, 2'-F, 2'-MOE, LNA, and cEt. In certain embodiments, the alternating modificatios are 2'-F and 2'-OMe. Such regions may be contiguous or may be interupted by differently modified nucleosides or conjugated nucleosides.

In certain embodiments, the alternating region of alternating modifications each consist of a single nucleoside (i.e., the patern is $(AB)_xA_y$ wheren A is a nucleoside having a sugar modification of a first type and B is a nucleoside having a sugar modification of a second type; x is 1-20 and y is 0 or 1). In certan embodiments, one or more alternating regions in an alternating motif includes more than a single nucleoside of a type. For example, oligonucleotides may include one or more regions of any of the following nucleoside motifs:

AABBAA;
ABBABB;
AABAAB;
ABBABAABB;
ABABAA;
AABABAB;
ABABAA;
ABBAABBABABAA;
BABBAABBABABAA; or
ABABBAABBABABAA;

wherein A is a nucleoside of a first type and B is a nucleoside of a second type. In certain embodiments, A and B are each selected from 2'-F, 2'-OMe, BNA, and MOE.

In certain embodiments, oligonucleotides having such an alternating motif also comprise a modified 5' terminal nucleoside, such as those of formula IIc or IIe.

In certain embodiments, oligonucleotides comprise a region having a 2-2-3 motif. Such regions comprises the following motif:

$-(A)_2-(B)_x-(A)_2-(C)_y-(A)_3-$

wherein: A is a first type of modifed nucleosde;

B and C, are nucleosides that are differently modified than A, however, B and C may have the same or different modifications as one another;

x and y are from 1 to 15.

In certain embodiments, A is a 2'-OMe modified nucleoside. In certain embodiments, B and C are both 2'-F modified nucleosides. In certain embodiments, A is a 2'-OMe modified nucleoside and B and C are both 2'-F modified nucleosides.

In certain embodiments, oligonucleosides have the following sugar motif:

5'- (Q)- $(AB)_xA_y$-$(D)_z$

wherein:

Q is a nucleoside comprising a stabilized phosphate moiety. In certain embodiments, Q is a nucleoside having Formula IIc or IIe;

A is a first type of modifed nucleoside;

B is a second type of modified nucleoside;

D is a modified nucleoside comprising a modification different from the nucleoside adjacent to it. Thus, if y is 0, then D must be differently modified than B and if y is 1, then D must be differently modified than A. In certain embodiments, D differs from both A and B.

X is 5-15;

Y is 0 or 1;

Z is 0-4.

In certain embodiments, oligonucleosides have the following sugar motif:

5'- (Q)- $(A)_x$-$(D)_z$

wherein:

Q is a nucleoside comprising a stabilized phosphate moiety. In certain embodiments, Q is a nucleoside having Formula IIc or IIe;

A is a first type of modifed nucleoside;

D is a modified nucleoside comprising a modification different from A.

X is 11-30;

Z is 0-4.

In certain embodiments A, B, C, and D in the above motifs are selected from: 2'-OMe, 2'-F, 2'-MOE, LNA, and cEt. In certain embodiments, D represents terminal nucleosides. In certain embodiments, such terminal nucleosides are not designed to hybridize to the target nucleic acid (though one or more might hybridize by chance). In certiain embodiments, the nucleobase of each D nucleoside is adenine, regardless of the identity of the nucleobase at the corresponding position of the target nucleic acid. In certain embodiments the nucleobase of each D nucleoside is thymine.

In certain embodiments, antisense compounds, including those particularly suited for use as ssRNA comprise modified internucleoside linkages arranged along the oligonucleotide or region thereof in a defined pattern or modified internucleoside linkage motif. In certain embodiments, oligonucleotides comprise a region having an alternating internucleoside linkage motif. In certain embodiments, oligonucleotides comprise a region of uniformly modified internucleoside linkages. In certain such embodiments, the

oligonucleotide comprises a region that is uniformly linked by phosphorothioate internucleoside linkages. In certain embodiments, the oligonucleotide is uniformly linked by phosphorothioate internucleoside linkages. In certain embodiments, each internucleoside linkage of the oligonucleotide is selected from phosphodiester and phosphorothioate. In certain embodiments, each internucleoside linkage of the oligonucleotide is selected from phosphodiester and phosphorothioate and at least one internucleoside linkage is phosphorothioate.

In certain embodiments, the oligonucleotide comprises at least 6 phosphorothioate internucleoside linkages. In certain embodiments, the oligonucleotide comprises at least 8 phosphorothioate internucleoside linkages. In certain embodiments, the oligonucleotide comprises at least 10 phosphorothioate internucleoside linkages. In certain embodiments, the oligonucleotide comprises at least one block of at least 6 consecutive phosphorothioate internucleoside linkages. In certain embodiments, the oligonucleotide comprises at least one block of at least 8 consecutive phosphorothioate internucleoside linkages. In certain embodiments, the oligonucleotide comprises at least one block of at least 10 consecutive phosphorothioate internucleoside linkages. In certain embodiments, the oligonucleotide comprises at least one block of at least one 12 consecutive phosphorothioate internucleoside linkages. In certain such embodiments, at least one such block is located at the 3' end of the oligonucleotide. In certain such embodiments, at least one such block is located within 3 nucleosides of the 3' end of the oligonucleotide.

Oligonucleotides having any of the various sugar motifs described herein, may have any linkage motif. For example, the oligonucleotides, including but not limited to those described above, may have a linkage motif selected from non-limiting the table below:

5' most linkage	Central region	3'-region
PS	Alternating PO/PS	6 PS
PS	Alternating PO/PS	7 PS
PS	Alternating PO/PS	8 PS

ii. siRNA compounds

In certain embodiments, antisense compounds are double-stranded RNAi compounds (siRNA). In such embodiments, one or both strands may comprise any modification motif described above for ssRNA. In certain embodiments, ssRNA compounds may be unmodified RNA. In certain embodiments, siRNA compounds may comprise unmodified RNA nucleosides, but modified internucleoside linkages.

Several embodiments relate to double-stranded compositions wherein each strand comprises a motif defined by the location of one or more modified or unmodified nucleosides. In certain embodiments, compositions are provided comprising a first and a second oligomeric compound that are fully or at least partially hybridized to form a duplex region and further comprising a region that is complementary to and hybridizes to a nucleic acid target. It is suitable that such a composition comprise a first oligomeric

compound that is an antisense strand having full or partial complementarity to a nucleic acid target and a second oligomeric compound that is a sense strand having one or more regions of complementarity to and forming at least one duplex region with the first oligomeric compound.

The compositions of several embodiments modulate gene expression by hybridizing to a nucleic acid target resulting in loss of its normal function. In some embodiments, the target nucleic acid is TMPRSS6. In certain embodiment, the degradation of the targeted TMPRSS6 is facilitated by an activated RISC complex that is formed with compositions of the invention.

Several embodiments are directed to double-stranded compositions wherein one of the strands is useful in, for example, influencing the preferential loading of the opposite strand into the RISC (or cleavage) complex. The compositions are useful for targeting selected nucleic acid molecules and modulating the expression of one or more genes. In some embodiments, the compositions of the present invention hybridize to a portion of a target RNA resulting in loss of normal function of the target RNA.

Certain embodiments are drawn to double-stranded compositions wherein both the strands comprises a hemimer motif, a fully modified motif, a positionally modified motif or an alternating motif. Each strand of the compositions of the present invention can be modified to fulfil a particular role in for example the siRNA pathway. Using a different motif in each strand or the same motif with different chemical modifications in each strand permits targeting the antisense strand for the RISC complex while inhibiting the incorporation of the sense strand. Within this model, each strand can be independently modified such that it is enhanced for its particular role. The antisense strand can be modified at the 5'-end to enhance its role in one region of the RISC while the 3'-end can be modified differentially to enhance its role in a different region of the RISC.

The double-stranded oligonucleotide molecules can be a double-stranded polynucleotide molecule comprising self-complementary sense and antisense regions, wherein the antisense region comprises nucleotide sequence that is complementary to nucleotide sequence in a target nucleic acid molecule or a portion thereof and the sense region having nucleotide sequence corresponding to the target nucleic acid sequence or a portion thereof. The double-stranded oligonucleotide molecules can be assembled from two separate oligonucleotides, where one strand is the sense strand and the other is the antisense strand, wherein the antisense and sense strands are self-complementary (i.e. each strand comprises nucleotide sequence that is complementary to nucleotide sequence in the other strand; such as where the antisense strand and sense strand form a duplex or double-stranded structure, for example wherein the double-stranded region is about 15 to about 30, e.g., about 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29 or 30 base pairs; the antisense strand comprises nucleotide sequence that is complementary to nucleotide sequence in a target nucleic acid molecule or a portion thereof and the sense strand comprises nucleotide sequence corresponding to the target nucleic acid sequence or a portion thereof (e.g., about 15 to about 25 or more nucleotides of the double-stranded oligonucleotide molecule are complementary to the target nucleic acid or a portion thereof). Alternatively, the double-stranded oligonucleotide is assembled from a single oligonucleotide, where the self-

complementary sense and antisense regions of the siRNA are linked by means of a nucleic acid based or non-nucleic acid-based linker(s).

The double-stranded oligonucleotide can be a polynucleotide with a duplex, asymmetric duplex, hairpin or asymmetric hairpin secondary structure, having self-complementary sense and antisense regions, wherein the antisense region comprises nucleotide sequence that is complementary to nucleotide sequence in a separate target nucleic acid molecule or a portion thereof and the sense region having nucleotide sequence corresponding to the target nucleic acid sequence or a portion thereof. The double-stranded oligonucleotide can be a circular single-stranded polynucleotide having two or more loop structures and a stem comprising self-complementary sense and antisense regions, wherein the antisense region comprises nucleotide sequence that is complementary to nucleotide sequence in a target nucleic acid molecule or a portion thereof and the sense region having nucleotide sequence corresponding to the target nucleic acid sequence or a portion thereof, and wherein the circular polynucleotide can be processed either in vivo or in vitro to generate an active siRNA molecule capable of mediating RNAi.

In certain embodiments, the double-stranded oligonucleotide comprises separate sense and antisense sequences or regions, wherein the sense and antisense regions are covalently linked by nucleotide or non-nucleotide linkers molecules as is known in the art, or are alternately non-covalently linked by ionic interactions, hydrogen bonding, van der waals interactions, hydrophobic interactions, and/or stacking interactions. In certain embodiments, the double-stranded oligonucleotide comprises nucleotide sequence that is complementary to nucleotide sequence of a target gene. In another embodiment, the double-stranded oligonucleotide interacts with nucleotide sequence of a target gene in a manner that causes inhibition of expression of the target gene.

As used herein, double-stranded oligonucleotides need not be limited to those molecules containing only RNA, but further encompasses chemically modified nucleotides and non-nucleotides. In certain embodiments, the short interfering nucleic acid molecules lack 2'-hydroxy (2'-OH) containing nucleotides. In certain embodiments short interfering nucleic acids optionally do not include any ribonucleotides (e.g., nucleotides having a 2'-OH group). Such double-stranded oligonucleotides that do not require the presence of ribonucleotides within the molecule to support RNAi can however have an attached linker or linkers or other attached or associated groups, moieties, or chains containing one or more nucleotides with 2'-OH groups. Optionally, double-stranded oligonucleotides can comprise ribonucleotides at about 5, 10, 20, 30, 40, or 50% of the nucleotide positions. As used herein, the term siRNA is meant to be equivalent to other terms used to describe nucleic acid molecules that are capable of mediating sequence specific RNAi, for example short interfering RNA (siRNA), double-stranded RNA (dsRNA), micro-RNA (miRNA), short hairpin RNA (shRNA), short interfering oligonucleotide, short interfering nucleic acid, short interfering modified oligonucleotide, chemically modified siRNA, post-transcriptional gene silencing RNA (ptgsRNA), and others. In addition, as used herein, the term RNAi is meant to be equivalent to other terms used to describe sequence specific RNA interference, such as post transcriptional gene silencing, translational inhibition, or

epigenetics. For example, double-stranded oligonucleotides can be used to epigenetically silence genes at both the post-transcriptional level and the pre-transcriptional level. In a non-limiting example, epigenetic regulation of gene expression by siRNA molecules of the invention can result from siRNA mediated modification of chromatin structure or methylation pattern to alter gene expression (see, for example, Verdel et al., 2004, Science, 303, 672-676; Pal-Bhadra et al., 2004, Science, 303, 669-672; Allshire, 2002, Science, 297, 1818-1819; Volpe et al., 2002, Science, 297, 1833-1837; Jenuwein, 2002, Science, 297, 2215-2218; and Hall et al., 2002, Science, 297, 2232-2237).

It is contemplated that compounds and compositions of several embodiments provided herein can target TMPRSS6 by a dsRNA-mediated gene silencing or RNAi mechanism, including, e.g., "hairpin" or stem-loop double-stranded RNA effector molecules in which a single RNA strand with self-complementary sequences is capable of assuming a double-stranded conformation, or duplex dsRNA effector molecules comprising two separate strands of RNA. In various embodiments, the dsRNA consists entirely of ribonucleotides or consists of a mixture of ribonucleotides and deoxynucleotides, such as the RNA/DNA hybrids disclosed, for example, by WO 00/63364, filed Apr. 19, 2000, or U.S. Ser. No. 60/130,377, filed Apr. 21, 1999. The dsRNA or dsRNA effector molecule may be a single molecule with a region of self-complementarity such that nucleotides in one segment of the molecule base pair with nucleotides in another segment of the molecule. In various embodiments, a dsRNA that consists of a single molecule consists entirely of ribonucleotides or includes a region of ribonucleotides that is complementary to a region of deoxyribonucleotides. Alternatively, the dsRNA may include two different strands that have a region of complementarity to each other.

In various embodiments, both strands consist entirely of ribonucleotides, one strand consists entirely of ribonucleotides and one strand consists entirely of deoxyribonucleotides, or one or both strands contain a mixture of ribonucleotides and deoxyribonucleotides. In certain embodiments, the regions of complementarity are at least 70, 80, 90, 95, 98, or 100% complementary to each other and to a target nucleic acid sequence. In certain embodiments, the region of the dsRNA that is present in a double-stranded conformation includes at least 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 50, 75,100, 200, 500, 1000, 2000 or 5000 nucleotides or includes all of the nucleotides in a cDNA or other target nucleic acid sequence being represented in the dsRNA. In some embodiments, the dsRNA does not contain any single stranded regions, such as single stranded ends, or the dsRNA is a hairpin. In other embodiments, the dsRNA has one or more single stranded regions or overhangs. In certain embodiments, RNA/DNA hybrids include a DNA strand or region that is an antisense strand or region (e.g, has at least 70, 80, 90, 95, 98, or 100% complementarity to a target nucleic acid) and an RNA strand or region that is a sense strand or region (e.g, has at least 70, 80, 90, 95, 98, or 100% identity to a target nucleic acid), and vice versa.

In various embodiments, the RNA/DNA hybrid is made in vitro using enzymatic or chemical synthetic methods such as those described herein or those described in WO 00/63364, filed Apr. 19, 2000, or U.S. Ser. No. 60/130,377, filed Apr. 21, 1999. In other embodiments, a DNA strand synthesized in vitro is

complexed with an RNA strand made in vivo or in vitro before, after, or concurrent with the transformation of the DNA strand into the cell. In yet other embodiments, the dsRNA is a single circular nucleic acid containing a sense and an antisense region, or the dsRNA includes a circular nucleic acid and either a second circular nucleic acid or a linear nucleic acid (see, for example, WO 00/63364, filed Apr. 19, 2000, or U.S. Ser. No. 60/130,377, filed Apr. 21, 1999.) Exemplary circular nucleic acids include lariat structures in which the free 5' phosphoryl group of a nucleotide becomes linked to the 2' hydroxyl group of another nucleotide in a loop back fashion.

In other embodiments, the dsRNA includes one or more modified nucleotides in which the 2' position in the sugar contains a halogen (such as fluorine group) or contains an alkoxy group (such as a methoxy group) which increases the half-life of the dsRNA in vitro or in vivo compared to the corresponding dsRNA in which the corresponding 2' position contains a hydrogen or an hydroxyl group. In yet other embodiments, the dsRNA includes one or more linkages between adjacent nucleotides other than a naturally-occurring phosphodiester linkage. Examples of such linkages include phosphoramide, phosphorothioate, and phosphorodithioate linkages. The dsRNAs may also be chemically modified nucleic acid molecules as taught in U.S. Pat. No. 6,673,661. In other embodiments, the dsRNA contains one or two capped strands, as disclosed, for example, by WO 00/63364, filed Apr. 19, 2000, or U.S. Ser. No. 60/130,377, filed Apr. 21, 1999.

In other embodiments, the dsRNA can be any of the at least partially dsRNA molecules disclosed in WO 00/63364, as well as any of the dsRNA molecules described in U.S. Provisional Application 60/399,998; and U.S. Provisional Application 60/419,532, and PCT/US2003/033466, the teaching of which is hereby incorporated by reference. Any of the dsRNAs may be expressed in vitro or in vivo using the methods described herein or standard methods, such as those described in WO 00/63364.

Occupancy

In certain embodiments, antisense compounds are not expected to result in cleavage or the target nucleic acid via RNase H or to result in cleavage or sequestration through the RISC pathway. In certain such embodiments, antisense activity may result from occupancy, wherein the presence of the hybridized antisense compound disrupts the activity of the target nucleic acid. In certain such embodiments, the antisense compound may be uniformly modified or may comprise a mix of modifications and/or modified and unmodified nucleosides.

Target Nucleic Acids, Target Regions and Nucleotide Sequences

Nucleotide sequences that encode TMPRSS6 include, without limitation, the following: GENBANK Accession NM_153609.2 (incorporated herein as SEQ ID NO: 1), the complement of GENBANK Accession NT_011520.12 truncated from 16850000 to 16897000 (incorporated herein as SEQ ID NO: 2), GENBANK Accession CR456446.1 (incorporated herein as SEQ ID NO: 3), GENBANK Accession No. BC039082.1

(incorporated herein as SEQ ID NO: 4), GENBANK Accession No. AY358398.1 (incorporated herein as SEQ ID NO: 5), or GENBANK Accession No. DB081153.1 (incorporated herein as SEQ ID NO: 6). In certain embodiments, an antisense compound described herein targets a nucleic acid sequence encoding TMPRSS6. In certain embodiments, an antisense compound described herein targets the sequence of any of SEQ ID NOs: 1-6.

It is understood that the sequence set forth in each SEQ ID NO in the examples contained herein is independent of any modification to a sugar moiety, an internucleoside linkage, or a nucleobase. As such, antisense compounds defined by a SEQ ID NO may comprise, independently, one or more modifications to a sugar moiety, an internucleoside linkage, or a nucleobase. Antisense compounds described by Isis Number (Isis No) indicate a combination of nucleobase sequence and motif.

In certain embodiments, a target region is a structurally defined region of the target nucleic acid. For example, a target region may encompass a 3' UTR, a 5' UTR, an exon, an intron, an exon/intron junction, a coding region, a translation initiation region, translation termination region, or other defined nucleic acid region. The structurally defined regions for TMPRSS6 can be obtained by accession number from sequence databases such as NCBI and such information is incorporated herein by reference. In certain embodiments, a target region may encompass the sequence from a 5' target site of one target segment within the target region to a 3' target site of another target segment within the target region.

In certain embodiments, a "target segment" is a smaller, sub-portion of a target region within a nucleic acid. For example, a target segment can be the sequence of nucleotides of a target nucleic acid to which one or more antisense compound is targeted. "5' target site" refers to the 5'-most nucleotide of a target segment. "3' target site" refers to the 3'-most nucleotide of a target segment.

Targeting includes determination of at least one target segment to which an antisense compound hybridizes, such that a desired effect occurs. In certain embodiments, the desired effect is a reduction in mRNA target nucleic acid levels. In certain embodiments, the desired effect is reduction of levels of protein encoded by the target nucleic acid or a phenotypic change associated with the target nucleic acid.

A target region may contain one or more target segments. Multiple target segments within a target region may be overlapping. Alternatively, they may be non-overlapping. In certain embodiments, target segments within a target region are separated by no more than about 300 nucleotides. In certain emodiments, target segments within a target region are separated by a number of nucleotides that is, is about, is no more than, is no more than about, 250, 200, 150, 100, 90, 80, 70, 60, 50, 40, 30, 20, or 10 nucleotides on the target nucleic acid, or is a range defined by any two of the preceeding values. In certain embodiments, target segments within a target region are separated by no more than, or no more than about, 5 nucleotides on the target nucleic acid. In certain embodiments, target segments are contiguous. Contemplated are target regions defined by a range having a starting nucleic acid that is any of the 5' target sites or 3' target sites listed herein.

Suitable target segments may be found within a 5' UTR, a coding region, a 3' UTR, an intron, an exon, or an exon/intron junction. Target segments containing a start codon or a stop codon are also suitable target segments. A suitable target segment may specifcally exclude a certain structurally defined region such as the start codon or stop codon.

The determination of suitable target segments may include a comparison of the sequence of a target nucleic acid to other sequences throughout the genome. For example, the BLAST algorithm may be used to identify regions of similarity amongst different nucleic acids. This comparison can prevent the selection of antisense compound sequences that may hybridize in a non-specific manner to sequences other than a selected target nucleic acid (i.e., non-target or off-target sequences).

There may be variation in activity (e.g., as defined by percent reduction of target nucleic acid levels) of the antisense compounds within an active target region. In certain embodiments, reductions in TMPRSS6 mRNA levels are indicative of inhibition of TMPRSS6 expression. Reductions in levels of a TMPRSS6 protein are also indicative of inhibition of TMPRSS6 expression. Further, phenotypic changes are indicative of inhibition of TMPRSS6 expression. For example, an increase in hepcidin expression levels can be indicative of inhibition of TMPRSS6 expression. In another example, a decrease in iron accumulation in tissues can be indicative of inhibition of TMPRSS6 expression. In another example, an increase in the percentage of saturation of transferrin can be indicative of inhibition of TMPRSS6 expression.

Hybridization

In some embodiments, hybridization occurs between an antisense compound disclosed herein and a TMPRSS6 nucleic acid. The most common mechanism of hybridization involves hydrogen bonding (e.g., Watson-Crick, Hoogsteen or reversed Hoogsteen hydrogen bonding) between complementary nucleobases of the nucleic acid molecules.

Hybridization can occur under varying conditions. Stringent conditions are sequence-dependent and are determined by the nature and composition of the nucleic acid molecules to be hybridized.

Methods of determining whether a sequence is specifically hybridizable to a target nucleic acid are well known in the art (Sambrook and Russell, Molecular Cloning: A Laboratory Manual, 3rd Ed., 2001). In certain embodiments, the antisense compounds provided herein are specifically hybridizable with a TMPRSS6 nucleic acid.

Complementarity

An antisense compound and a target nucleic acid are complementary to each other when a sufficient number of nucleobases of the antisense compound can hydrogen bond with the corresponding nucleobases of the target nucleic acid, such that a desired effect will occur (e.g., antisense inhibition of a target nucleic acid, such as a TMPRSS6 nucleic acid).

Non-complementary nucleobases between an antisense compound and a TMPRSS6 nucleic acid may

be tolerated provided that the antisense compound remains able to specifically hybridize to the TMPRSS6 nucleic acid. Moreover, an antisense compound may hybridize over one or more segments of a TMPRSS6 nucleic acid such that intervening or adjacent segments are not involved in the hybridization event (e.g., a loop structure, mismatch or hairpin structure).

In certain embodiments, the antisense compounds provided herein, or a specified portion thereof, are, or are at least 70%, at least 80%, at least 85%, at least 86%, at least 87%, at least 88%, at least 89%, at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% complementary to a TMPRSS6 nucleic acid, a target region, target segment, or specified portion thereof. Percent complementarity of an antisense compound with a target nucleic acid can be determined using routine methods. For example, an antisense compound in which 18 of 20 nucleobases of the antisense compound are complementary to a target region, and would therefore specifically hybridize, would represent 90 percent complementarity. In this example, the remaining noncomplementary nucleobases may be clustered or interspersed with complementary nucleobases and need not be contiguous to each other or to complementary nucleobases. As such, an antisense compound which is 18 nucleobases in length having 4 (four) noncomplementary nucleobases which are flanked by two regions of complete complementarity with the target nucleic acid would have 77.8% overall complementarity with the target nucleic acid and would thus fall within the scope of the present invention.

Percent complementarity of an antisense compound with a region of a target nucleic acid can be determined routinely using BLAST programs (basic local alignment search tools) and PowerBLAST programs known in the art (Altschul et al., J. Mol. Biol., 1990, 215, 403 410; Zhang and Madden, Genome Res., 1997, 7, 649 656). Percent homology, sequence identity or complementarity, can be determined by, for example, the Gap program (Wisconsin Sequence Analysis Package, Version 8 for Unix, Genetics Computer Group, University Research Park, Madison Wis.), using default settings, which uses the algorithm of Smith and Waterman (Adv. Appl. Math., 1981, 2, 482 489).

In certain embodiments, the antisense compounds provided herein, or specified portions thereof, are fully complementary (i.e. 100% complementary) to a target nucleic acid, or specified portion thereof. For example, antisense compound may be fully complementary to a TMPRSS6 nucleic acid, or a target region, or a target segment or target sequence thereof. As used herein, "fully complementary" means each nucleobase of an antisense compound is capable of precise base pairing with the corresponding nucleobases of a target nucleic acid. For example, a 20 nucleobase antisense compound is fully complementary to a target sequence that is 400 nucleobases long, so long as there is a corresponding 20 nucleobase portion of the target nucleic acid that is fully complementary to the antisense compound. Fully complementary can also be used in reference to a specified portion of the first and /or the second nucleic acid. For example, a 20 nucleobase portion of a 30 nucleobase antisense compound can be "fully complementary" to a target sequence that is 400 nucleobases long. The 20 nucleobase portion of the 30 nucleobase oligonucleotide is fully complementary to the target sequence if the target sequence has a corresponding 20 nucleobase portion wherein each nucleobase

is complementary to the 20 nucleobase portion of the antisense compound. At the same time, the entire 30 nucleobase antisense compound may or may not be fully complementary to the target sequence, depending on whether the remaining 10 nucleobases of the antisense compound are also complementary to the target sequence.

The location of a non-complementary nucleobase may be at the 5' end or 3' end of the antisense compound. Alternatively, the non-complementary nucleobase or nucleobases may be at an internal position of the antisense compound. When two or more non-complementary nucleobases are present, they may be contiguous (i.e. linked) or non-contiguous. In one embodiment, a non-complementary nucleobase is located in the wing segment of a gapmer antisense oligonucleotide.

In certain embodiments, antisense compounds that are, or are up to, 12, 13, 14, 15, 16, 17, 18, 19, or 20 nucleobases in length comprise no more than 4, no more than 3, no more than 2, or no more than 1 non-complementary nucleobase(s) relative to a target nucleic acid, such as a TMPRSS6 nucleic acid, or specified portion thereof.

In certain embodiments, antisense compounds that are, or are up to, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 nucleobases in length comprise no more than 6, no more than 5, no more than 4, no more than 3, no more than 2, or no more than 1 non-complementary nucleobase(s) relative to a target nucleic acid, such as a TMPRSS6 nucleic acid, or specified portion thereof.

The antisense compounds provided herein also include those which are complementary to a portion of a target nucleic acid. As used herein, "portion" refers to a defined number of contiguous (i.e. linked) nucleobases within a region or segment of a target nucleic acid. A "portion" can also refer to a defined number of contiguous nucleobases of an antisense compound. In certain embodiments, the antisense compounds, are complementary to at least an 8 nucleobase portion of a target segment. In certain embodiments, the antisense compounds are complementary to at least a 12 nucleobase portion of a target segment. In certain embodiments, the antisense compounds are complementary to at least a 15 nucleobase portion of a target segment. Also contemplated are antisense compounds that are complementary to at least a 9, at least a 10, at least an 11, at least a 12, at least a 13, at least a 14, at least a 15, at least a 16, at least a 17, at least an 18, at least a 19, at least a 20, or more nucleobase portion of a target segment, or a range defined by any two of these values.

Identity

The antisense compounds provided herein may also have a defined percent identity to a particular nucleotide sequence, SEQ ID NO, or compound represented by a specific Isis number, or portion thereof. As used herein, an antisense compound 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 a disclosed DNA sequence would be considered identical to the DNA sequence since both uracil and thymidine pair with adenine. Shortened and lengthened versions of the antisense compounds described herein as well as

compounds having non-identical bases relative to the antisense compounds provided herein also are contemplated. The non-identical bases may be adjacent to each other or dispersed throughout the antisense compound. Percent identity of an antisense compound is calculated according to the number of bases that have identical base pairing relative to the sequence to which it is being compared.

In certain embodiments, the antisense compounds, or portions thereof, are at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% or 100% identical to one or more of the antisense compounds or SEQ ID NOs, or a portion thereof, disclosed herein.

Modifications

A nucleoside is a base-sugar combination. The nucleobase (also known as base) portion of the nucleoside is normally a heterocyclic base moiety. 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. Oligonucleotides are formed through the covalent linkage of adjacent nucleosides to one another, to form a linear polymeric oligonucleotide. Within the oligonucleotide structure, the phosphate groups are commonly referred to as forming the internucleoside linkages of the oligonucleotide.

Modifications to antisense compounds encompass substitutions or changes to internucleoside linkages, sugar moieties, or nucleobases. Modified antisense compounds are often preferred over native forms because of desirable properties such as, for example, enhanced cellular uptake, enhanced affinity for nucleic acid target, increased stability in the presence of nucleases, or increased inhibitory activity.

Chemically modified nucleosides may also be employed to increase the binding affinity of a shortened or truncated antisense oligonucleotide for its target nucleic acid. Consequently, comparable results can often be obtained with shorter antisense compounds that have such chemically modified nucleosides.

Modified Internucleoside Linkages

The naturally occuring internucleoside linkage of RNA and DNA is a 3' to 5' phosphodiester linkage. Antisense compounds having one or more modified, i.e. non-naturally occurring, internucleoside linkages are often selected over antisense compounds having naturally occurring internucleoside linkages because of desirable properties such as, for example, enhanced cellular uptake, enhanced affinity for target nucleic acids, and increased stability in the presence of nucleases.

Oligonucleotides having modified internucleoside linkages include internucleoside linkages that retain a phosphorus atom as well as internucleoside linkages that do not have a phosphorus atom. Representative phosphorus containing internucleoside linkages include, but are not limited to, phosphodiesters, phosphotriesters, methylphosphonates, phosphoramidate, and phosphorothioates. Methods of preparation of phosphorous-containing and non-phosphorous-containing linkages are well known.

In certain embodiments, antisense compounds targeted to a TMPRSS6 nucleic acid comprise one or more modified internucleoside linkages. In certain embodiments, at least one of the modified internucleoside linkages are phosphorothioate linkages. In certain embodiments, each internucleoside linkage of an antisense compound is a phosphorothioate internucleoside linkage.

Modified Sugar Moieties

Antisense compounds of the invention can optionally contain one or more nucleosides wherein the sugar group has been modified. Such sugar modified nucleosides may impart enhanced nuclease stability, increased binding affinity, or some other beneficial biological property to the antisense compounds. In certain embodiments, nucleosides comprise chemically modified ribofuranose ring moieties. Examples of chemically modified ribofuranose rings include without limitation, addition of substitutent groups (including 5' and 2' substituent groups, bridging of non-geminal ring atoms to form bicyclic nucleic acids (BNA), replacement of the ribosyl ring oxygen atom with S, N(R), or $C(R_1)(R_2)$ (R, R_1 and R_2 are each independently H, C_1-C_{12} alkyl or a protecting group) and combinations thereof. Examples of chemically modified sugars include 2'-F-5'-methyl substituted nucleoside (see PCT International Application WO 2008/101157 Published on 8/21/08 for other disclosed 5',2'-bis substituted nucleosides) or replacement of the ribosyl ring oxygen atom with S with further substitution at the 2'-position (see published U.S. Patent Application US2005-0130923, published on June 16, 2005) or alternatively 5'-substitution of a BNA (see PCT International Application WO 2007/134181 Published on 11/22/07 wherein LNA is substituted with for example a 5'-methyl or a 5'-vinyl group).

Examples of nucleosides having modified sugar moieties include without limitation nucleosides comprising 5'-vinyl, 5'-methyl (*R* or *S*), 4'-S, 2'-F, 2'-OCH_3, 2'-OCH_2CH_3, 2'-OCH_2CH_2F and 2'-$O(CH_2)_2OCH_3$ substituent groups. The substituent at the 2' position can also be selected from allyl, amino, azido, thio, O-allyl, O-C_1-C_{10} alkyl, OCF_3, OCH_2F, $O(CH_2)_2SCH_3$, $O(CH_2)_2$-O-$N(R_m)(R_n)$, O-CH_2-C(=O)-$N(R_m)(R_n)$, and O-CH_2-C(=O)-$N(R_l)$-$(CH_2)_2$-$N(R_m)(R_n)$, where each R_l, R_m and R_n is, independently, H or substituted or unsubstituted C_1-C_{10} alkyl.

As used herein, "bicyclic nucleosides" refer to modified nucleosides comprising a bicyclic sugar moiety. Examples of bicyclic nucleic acids (BNAs) include without limitation nucleosides comprising a bridge between the 4' and the 2' ribosyl ring atoms. In certain embodiments, antisense compounds provided herein include one or more BNA nucleosides wherein the bridge comprises one of the formulas: 4'-(CH_2)-O-2' (LNA); 4'-(CH_2)-S-2'; 4'-$(CH_2)_2$-O-2' (ENA); 4'-$CH(CH_3)$-O-2' (cEt) and 4'-$CH(CH_2OCH_3)$-O-2' (and analogs thereof see U.S. Patent 7,399,845, issued on July 15, 2008); 4'-$C(CH_3)(CH_3)$-O-2' (and analogs thereof see PCT/US2008/068922 published as WO/2009/006478, published January 8, 2009); 4'-CH_2-$N(OCH_3)$-2' (and analogs thereof see PCT/US2008/064591 published as WO/2008/150729, published December 11, 2008); 4'-CH_2-O-$N(CH_3)$-2' (see published U.S. Patent Application US2004-0171570, published September 2, 2004); 4'-CH_2-N(R)-O-2', wherein R is H, C_1-C_{12} alkyl, or a protecting group (see U.S. Patent 7,427,672, issued on September 23, 2008); 4'-CH_2-$C(H)(CH_3)$-2' (see Zhou *et al., J. Org. Chem.,*

2009, *74*, 118-134); and 4'-CH_2-C(=CH_2)-2' (and analogs thereof see PCT/US2008/066154 published as WO 2008/154401, published on December 8, 2008).

Further bicyclic nucleosides have been reported in published literature (see for example: Srivastava et al., *J. Am. Chem. Soc.*, 2007, *129(26)* 8362-8379; Frieden *et al., Nucleic Acids Research,* 2003, *21*, 6365-6372; Elayadi *et al., Curr. Opinion Invens. Drugs,* 2001, *2*, 558-561; Braasch *et al., Chem. Biol.,* 2001, *8*, 1-7; Orum *et al., Curr. Opinion Mol. Ther.,* 2001, *3*, 239-243; Wahlestedt et al., *Proc. Natl. Acad. Sci. U. S. A.*, 2000, *97*, 5633-5638; Singh et al., *Chem. Commun.*, 1998, *4*, 455-456; Koshkin et al., *Tetrahedron*, 1998, *54*, 3607-3630; Kumar et al., *Bioorg. Med. Chem. Lett.*, 1998, *8*, 2219-2222; Singh et al., *J. Org. Chem.*, 1998, *63*, 10035-10039; U.S. Patents Nos.: 7,399,845; 7,053,207; 7,034,133; 6,794,499; 6,770,748; 6,670,461; 6,525,191; 6,268,490; U.S. Patent Publication Nos.: US2008-0039618; US2007-0287831; US2004-0171570; U.S. Patent Applications, Serial Nos.: 12/129,154; 61/099,844; 61/097,787; 61/086,231; 61/056,564; 61/026,998; 61/026,995; 60/989,574; International applications WO 2007/134181; WO 2005/021570; WO 2004/106356; WO 99/14226; and PCT International Applications Nos.: PCT/US2008/068922; PCT/US-2008/066154; and PCT/US2008/064591). Each of the foregoing bicyclic nucleosides can be prepared having one or more stereochemical sugar configurations including for example α-L-ribofuranose and β-D-ribofuranose (see PCT international application PCT/DK98/00393, published on March 25, 1999 as WO 99/14226).

As used herein, "monocyclic nucleosides" refer to nucleosides comprising modified sugar moieties that are not bicyclic sugar moieties. In certain embodiments, the sugar moiety, or sugar moiety analogue, of a nucleoside may be modified or substituted at any position.

As used herein, "4'-2' bicyclic nucleoside" or "4' to 2' bicyclic nucleoside" refers to a bicyclic nucleoside comprising a furanose ring comprising a bridge connecting two carbon atoms of the furanose ring connects the 2' carbon atom and the 4' carbon atom of the sugar ring.

In certain embodiments, bicyclic sugar moieties of BNA nucleosides include, but are not limited to, compounds having at least one bridge between the 4' and the 2' carbon atoms of the pentofuranosyl sugar moiety including without limitation, bridges comprising 1 or from 1 to 4 linked groups independently selected from -$[C(R_a)(R_b)]_n$-, -$C(R_a)$=$C(R_b)$-, -$C(R_a)$=N-, -C(=NR_a)-, -C(=O)-, -C(=S)-, -O-, -$Si(R_a)_2$-, -$S(=O)_x$-, and -$N(R_a)$-; wherein: x is 0, 1, or 2; n is 1, 2, 3, or 4; each R_a and R_b is, independently, H, a protecting group, hydroxyl, C_1-C_{12} alkyl, substituted C_1-C_{12} alkyl, C_2-C_{12} alkenyl, substituted C_2-C_{12} alkenyl, C_2-C_{12} alkynyl, substituted C_2-C_{12} alkynyl, C_5-C_{20} aryl, substituted C_5-C_{20} aryl, heterocycle radical, substituted heterocycle radical, heteroaryl, substituted heteroaryl, C_5-C_7 alicyclic radical, substituted C_5-C_7 alicyclic radical, halogen, OJ_1, NJ_1J_2, SJ_1, N_3, $COOJ_1$, acyl (C(=O)-H), substituted acyl, CN, sulfonyl ($S(=O)_2$-J_1), or sulfoxyl (S(=O)-J_1); and

each J_1 and J_2 is, independently, H, C_1-C_{12} alkyl, substituted C_1-C_{12} alkyl, C_2-C_{12} alkenyl, substituted C_2-C_{12} alkenyl, C_2-C_{12} alkynyl, substituted C_2-C_{12} alkynyl, C_5-C_{20} aryl, substituted C_5-C_{20} aryl, acyl (C(=O)-

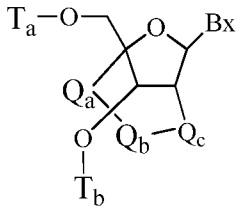
H), substituted acyl, a heterocycle radical, a substituted heterocycle radical, C_1-C_{12} aminoalkyl, substituted C_1-C_{12} aminoalkyl or a protecting group.

In certain embodiments, the bridge of a bicyclic sugar moiety is , -[$C(R_a)(R_b)]_n$-, -[$C(R_a)(R_b)]_n$-O- , -$C(R_aR_b)$-N(R)-O- or –$C(R_aR_b)$-O-N(R)-. In certain embodiments, the bridge is 4'-CH_2-2', 4'-$(CH_2)_2$-2', 4'-$(CH_2)_3$-2', 4'-CH_2-O-2', 4'-$(CH_2)_2$-O-2', 4'-CH_2-O-N(R)-2' and 4'-CH_2-N(R)-O-2'- wherein each R is, independently, H, a protecting group or C_1-C_{12} alkyl.

In certain embodiments, bicyclic nucleosides are further defined by isomeric configuration. For example, a nucleoside comprising a 4'-(CH_2)-O-2' bridge, may be in the α-L configuration or in the β-D configuration. Previously, α-L-methyleneoxy (4'-CH_2-O-2') BNA's have been incorporated into antisense oligonucleotides that showed antisense activity (Frieden *et al., Nucleic Acids Research,* 2003, *21*, 6365-6372).

In certain embodiments, bicyclic nucleosides include those having a 4' to 2' bridge wherein such bridges include without limitation, α-L-4'-(CH_2)-O-2', β-D-4'-CH_2-O-2', 4'-$(CH_2)_2$-O-2', 4'-CH_2-O-N(R)-2', 4'-CH_2-N(R)-O-2', 4'-$CH(CH_3)$-O-2', 4'-CH_2-S-2', 4'-CH_2-N(R)-2', 4'-CH_2-$CH(CH_3)$-2', and 4'-$(CH_2)_3$-2', wherein R is H, a protecting group or C_1-C_{12} alkyl.

In certain embodiment, bicyclic nucleosides have the formula:



wherein:

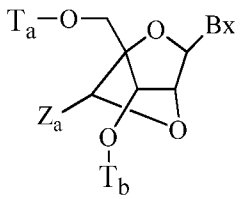
Bx is a heterocyclic base moiety;

$-Q_a-Q_b-Q_c-$ is $-CH_2-N(R_c)-CH_2-$, $-C(=O)-N(R_c)-CH_2-$, $-CH_2-O-N(R_c)-$, $-CH_2-N(R_c)-O-$ or $-N(R_c)-O-CH_2$;

R_c is C_1-C_{12} alkyl or an amino protecting group; and

T_a and T_b are each, independently H, a hydroxyl protecting group, a conjugate group, a reactive phosphorus group, a phosphorus moiety or a covalent attachment to a support medium.

In certain embodiments, bicyclic nucleosides have the formula:



wherein:

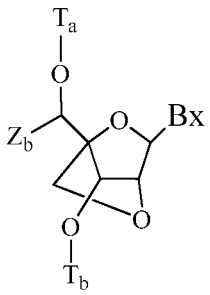
Bx is a heterocyclic base moiety;

T_a and T_b are each, independently H, a hydroxyl protecting group, a conjugate group, a reactive phosphorus group, a phosphorus moiety or a covalent attachment to a support medium;

Z_a is C_1-C_6 alkyl, C_2-C_6 alkenyl, C_2-C_6 alkynyl, substituted C_1-C_6 alkyl, substituted C_2-C_6 alkenyl, substituted C_2-C_6 alkynyl, acyl, substituted acyl, substituted amide, thiol or substituted thiol.

In one embodiment, each of the substituted groups, is, independently, mono or poly substituted with substituent groups independently selected from halogen, oxo, hydroxyl, OJ_c, NJ_cJ_d, SJ_c, N_3, $OC(=X)J_c$, and $NJ_eC(=X)NJ_cJ_d$, wherein each J_c, J_d and J_e is, independently, H, C_1-C_6 alkyl, or substituted C_1-C_6 alkyl and X is O or NJ_c.

In certain embodiments, bicyclic nucleosides have the formula:



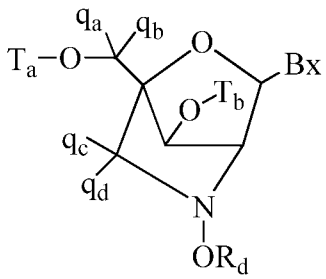
wherein:

Bx is a heterocyclic base moiety;

T_a and T_b are each, independently H, a hydroxyl protecting group, a conjugate group, a reactive phosphorus group, a phosphorus moiety or a covalent attachment to a support medium;

Z_b is C_1-C_6 alkyl, C_2-C_6 alkenyl, C_2-C_6 alkynyl, substituted C_1-C_6 alkyl, substituted C_2-C_6 alkenyl, substituted C_2-C_6 alkynyl or substituted acyl (C(=O)-).

In certain embodiments, bicyclic nucleosides have the formula:



wherein:

Bx is a heterocyclic base moiety;

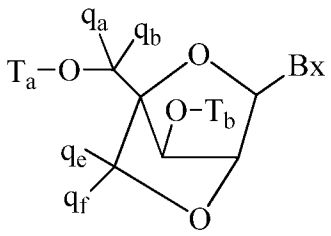
T_a and T_b are each, independently H, a hydroxyl protecting group, a conjugate group, a reactive phosphorus group, a phosphorus moiety or a covalent attachment to a support medium;

R_d is C_1-C_6 alkyl, substituted C_1-C_6 alkyl, C_2-C_6 alkenyl, substituted C_2-C_6 alkenyl, C_2-C_6 alkynyl or substituted C_2-C_6 alkynyl;

each q_a, q_b, q_c and q_d is, independently, H, halogen, C_1-C_6 alkyl, substituted C_1-C_6 alkyl, C_2-C_6 alkenyl, substituted C_2-C_6 alkenyl, C_2-C_6 alkynyl or substituted C_2-C_6 alkynyl, C_1-C_6 alkoxyl, substituted C_1-

C_6 alkoxyl, acyl, substituted acyl, C_1-C_6 aminoalkyl or substituted C_1-C_6 aminoalkyl;

In certain embodiments, bicyclic nucleosides have the formula:



wherein:

Bx is a heterocyclic base moiety;

T_a and T_b are each, independently H, a hydroxyl protecting group, a conjugate group, a reactive phosphorus group, a phosphorus moiety or a covalent attachment to a support medium;

q_a, q_b, q_e and q_f are each, independently, hydrogen, halogen, C_1-C_{12} alkyl, substituted C_1-C_{12} alkyl, C_2-C_{12} alkenyl, substituted C_2-C_{12} alkenyl, C_2-C_{12} alkynyl, substituted C_2-C_{12} alkynyl, C_1-C_{12} alkoxy, substituted C_1-C_{12} alkoxy, OJ_j, SJ_j, SOJ_j, SO_2J_j, NJ_jJ_k, N_3, CN, $C(=O)OJ_j$, $C(=O)NJ_jJ_k$, $C(=O)J_j$, $O\text{-}C(=O)NJ_jJ_k$, $N(H)C(=NH)NJ_jJ_k$, $N(H)C(=O)NJ_jJ_k$ or $N(H)C(=S)NJ_jJ_k$;

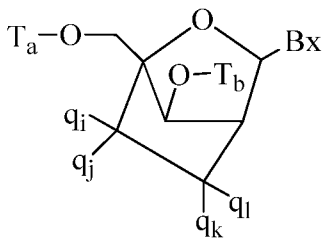
or q_e and q_f together are $=C(q_g)(q_h)$;

q_g and q_h are each, independently, H, halogen, C_1-C_{12} alkyl or substituted C_1-C_{12} alkyl.

The synthesis and preparation of adenine, cytosine, guanine, 5-methyl-cytosine, thymine and uracil bicyclic nucleosides having a 4'-CH_2-O-2' bridge, along with their oligomerization, and nucleic acid recognition properties have been described (Koshkin et al., *Tetrahedron*, 1998, *54*, 3607-3630). The synthesis of bicyclic nucleosides has also been described in WO 98/39352 and WO 99/14226.

Analogs of various bicyclic nucleosides that have 4' to 2' bridging groups such as 4'-CH_2-O-2' and 4'-CH_2-S-2', have also been prepared (Kumar *et al., Bioorg. Med. Chem. Lett.*, 1998, *8*, 2219-2222). Preparation of oligodeoxyribonucleotide duplexes comprising bicyclic nucleosides for use as substrates for nucleic acid polymerases has also been described (Wengel et al., WO 99/14226). Furthermore, synthesis of 2'-amino-BNA, a novel conformationally restricted high-affinity oligonucleotide analog has been described in the art (Singh et al., *J. Org. Chem.*, 1998, *63*, 10035-10039). In addition, 2'-amino- and 2'-methylamino-BNA's have been prepared and the thermal stability of their duplexes with complementary RNA and DNA strands has been previously reported.

In certain embodiments, bicyclic nucleosides have the formula:



wherein:

Bx is a heterocyclic base moiety;

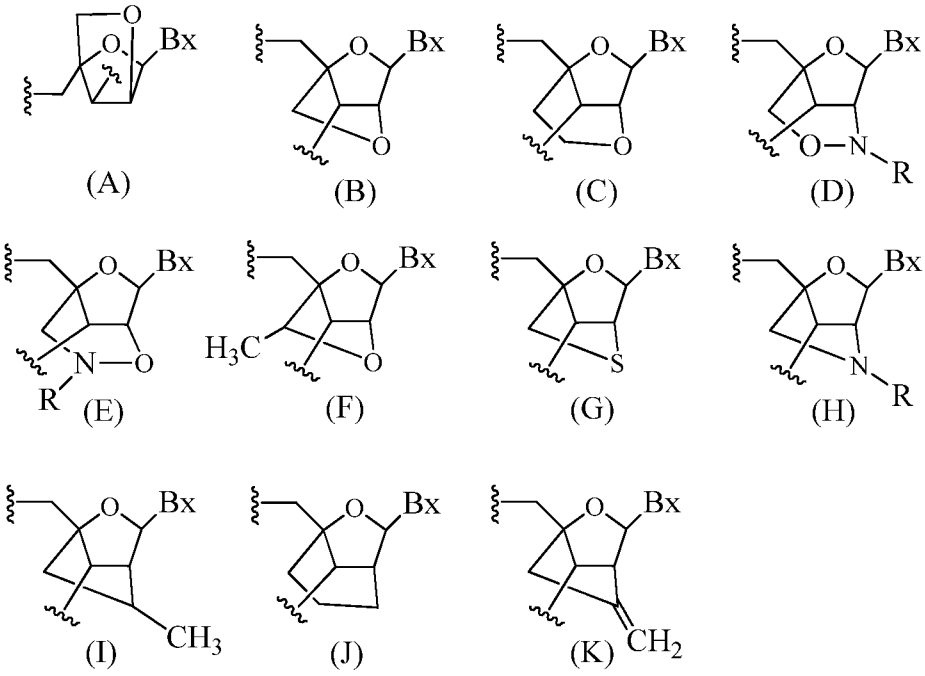
T_a and T_b are each, independently H, a hydroxyl protecting group, a conjugate group, a reactive phosphorus group, a phosphorus moiety or a covalent attachment to a support medium;

each q_i, q_j, q_k and q_l is, independently, H, halogen, C_1-C_{12} alkyl, substituted C_1-C_{12} alkyl, C_2-C_{12} alkenyl, substituted C_2-C_{12} alkenyl, C_2-C_{12} alkynyl, substituted C_2-C_{12} alkynyl, C_1-C_{12} alkoxyl, substituted C_1-C_{12} alkoxyl, OJ_j, SJ_j, SOJ_j, SO_2J_j, NJ_jJ_k, N_3, CN, $C(=O)OJ_j$, $C(=O)NJ_jJ_k$, $C(=O)J_j$, $O\text{-}C(=O)NJ_jJ_k$, $N(H)C(=NH)NJ_jJ_k$, $N(H)C(=O)NJ_jJ_k$ or $N(H)C(=S)NJ_jJ_k$; and

q_i and q_j or q_l and q_k together are $=C(q_g)(q_h)$, wherein q_g and q_h are each, independently, H, halogen, C_1-C_{12} alkyl or substituted C_1-C_{12} alkyl.

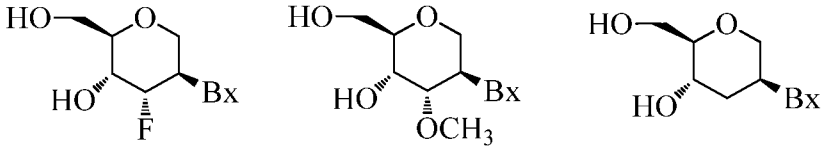
One carbocyclic bicyclic nucleoside having a 4'-$(CH_2)_3$-2' bridge and the alkenyl analog bridge 4'-CH=CH-CH_2-2' have been described (Frier *et al., Nucleic Acids Research*, 1997, *25*(*22*), 4429-4443 and Albaek *et al., J. Org. Chem.*, 2006, *71*, 7731-7740). The synthesis and preparation of carbocyclic bicyclic nucleosides along with their oligomerization and biochemical studies have also been described (Srivastava *et al., J. Am. Chem. Soc.* 2007, *129(26)*, 8362-8379).

In certain embodiments, bicyclic nucleosides include, but are not limited to, (A) α-L-methyleneoxy (4'-CH_2-O-2') BNA , (B) β-D-methyleneoxy (4'-CH_2-O-2') BNA , (C) ethyleneoxy (4'-$(CH_2)_2$-O-2') BNA, (D) aminooxy (4'-CH_2-O-N(R)-2') BNA, (E) oxyamino (4'-CH_2-N(R)-O-2') BNA, (F) methyl(methyleneoxy) (4'-$CH(CH_3)$-O-2') BNA (also referred to as constrained ethyl or cEt), (G) methylene-thio (4'-CH_2-S-2') BNA, (H) methylene-amino (4'-CH_2-N(R)-2') BNA, (I) methyl carbocyclic (4'-CH_2-$CH(CH_3)$-2') BNA, (J) propylene carbocyclic (4'-$(CH_2)_3$-2') BNA, and (K) vinyl BNA as depicted below.

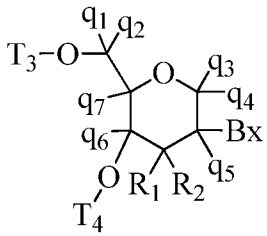


wherein Bx is the base moiety and R is, independently, H, a protecting group, C_1-C_6 alkyl or C_1-C_6 alkoxy.

As used herein, the term "modified tetrahydropyran nucleoside" or "modified THP nucleoside" means a nucleoside having a six-membered tetrahydropyran "sugar" substituted for the pentofuranosyl residue in normal nucleosides and can be referred to as a sugar surrogate. Modified THP nucleosides include, but are not limited to, what is referred to in the art as hexitol nucleic acid (HNA), anitol nucleic acid (ANA), manitol nucleic acid (MNA) (see Leumann, *Bioorg. Med. Chem.*, 2002, *10*, 841-854) or fluoro HNA (F-HNA) having a tetrahydropyranyl ring system as illustrated below.



In certain embodiment, sugar surrogates are selected having the formula:



wherein:

Bx is a heterocyclic base moiety;

T_3 and T_4 are each, independently, an internucleoside linking group linking the tetrahydropyran nucleoside analog to the oligomeric compound or one of T_3 and T_4 is an internucleoside linking group linking the tetrahydropyran nucleoside analog to an oligomeric compound or oligonucleotide and the other of T_3 and T_4 is H, a hydroxyl protecting group, a linked conjugate group or a 5' or 3'-terminal group;

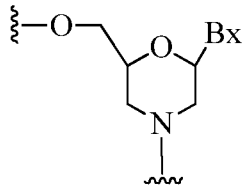
q_1, q_2, q_3, q_4, q_5, q_6 and q_7 are each independently, H, C_1-C_6 alkyl, substituted C_1-C_6 alkyl, C_2-C_6 alkenyl, substituted C_2-C_6 alkenyl, C_2-C_6 alkynyl or substituted C_2-C_6 alkynyl; and

one of R_1 and R_2 is hydrogen and the other is selected from halogen, substituted or unsubstituted alkoxy, NJ_1J_2, SJ_1, N_3, $OC(=X)J_1$, $OC(=X)NJ_1J_2$, $NJ_3C(=X)NJ_1J_2$ and CN, wherein X is O, S or NJ_1 and each J_1, J_2 and J_3 is, independently, H or C_1-C_6 alkyl.

In certain embodiments, q_1, q_2, q_3, q_4, q_5, q_6 and q_7 are each H. In certain embodiments, at least one of q_1, q_2, q_3, q_4, q_5, q_6 and q_7 is other than H. In certain embodiments, at least one of q_1, q_2, q_3, q_4, q_5, q_6 and q_7 is methyl. In certain embodiments, THP nucleosides are provided wherein one of R_1 and R_2 is F. In certain embodiments, R_1 is fluoro and R_2 is H; R_1 is methoxy and R_2 is H, and R_1 is methoxyethoxy and R_2 is H.

In certain embodiments, sugar surrogates comprise rings having more than 5 atoms and more than one heteroatom. For example nucleosides comprising morpholino sugar moieties and their use in oligomeric compounds has been reported (see for example: Braasch *et al.*, *Biochemistry*, 2002, *41*, 4503-4510; and U.S.

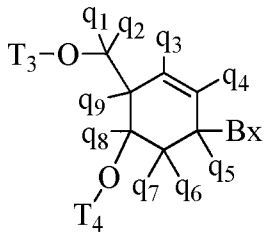
Patents 5,698,685; 5,166,315; 5,185,444; and 5,034,506). As used here, the term "morpholino" means a sugar surrogate having the following formula:



In certain embodiments, morpholinos may be modified, for example by adding or altering various substituent groups from the above morpholino structure. Such sugar surrogates are referred to herein as "modifed morpholinos."

Combinations of modifications are also provided without limitation, such as 2'-F-5'-methyl substituted nucleosides (see PCT International Application WO 2008/101157 published on 8/21/08 for other disclosed 5', 2'-bis substituted nucleosides) and replacement of the ribosyl ring oxygen atom with S and further substitution at the 2'-position (see published U.S. Patent Application US2005-0130923, published on June 16, 2005) or alternatively 5'-substitution of a bicyclic nucleic acid (see PCT International Application WO 2007/134181, published on 11/22/07 wherein a 4'-CH_2-O-2' bicyclic nucleoside is further substituted at the 5' position with a 5'-methyl or a 5'-vinyl group). The synthesis and preparation of carbocyclic bicyclic nucleosides along with their oligomerization and biochemical studies have also been described (*see, e.g.,* Srivastava *et al., J. Am. Chem. Soc.* 2007, *129(26)*, 8362-8379).

In certain embodiments, antisense compounds comprise one or more modified cyclohexenyl nucleosides, which is a nucleoside having a six-membered cyclohexenyl in place of the pentofuranosyl residue in naturally occurring nucleosides. Modified cyclohexenyl nucleosides include, but are not limited to those described in the art (see for example commonly owned, published PCT Application WO 2010/036696, published on April 10, 2010, Robeyns *et al., J. Am. Chem. Soc.,* 2008, *130(6)*, 1979-1984; Horváth *et al., Tetrahedron Letters,* 2007, *48*, 3621-3623; Nauwelaerts *et al., J. Am. Chem. Soc*., 2007, *129(30)*, 9340-9348; Gu *et al.,*, *Nucleosides, Nucleotides & Nucleic Acids*, 2005, *24(5-7)*, 993-998; Nauwelaerts *et al., Nucleic Acids Research,* 2005, *33(8)*, 2452-2463; Robeyns et al., *Acta Crystallographica, Section F: Structural Biology and Crystallization Communications,* 2005, *F61(6)*, 585-586; Gu *et al., Tetrahedron*, 2004, *60(9)*, 2111-2123; Gu *et al., Oligonucleotides*, 2003, *13(6)*, 479-489; Wang *et al., J. Org. Chem.,* 2003, *68*, 4499-4505; Verbeure *et al., Nucleic Acids Research*, 2001, *29(24)*, 4941-4947; Wang *et al., J. Org. Chem.,* 2001, *66*, 8478-82; Wang *et al., Nucleosides, Nucleotides & Nucleic Acids*, 2001, *20(4-7)*, 785-788; Wang *et al., J. Am. Chem.,* 2000, *122*, 8595-8602; Published PCT application, WO 06/047842; and Published PCT Application WO 01/049687; the text of each is incorporated by reference herein, in their entirety). Certain modified cyclohexenyl nucleosides have Formula X.



X

wherein independently for each of said at least one cyclohexenyl nucleoside analog of Formula X:

Bx is a heterocyclic base moiety;

T_3 and T_4 are each, independently, an internucleoside linking group linking the cyclohexenyl nucleoside analog to an antisense compound or one of T_3 and T_4 is an internucleoside linking group linking the tetrahydropyran nucleoside analog to an antisense compound and the other of T_3 and T_4 is H, a hydroxyl protecting group, a linked conjugate group, or a 5'-or 3'-terminal group; and

q_1, q_2, q_3, q_4, q_5, q_6, q_7, q_8 and q_9 are each, independently, H, C_1-C_6 alkyl, substituted C_1-C_6 alkyl, C_2-C_6 alkenyl, substituted C_2-C_6 alkenyl, C_2-C_6 alkynyl, substituted C_2-C_6 alkynyl or other sugar substituent group.

Many other monocyclic, bicyclic and tricyclic ring systems are known in the art and are suitable as sugar surrogates that can be used to modify nucleosides for incorporation into oligomeric compounds as provided herein (see for example review article: Leumann, Christian J. *Bioorg. & Med. Chem.*, 2002, *10*, 841-854). Such ring systems can undergo various additional substitutions to further enhance their activity.

As used herein, "2'-modified sugar" means a furanosyl sugar modified at the 2' position. In certain embodiments, such modifications include substituents selected from: a halide, including, but not limited to substituted and unsubstituted alkoxy, substituted and unsubstituted thioalkyl, substituted and unsubstituted amino alkyl, substituted and unsubstituted alkyl, substituted and unsubstituted allyl, and substituted and unsubstituted alkynyl. In certain embodiments, 2' modifications are selected from substituents including, but not limited to: $O[(CH_2)_nO]_mCH_3$, $O(CH_2)_nNH_2$, $O(CH_2)_nCH_3$, $O(CH_2)_nF$, $O(CH_2)_nONH_2$, $OCH_2C(=O)N(H)CH_3$, and $O(CH_2)_nON[(CH_2)_nCH_3]_2$, where n and m are from 1 to about 10. Other 2'-substituent groups can also be selected from: C_1-C_{12} alkyl, substituted alkyl, alkenyl, alkynyl, alkaryl, aralkyl, O-alkaryl or O-aralkyl, SH, SCH_3, OCN, Cl, Br, CN, F, CF_3, OCF_3, $SOCH_3$, SO_2CH_3, ONO_2, NO_2, N_3, NH_2, heterocycloalkyl, heterocycloalkaryl, aminoalkylamino, polyalkylamino, substituted silyl, an RNA cleaving group, a reporter group, an intercalator, a group for improving pharmacokinetic properties, or a group for improving the pharmacodynamic properties of an antisense compound, and other substituents having similar properties. In certain embodiments, modifed nucleosides comprise a 2'-MOE side chain (Baker *et al.*, *J. Biol. Chem.*, 1997, *272*, 11944-12000). Such 2'-MOE substitution have been described as having improved binding affinity compared to unmodified nucleosides and to other modified nucleosides, such as 2'- *O*-methyl, *O*-propyl, and *O*-aminopropyl. Oligonucleotides having the 2'-MOE substituent also have been shown to be antisense inhibitors of gene expression with promising features for *in vivo* use (Martin, *Helv.*

Chim. Acta, 1995, *78*, 486-504; Altmann *et al.*, *Chimia*, 1996, *50*, 168-176; Altmann *et al.*, *Biochem. Soc. Trans.*, 1996, *24*, 630-637; and Altmann *et al.*, *Nucleosides Nucleotides*, 1997, *16*, 917-926).

As used herein, "2'-modified" or "2'-substituted" refers to a nucleoside comprising a sugar comprising a substituent at the 2' position other than H or OH. 2'-modified nucleosides, include, but are not limited to, nucleosides with non-bridging 2'substituents, such as allyl, amino, azido, thio, O-allyl, O-C_1-C_{10} alkyl, -OCF_3, O-$(CH_2)_2$-O-CH_3, 2'-O$(CH_2)_2SCH_3$, O-$(CH_2)_2$-O-N$(R_m)(R_n)$, or O-CH_2-C(=O)-N$(R_m)(R_n)$, where each R_m and R_n is, independently, H or substituted or unsubstituted C_1-C_{10} alkyl. 2'-modifed nucleosides may further comprise other modifications, for example at other positions of the sugar and/or at the nucleobase.

As used herein, "2'-F" refers to a nucleoside comprising a sugar comprising a fluoro group at the 2' position of the sugar ring.

As used herein, "2'-OMe" or "2'-OCH_3", "2'-O-methyl" or "2'-methoxy" each refers to a nucleoside comprising a sugar comprising an -OCH_3 group at the 2' position of the sugar ring.

As used herein, "MOE" or "2'-MOE" or "2'-$OCH_2CH_2OCH_3$" or "2'-O-methoxyethyl" each refers to a nucleoside comprising a sugar comprising a -$OCH_2CH_2OCH_3$ group at the 2' position of the sugar ring.

Methods for the preparations of modified sugars are well known to those skilled in the art. Some representative U.S. patents that teach the preparation of such modified sugars include without limitation, U.S.: 4,981,957; 5,118,800; 5,319,080; 5,359,044; 5,393,878; 5,446,137; 5,466,786; 5,514,785; 5,519,134; 5,567,811; 5,576,427; 5,591,722; 5,597,909; 5,610,300; 5,627,053; 5,639,873; 5,646,265; 5,670,633; 5,700,920; 5,792,847 and 6,600,032 and International Application PCT/US2005/019219, filed June 2, 2005 and published as WO 2005/121371 on December 22, 2005, and each of which is herein incorporated by reference in its entirety.

As used herein, "oligonucleotide" refers to a compound comprising a plurality of linked nucleosides. In certain embodiments, one or more of the plurality of nucleosides is modified. In certain embodiments, an oligonucleotide comprises one or more ribonucleosides (RNA) and/or deoxyribonucleosides (DNA).

In nucleotides having modified sugar moieties, the nucleobase moieties (natural, modified or a combination thereof) are maintained for hybridization with an appropriate nucleic acid target.

In certain embodiments, antisense compounds comprise one or more nucleosides having modified sugar moieties. In certain embodiments, the modified sugar moiety is 2'-MOE. In certain embodiments, the 2'-MOE modified nucleosides are arranged in a gapmer motif. In certain embodiments, the modified sugar moiety is a bicyclic nucleoside having a (4'-$CH(CH_3)$-O-2') bridging group. In certain embodiments, the (4'-$CH(CH_3)$-O-2') modified nucleosides are arranged throughout the wings of a gapmer motif.

Modified Nucleobases

Nucleobase (or base) modifications or substitutions are structurally distinguishable from, yet functionally interchangeable with, naturally occurring or synthetic unmodified nucleobases. Both natural and

modified nucleobases are capable of participating in hydrogen bonding. Such nucleobase modifications may impart nuclease stability, binding affinity or some other beneficial biological property to antisense compounds. Modified nucleobases include synthetic and natural nucleobases such as, for example, 5-methylcytosine (5-me-C). Certain nucleobase substitutions, including 5-methylcytosine substitutions, are particularly useful for increasing the binding affinity of an antisense compound for a target nucleic acid. For example, 5-methylcytosine substitutions have been shown to increase nucleic acid duplex stability by 0.6-1.2°C (Sanghvi, Y.S., Crooke, S.T. and Lebleu, B., eds., *Antisense Research and Applications*, CRC Press, Boca Raton, 1993, pp. 276-278).

Additional unmodified nucleobases include 5-hydroxymethyl cytosine, xanthine, hypoxanthine, 2-aminoadenine, 6-methyl and other alkyl derivatives of adenine and guanine, 2-propyl and other alkyl derivatives of adenine and guanine, 2-thiouracil, 2-thiothymine and 2-thiocytosine, 5-halouracil and cytosine, 5-propynyl (-C≡C-CH_3) uracil and cytosine and other alkynyl derivatives of pyrimidine bases, 6-azo uracil, cytosine and thymine, 5-uracil (pseudouracil), 4-thiouracil, 8-halo, 8-amino, 8-thiol, 8-thioalkyl, 8-hydroxyl and other 8-substituted adenines and guanines, 5-halo particularly 5-bromo, 5-trifluoromethyl and other 5-substituted uracils and cytosines, 7-methylguanine and 7-methyladenine, 2-F-adenine, 2-amino-adenine, 8-azaguanine and 8-azaadenine, 7-deazaguanine and 7-deazaadenine and 3-deazaguanine and 3-deazaadenine.

Heterocyclic base moieties may also include those in which the purine or pyrimidine base is replaced with other heterocycles, for example 7-deaza-adenine, 7-deazaguanosine, 2-aminopyridine and 2-pyridone. Nucleobases that are particularly useful for increasing the binding affinity of antisense compounds include 5-substituted pyrimidines, 6-azapyrimidines and N-2, N-6 and O-6 substituted purines, including 2 aminopropyladenine, 5-propynyluracil and 5-propynylcytosine.

In certain embodiments, antisense compounds targeted to a TMPRSS6 nucleic acid comprise one or more modified nucleobases. In certain embodiments, gap-widened antisense oligonucleotides targeted to a TMPRSS6 nucleic acid comprise one or more modified nucleobases. In certain embodiments, at least one of the modified nucleobases is 5-methylcytosine. In certain embodiments, each cytosine is a 5-methylcytosine.

Compositions and Methods for Formulating Pharmaceutical Compositions

Antisense oligonucleotides may be admixed with pharmaceutically acceptable active or inert substance for the preparation of pharmaceutical compositions or formulations. Compositions and methods for the formulation of pharmaceutical compositions are dependent upon a number of criteria, including, but not limited to, route of administration, extent of disease, or dose to be administered.

Antisense compound targeted to a TMPRSS6 nucleic acid can be utilized in pharmaceutical compositions by combining the antisense compound with a suitable pharmaceutically acceptable diluent or carrier. A pharmaceutically acceptable diluent includes water e.g., water-for-injection (WFI). A pharmaceutically acceptable diluent includes saline e.g., phosphate-buffered saline (PBS). Water or saline is a

diluent suitable for use in compositions to be delivered parenterally. Accordingly, in one embodiment, employed in the methods described herein is a pharmaceutical composition comprising an antisense compound targeted to a TMPRSS6 nucleic acid and a pharmaceutically acceptable diluent. In certain embodiments, the pharmaceutically acceptable diluent is water or saline. In certain embodiments, the antisense compound is an antisense oligonucleotide.

Pharmaceutical compositions comprising antisense compounds encompass any pharmaceutically acceptable salts, esters, or salts of such esters, or any other oligonucleotide 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 herein is also drawn to pharmaceutically acceptable salts of antisense compounds, prodrugs, pharmaceutically acceptable salts of such prodrugs, and other bioequivalents. Suitable pharmaceutically acceptable salts include, but are not limited to, sodium and potassium salts.

Pharmaceutically acceptable salts of the compounds described herein may be prepared by methods well-known in the art. For a review of pharmaceutically acceptable salts, see Stahl and Wermuth, Handbook of Pharmaceutical Salts: Properties, Selection and Use (Wiley-VCH, Weinheim, Germany, 2002). Sodium salts of antisense oligonucleotides are useful and are well accepted for therapeutic administration to humans. Accordingly, in one embodiment the compounds described herein are in the form of a sodium salt.

A prodrug can include the incorporation of additional nucleosides at one or both ends of an antisense compound which are cleaved by endogenous nucleases within the body, to form the active antisense compound.

Dosing

In certain embodiments, pharmaceutical compositions are administered according to a dosing regimen (e.g., dose, dose frequency, and duration) wherein the dosing regimen can be selected to achieve a desired effect. The desired effect can be, for example, reduction of TMPRSS6 or the prevention, reduction, amelioration or slowing the progression of a disease, disorder or condition associated with TMPRSS6.

In certain embodiments, the variables of the dosing regimen are adjusted to result in a desired concentration of pharmaceutical composition in a subject. "Concentration of pharmaceutical composition" as used with regard to dose regimen can refer to the compound, oligonucleotide, or active ingredient of the pharmaceutical composition. For example, in certain embodiments, dose and dose frequency are adjusted to provide a tissue concentration or plasma concentration of a pharmaceutical composition at an amount sufficient to achieve a desired effect.

Dosing is dependent on severity and responsiveness of the disease state to be treated, with the course of treatment lasting from several days to several months, or until a cure is effected or a diminution of the disease state is achieved. Dosing is also dependent on drug potency and metabolism. In certain embodiments, dosage is from 0.01μg to 100mg per kg of body weight, or within a range of 0.001mg to 1000mg dosing, and

may be given once or more daily, weekly, biweekly, monthly, quarterly, semi-annually or yearly, or even once every 2 to 20 years. Following successful treatment, it may be desirable to have the patient undergo maintenance therapy to prevent the recurrence of the disease state, wherein the oligonucleotide is administered in maintenance doses, ranging from 0.01μg to 100mg per kg of body weight, once or more daily, to once every 20 years or ranging from 0.001mg to 1000mg dosing.

Administration

The compounds or pharmaceutical compositions of the present invention can be administered in a number of ways depending upon whether local or systemic treatment is desired and upon the area to be treated. Administration can be inhaled (i.e., pulmonary), enteral (i.e., enteric), parenteral or topical.

In certain embodiments, the compounds and compositions as described herein are administered parenterally. Parenteral administration includes, but is not limited to, intravenous, intra-arterial, subcutaneous, intraperitoneal, intraocular, intramuscular, intracranial, intrathecal, intramedullary, intraventricular or intratumoral injection or infusion. Parenteral administration also includes intranasal administration.

In certain embodiments, parenteral administration is by infusion. Infusion can be chronic or continuous or short or intermittent. In certain embodiments, infused pharmaceutical agents are delivered with a pump.

In certain embodiments, parenteral administration is by injection. The injection can be delivered with a syringe or a pump. In certain embodiments, the injection is a bolus injection. In certain embodiments, the injection is administered directly to a tissue or organ.

In certain embodiments, formulations for parenteral administration can include sterile aqueous solutions which can also contain buffers, diluents and other suitable additives such as, but not limited to, penetration enhancers, carrier compounds and other pharmaceutically acceptable carriers or excipients.

In certain embodiments, the compounds and compositions as described herein are administered enterally. Enteric administration includes, but is not limited to, oral, transmucosal, intestinal or rectal (e.g., suppository, enema). In certain embodiments, formulations for enteral administration of the compounds or compositions can include, but is not limited to, pharmaceutical carriers, excipients, powders or granules, microparticulates, nanoparticulates, suspensions or solutions in water or non-aqueous media, capsules, gel capsules, sachets, tablets or minitablets. Thickeners, flavoring agents, diluents, emulsifiers, dispersing aids or binders can be desirable. In certain embodiments, enteral formulations are those in which compounds provided herein are administered in conjunction with one or more penetration enhancers, surfactants and chelators.

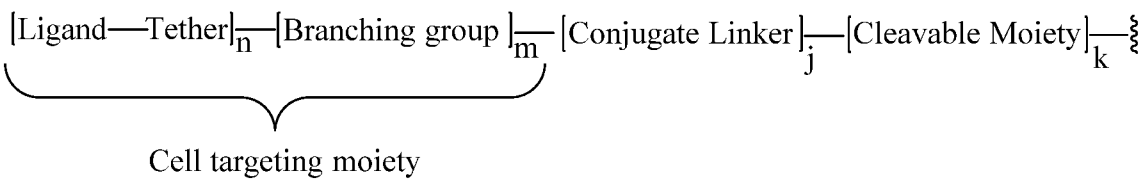
In certain embodiments, administration includes pulmonary administration. In certain embodiments, pulmonary administration comprises delivery of aerosolized oligonucleotide to the lung of a subject by inhalation. Following inhalation by a subject of aerosolized oligonucleotide, oligonucleotide distributes to

cells of both normal and inflamed lung tissue, including alveolar macrophages, eosinophils, epithelium, blood vessel endothelium, and bronchiolar epithelium. A suitable device for the delivery of a pharmaceutical composition comprising a modified oligonucleotide includes, but is not limited to, a standard nebulizer device. Additional suitable devices include dry powder inhalers or metered dose inhalers.

In certain embodiments, pharmaceutical compositions are administered to achieve local rather than systemic exposures. For example, pulmonary administration delivers a pharmaceutical composition to the lung, with minimal systemic exposure.

Conjugated Antisense Compounds

In certain embodiments, the oligonucleotides or oligomeric compounds as provided herein are modified by covalent attachment of one or more conjugate groups. In general, conjugate groups modify one or more properties of the attached oligonucleotide or oligomeric compound including but not limited to pharmacodynamics, pharmacokinetics, stability, binding, absorption, cellular distribution, cellular uptake, charge and clearance. As used herein, "conjugate group" means a radical group comprising a group of atoms that are attached to an oligonucleotide or oligomeric compound. In general, conjugate groups modify one or more properties of the compound to which they are attached, including, but not limited to pharmacodynamic, pharmacokinetic, binding, absorption, cellular distribution, cellular uptake, charge and/or clearance properties. Conjugate groups are routinely used in the chemical arts and can include a conjugate linker that covalently links the conjugate group to an oligonucleotide or oligomeric compound. In certain embodiments, conjugate groups include a cleavable moiety that covalently links the conjugate group to an oligonucleotide or oligomeric compound. In certain embodiments, conjugate groups include a conjugate linker and a cleavable moiety to covalently link the conjugate group to an oligonucleotide or oligomeric compound. In certain embodiments, a conjugate group has the general formula:



wherein n is from 1 to about 3, m is 0 when n is 1 or m is 1 when n is 2 or 3, j is 1 or 0, k is 1 or 0 and the sum of j and k is at least one.

In certain embodiments, n is 1, j is 1 and k is 0. In certain embodiments, n is 1, j is 0 and k is 1. In certain embodiments, n is 1, j is 1 and k is 1. In certain embodiments, n is 2, j is 1 and k is 0. In certain embodiments, n is 2, j is 0 and k is 1. In certain embodiments, n is 2, j is 1 and k is 1. In certain embodiments, n is 3, j is 1 and k is 0. In certain embodiments, n is 3, j is 0 and k is 1. In certain embodiments, n is 3, j is 1 and k is 1.

Conjugate groups are shown herein as radicals, providing a bond for forming covalent attachment to an oligomeric compound such as an oligonucleotide. In certain embodiments, the point of attachment on the oligomeric compound is at the 3'-terminal nucleoside or modified nucleoside. In certain embodiments, the point of attachment on the oligomeric compound is the 3'-oxygen atom of the 3'-hydroxyl group of the 3' terminal nucleoside or modified nucleoside. In certain embodiments, the point of attachment on the oligomeric compound is at the 5'-terminal nucleoside or modified nucleoside. In certain embodiments the point of attachment on the oligomeric compound is the 5'-oxygen atom of the 5'-hydroxyl group of the 5'-terminal nucleoside or modified nucleoside. In certain embodiments, the point of attachment on the oligomeric compound is at any reactive site on a nucleoside, a modified nucleoside or an internucleoside linkage.

As used herein, "cleavable moiety" and "cleavable bond" mean a cleavable bond or group of atoms that is capable of being split or cleaved under certain physiological conditions. In certain embodiments, a cleavable moiety is a cleavable bond. In certain embodiments, a cleavable moiety comprises a cleavable bond. In certain embodiments, a cleavable moiety is a group of atoms. In certain embodiments, a cleavable moiety is selectively cleaved inside a cell or sub-cellular compartment, such as a lysosome. In certain embodiments, a cleavable moiety is selectively cleaved by endogenous enzymes, such as nucleases. In certain embodiments, a cleavable moiety comprises a group of atoms having one, two, three, four, or more than four cleavable bonds.

In certain embodiments, conjugate groups comprise a cleavable moiety. In certain such embodiments, the cleavable moiety covalently attaches the oligomeric compound to the conjugate linker. In certain such embodiments, the cleavable moiety covalently attaches the oligomeric compound to the cell-targeting moiety.

In certain embodiments, a cleavable bond is selected from among: an amide, a polyamide, an ester, an ether, one or both esters of a phosphodiester, a phosphate ester, a carbamate, a di-sulfide, or a peptide. In certain embodiments, a cleavable bond is one of the esters of a phosphodiester. In certain embodiments, a cleavable bond is one or both esters of a phosphodiester. In certain embodiments, the cleavable moiety is a phosphodiester linkage between an oligomeric compound and the remainder of the conjugate group. In certain embodiments, the cleavable moiety comprises a phosphodiester linkage that is located between an oligomeric compound and the remainder of the conjugate group. In certain embodiments, the cleavable moiety comprises a phosphate or phosphodiester. In certain embodiments, the cleavable moiety is attached to the conjugate linker by either a phosphodiester or a phosphorothioate linkage. In certain embodiments, the cleavable moiety is attached to the conjugate linker by a phosphodiester linkage. In certain embodiments, the conjugate group does not include a cleavable moiety.

In certain embodiments, the cleavable moiety is a cleavable nucleoside or a modified nucleoside. In certain embodiments, the nucleoside or modified nucleoside comprises an optionally protected heterocyclic base selected from a purine, substituted purine, pyrimidine or substituted pyrimidine. In certain

embodiments, the cleavable moiety is a nucleoside selected from uracil, thymine, cytosine, 4-N-benzoylcytosine, 5-methylcytosine, 4-N-benzoyl-5-methylcytosine, adenine, 6-N-benzoyladenine, guanine and 2-N-isobutyrylguanine.

In certain embodiments, the cleavable moiety is 2'-deoxy nucleoside that is attached to either the 3' or 5'-terminal nucleoside of an oligomeric compound by a phosphodiester linkage and covalently attached to the remainder of the conjugate group by a phosphodiester or phosphorothioate linkage. In certain embodiments, the cleavable moiety is 2'-deoxy adenosine that is attached to either the 3' or 5'-terminal nucleoside of an oligomeric compound by a phosphodiester linkage and covalently attached to the remainder of the conjugate group by a phosphodiester or phosphorothioate linkage. In certain embodiments, the cleavable moiety is 2'-deoxy adenosine that is attached to the 3'-oxygen atom of the 3'-hydroxyl group of the 3'-terminal nucleoside or modified nucleoside by a phosphodiester linkage. In certain embodiments, the cleavable moiety is 2'-deoxy adenosine that is attached to the 5'-oxygen atom of the 5'-hydroxyl group of the 5'-terminal nucleoside or modified nucleoside by a phosphodiester linkage. In certain embodiments, the cleavable moiety is attached to a 2'-position of a nucleoside or modified nucleoside of an oligomeric compound.

As used herein, "conjugate linker" in the context of a conjugate group means a portion of a conjugate group comprising any atom or group of atoms that covalently link the cell-targeting moiety to the oligomeric compound either directly or through the cleavable moiety. In certain embodiments, the conjugate linker comprises groups selected from alkyl, amino, oxo, amide, disulfide, polyethylene glycol, ether, thioether (-S-) and hydroxylamino (-O-N(H)-). In certain embodiments, the conjugate linker comprises groups selected from alkyl, amino, oxo, amide and ether groups. In certain embodiments, the conjugate linker comprises groups selected from alkyl and amide groups. In certain embodiments, the conjugate linker comprises groups selected from alkyl and ether groups. In certain embodiments, the conjugate linker comprises at least one phosphorus linking group. In certain embodiments, the conjugate linker comprises at least one phosphodiester group. In certain embodiments, the conjugate linker includes at least one neutral linking group.

In certain embodiments, the conjugate linker is covalently attached to the oligomeric compound. In certain embodiments, the conjugate linker is covalently attached to the oligomeric compound and the branching group. In certain embodiments, the conjugate linker is covalently attached to the oligomeric compound and a tethered ligand. In certain embodiments, the conjugate linker is covalently attached to the cleavable moiety. In certain embodiments, the conjugate linker is covalently attached to the cleavable moiety and the branching group. In certain embodiments, the conjugate linker is covalently attached to the cleavable moiety and a tethered ligand. In certain embodiments, the conjugate linker includes one or more cleavable bonds. In certain embodiments, the conjugate group does not include a conjugate linker.

As used herein, "branching group" means a group of atoms having at least 3 positions that are capable of forming covalent linkages to two or more tether-ligands and the remainder of the conjugate group. In general a branching group provides a plurality of reactive sites for connecting tethered ligands to the

oligomeric compound through the conjugate linker and/or the cleavable moiety. In certain embodiments, the branching group comprises groups selected from alkyl, amino, oxo, amide, disulfide, polyethylene glycol, ether, thioether and hydroxylamino groups. In certain embodiments, the branching group comprises a branched aliphatic group comprising groups selected from alkyl, amino, oxo, amide, disulfide, polyethylene glycol, ether, thioether and hydroxylamino groups. In certain such embodiments, the branched aliphatic group comprises groups selected from alkyl, amino, oxo, amide and ether groups. In certain such embodiments, the branched aliphatic group comprises groups selected from alkyl, amino and ether groups. In certain such embodiments, the branched aliphatic group comprises groups selected from alkyl and ether groups. In certain embodiments, the branching group comprises a mono or polycyclic ring system.

In certain embodiments, the branching group is covalently attached to the conjugate linker. In certain embodiments, the branching group is covalently attached to the cleavable moiety. In certain embodiments, the branching group is covalently attached to the conjugate linker and each of the tethered ligands. In certain embodiments, the branching group comprises one or more cleavable bond. In certain embodiments, the conjugate group does not include a branching group.

In certain embodiments, conjugate groups as provided herein include a cell-targeting moiety that has at least one tethered ligand. In certain embodiments, the cell-targeting moiety comprises two tethered ligands covalently attached to a branching group. In certain embodiments, the cell-targeting moiety comprises three tethered ligands covalently attached to a branching group.

As used herein, "tether" means a group of atoms that connect a ligand to the remainder of the conjugate group. In certain embodiments, each tether is a linear aliphatic group comprising one or more groups selected from alkyl, substituted alkyl, ether, thioether, disulfide, amino, oxo, amide, phosphodiester and polyethylene glycol groups in any combination. In certain embodiments, each tether is a linear aliphatic group comprising one or more groups selected from alkyl, ether, thioether, disulfide, amino, oxo, amide and polyethylene glycol groups in any combination. In certain embodiments, each tether is a linear aliphatic group comprising one or more groups selected from alkyl, substituted alkyl, phosphodiester, ether and amino, oxo, amide groups in any combination. In certain embodiments, each tether is a linear aliphatic group comprising one or more groups selected from alkyl, ether and amino, oxo, amide groups in any combination. In certain embodiments, each tether is a linear aliphatic group comprising one or more groups selected from alkyl, amino and oxo groups in any combination. In certain embodiments, each tether is a linear aliphatic group comprising one or more groups selected from alkyl and oxo groups in any combination. In certain embodiments, each tether is a linear aliphatic group comprising one or more groups selected from alkyl and phosphodiester in any combination. In certain embodiments, each tether comprises at least one phosphorus linking group or neutral linking group.

In certain embodiments, tethers include one or more cleavable bond. In certain embodiments, each tethered ligand is attached to a branching group. In certain embodiments, each tethered ligand is attached to a branching group through an amide group. In certain embodiments, each tethered ligand is attached to a

branching group through an ether group. In certain embodiments, each tethered ligand is attached to a branching group through a phosphorus linking group or neutral linking group. In certain embodiments, each tethered ligand is attached to a branching group through a phosphodiester group. In certain embodiments, each tether is attached to a ligand through either an amide or an ether group. In certain embodiments, each tether is attached to a ligand through an ether group.

In certain embodiments, each tether comprises from about 8 to about 20 atoms in chain length between the ligand and the branching group. In certain embodiments, each tether comprises from about 10 to about 18 atoms in chain length between the ligand and the branching group. In certain embodiments, each tether comprises about 13 atoms in chain length.

In certain embodiments, the present disclosure provides ligands wherein each ligand is covalently attached to the remainder of the conjugate group through a tether. In certain embodiments, each ligand is selected to have an affinity for at least one type of receptor on a target cell. In certain embodiments, ligands are selected that have an affinity for at least one type of receptor on the surface of a mammalian liver cell. In certain embodiments, ligands are selected that have an affinity for the hepatic asialoglycoprotein receptor (ASGP-R). In certain embodiments, each ligand is a carbohydrate. In certain embodiments, each ligand is, independently selected from galactose, N-acetyl galactoseamine, mannose, glucose, glucosamone and fucose. In certain embodiments, each ligand is N-acetyl galactoseamine (GalNAc). In certain embodiments, the targeting moiety comprises 1 to 3 ligands. In certain embodiments, the targeting moiety comprises 3 ligands. In certain embodiments, the targeting moiety comprises 2 ligands. In certain embodiments, the targeting moiety comprises 1 ligand. In certain embodiments, the targeting moiety comprises 3 N-acetyl galactoseamine ligands. In certain embodiments, the targeting moiety comprises 2 N-acetyl galactoseamine ligands. In certain embodiments, the targeting moiety comprises 1 N-acetyl galactoseamine ligand.

In certain embodiments, each ligand is a carbohydrate, carbohydrate derivative, modified carbohydrate, multivalent carbohydrate cluster, polysaccharide, modified polysaccharide, or polysaccharide derivative. In certain embodiments, each ligand is an amino sugar or a thio sugar. For example, amino sugars may be selected from any number of compounds known in the art, for example glucosamine, sialic acid, α-D-galactosamine, N-Acetylgalactosamine, 2-acetamido-2-deoxy-D-galactopyranose (GalNAc), 2-Amino-3-*O*-[(*R*)-1-carboxyethyl]-2-deoxy-β-D-glucopyranose (β-muramic acid), 2-Deoxy-2-methylamino-L-glucopyranose, 4,6-Dideoxy-4-formamido-2,3-di-*O*-methyl-D-mannopyranose, 2-Deoxy-2-sulfoamino-D-glucopyranose and *N*-sulfo-D-glucosamine, and *N*-Glycoloyl-α-neuraminic acid. For example, thio sugars may be selected from the group consisting of 5-Thio-β-D-glucopyranose, Methyl 2,3,4-tri-*O*-acetyl-1-thio-6-*O*-trityl-α-D-glucopyranoside, 4-Thio-β-D-galactopyranose, and ethyl 3,4,6,7-tetra-*O*-acetyl-2-deoxy-1,5-dithio-α-D-*gluco*-heptopyranoside.

In certain embodiments, conjugate groups as provided herein comprise a carbohydrate cluster. As used herein, "carbohydrate cluster" means a portion of a conjugate group wherein two or more carbohydrate residues are attached to a branching group through tether groups. (*see*, e.g., Maier et al., "Synthesis of

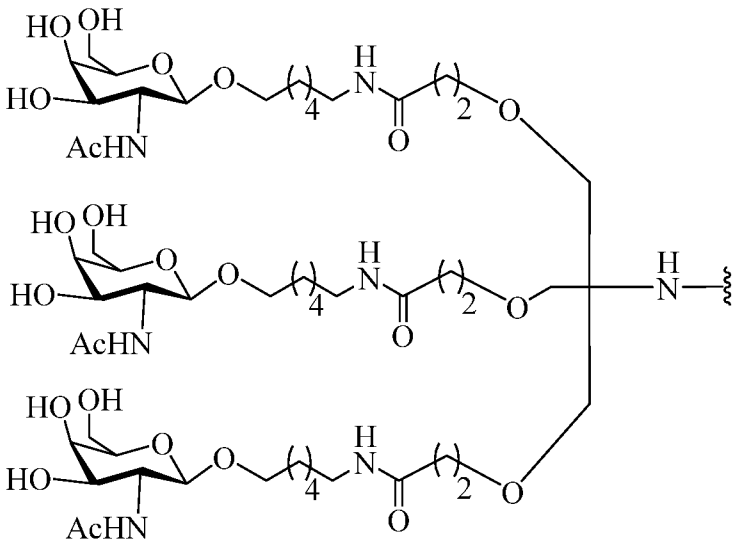
Antisense Oligonucleotides Conjugated to a Multivalent Carbohydrate Cluster for Cellular Targeting," *Bioconjugate Chemistry*, 2003, (14): 18-29, which is incorporated herein by reference in its entirety, or Rensen et al., "Design and Synthesis of Novel *N*-Acetylgalactosamine-Terminated Glycolipids for Targeting of Lipoproteins to the Hepatic Asiaglycoprotein Receptor," *J. Med. Chem.* 2004, (47): 5798-5808, for examples of carbohydrate conjugate clusters).

As used herein, "modified carbohydrate" means any carbohydrate having one or more chemical modifications relative to naturally occurring carbohydrates.

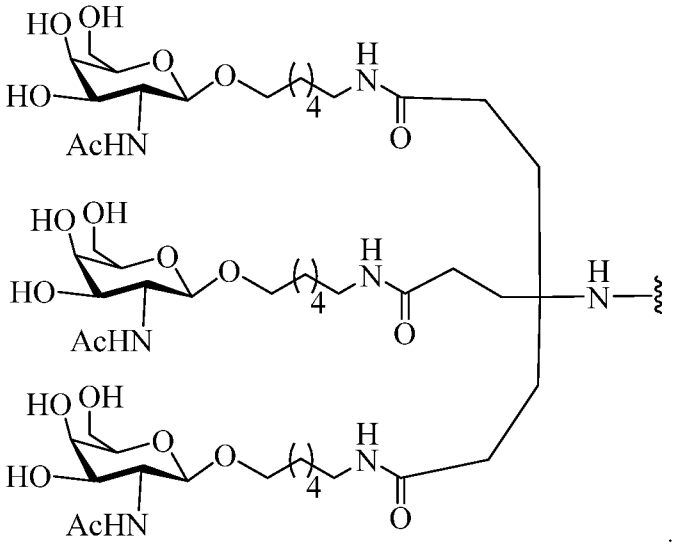
As used herein, "carbohydrate derivative" means any compound which may be synthesized using a carbohydrate as a starting material or intermediate.

As used herein, "carbohydrate" means a naturally occurring carbohydrate, a modified carbohydrate, or a carbohydrate derivative.

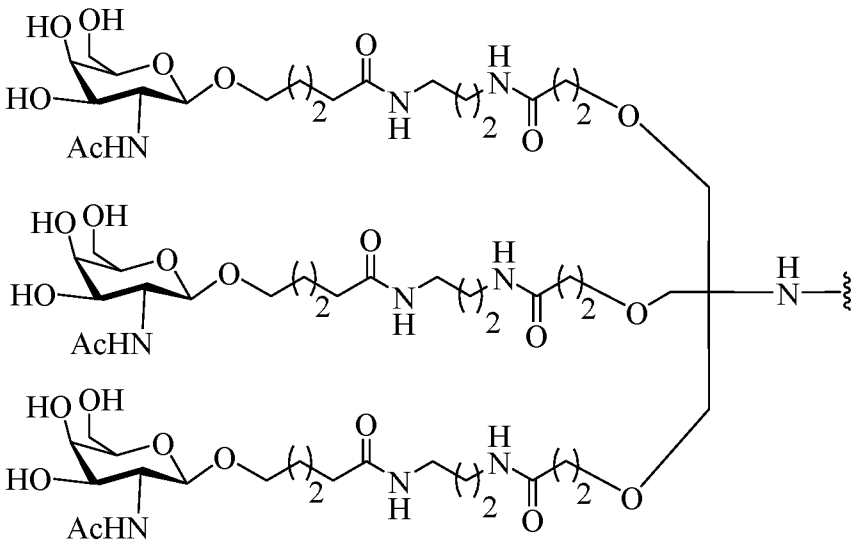
In certain embodiments, conjugate groups are provided wherein the cell-targeting moiety has the formula:



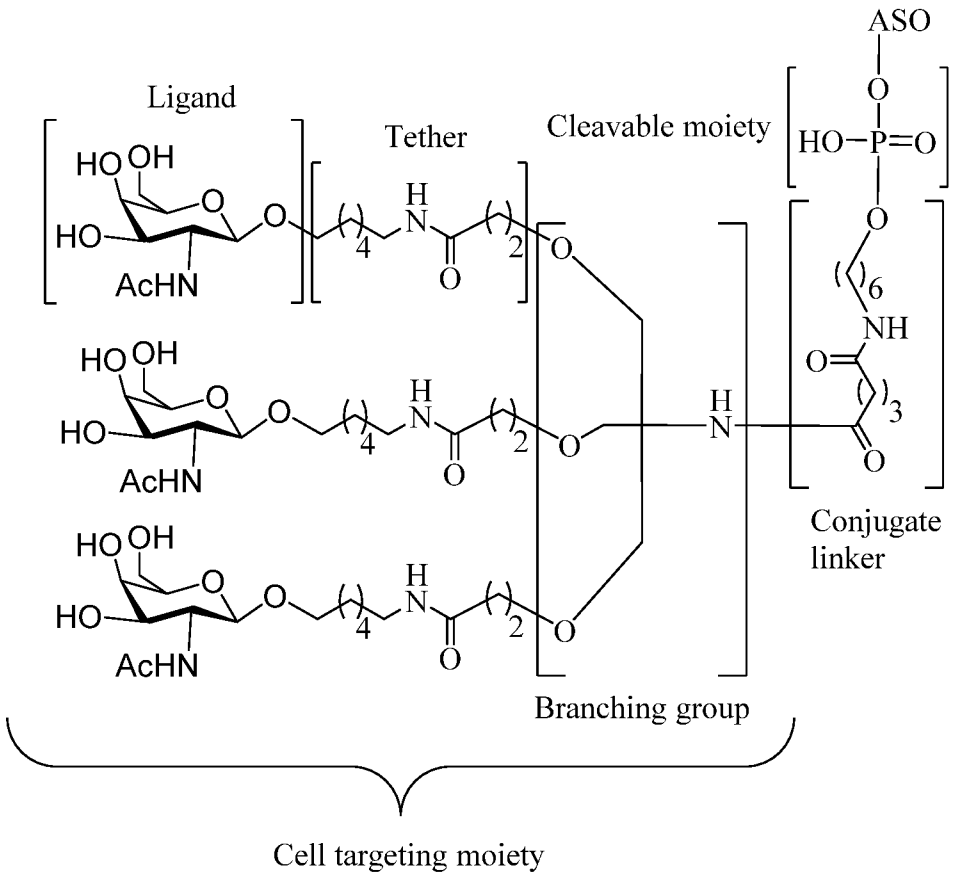
In certain embodiments, conjugate groups are provided wherein the cell-targeting moiety has the formula:



In certain embodiments, conjugate groups are provided wherein the cell-targeting moiety has the formula:



In certain embodiments, conjugate groups have the formula:



Representative United States patents, United States patent application publications, and international patent application publications that teach the preparation of certain of the above noted conjugate groups, conjugated oligomeric compounds such as antisense compounds comprising a conjugate group, tethers, conjugate linkers, branching groups, ligands, cleavable moieties as well as other modifications include without limitation, US 5,994,517, US 6,300,319, US 6,660,720, US 6,906,182, US 7,262,177, US 7,491,805, US 8,106,022, US 7,723,509, US 2006/0148740, US 2011/0123520, WO 2013/033230 and WO 2012/037254, each of which is incorporated by reference herein in its entirety.

Representative publications that teach the preparation of certain of the above noted conjugate groups, conjugated oligomeric compounds such as antisense compounds comprising a conjugate group, tethers, conjugate linkers, branching groups, ligands, cleavable moieties as well as other modifications include without limitation, BIESSEN et al., "The Cholesterol Derivative of a Triantennary Galactoside with High Affinity for the Hepatic Asialoglycoprotein Receptor: a Potent Cholesterol Lowering Agent" J. Med. Chem. (1995) 38:1846-1852, BIESSEN et al., "Synthesis of Cluster Galactosides with High Affinity for the Hepatic Asialoglycoprotein Receptor" J. Med. Chem. (1995) 38:1538-1546, LEE et al., "New and more efficient multivalent glyco-ligands for asialoglycoprotein receptor of mammalian hepatocytes" Bioorganic & Medicinal Chemistry (2011) 19:2494-2500, RENSEN et al., "Determination of the Upper Size Limit for

Uptake and Processing of Ligands by the Asialoglycoprotein Receptor on Hepatocytes in Vitro and in Vivo" J. Biol. Chem. (2001) 276(40):37577-37584, RENSEN et al., "Design and Synthesis of Novel N-Acetylgalactosamine-Terminated Glycolipids for Targeting of Lipoproteins to the Hepatic Asialoglycoprotein Receptor" J. Med. Chem. (2004) 47:5798-5808, SLIEDREGT et al., "Design and Synthesis of Novel Amphiphilic Dendritic Galactosides for Selective Targeting of Liposomes to the Hepatic Asialoglycoprotein Receptor" J. Med. Chem. (1999) 42:609-618, and Valentijn *et al.,* "Solid-phase synthesis of lysine-based cluster galactosides with high affinity for the Asialoglycoprotein Receptor" *Tetrahedron,* 1997, *53(2),* 759-770, each of which is incorporated by reference herein in its entirety.

In certain embodiments, conjugate groups include without limitation, intercalators, reporter molecules, polyamines, polyamides, polyethylene glycols, thioethers, polyethers, cholesterols, thiocholesterols, cholic acid moieties, folate, lipids, phospholipids, biotin, phenazine, phenanthridine, anthraquinone, adamantane, acridine, fluoresceins, rhodamines, coumarins and dyes. Certain conjugate groups have been described previously, for example: cholesterol moiety (Letsinger et al., Proc. Natl. Acad. Sci. USA, 1989, 86, 6553-6556), cholic acid (Manoharan et al., Bioorg. Med. Chem. Let., 1994, 4, 1053-1060), a thioether, e.g., hexyl-S-tritylthiol (Manoharan et al., Ann. N.Y. Acad. Sci., 1992, 660, 306-309; Manoharan et al., Bioorg. Med. Chem. Let., 1993, 3, 2765-2770), a thiocholesterol (Oberhauser et al., Nucl. Acids Res., 1992, 20, 533-538), an aliphatic chain, e.g., do-decan-diol or undecyl residues (Saison-Behmoaras et al., EMBO J., 1991, 10, 1111-1118; Kabanov et al., FEBS Lett., 1990, 259, 327-330; Svinarchuk et al., Biochimie, 1993, 75, 49-54), a phospholipid, e.g., di-hexadecyl-rac-glycerol or triethyl-ammonium 1,2-di-O-hexadecyl-rac-glycero-3-H-phosphonate (Manoharan et al., Tetrahedron Lett., 1995, 36, 3651-3654; Shea et al., Nucl. Acids Res., 1990, 18, 3777-3783), a polyamine or a polyethylene glycol chain (Manoharan et al., Nucleosides & Nucleotides, 1995, 14, 969-973), or adamantane acetic acid (Manoharan et al., Tetrahedron Lett., 1995, 36, 3651-3654), a palmityl moiety (Mishra et al., Biochim. Biophys. Acta, 1995, 1264, 229-237), or an octadecylamine or hexylamino-carbonyl-oxycholesterol moiety (Crooke et al., J. Pharmacol. Exp. Ther., 1996, 277, 923-937).

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

Some nonlimiting examples of conjugate linkers include pyrrolidine, 8-amino-3,6-dioxaoctanoic acid (ADO), succinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate (SMCC) and 6-aminohexanoic acid (AHEX or AHA). Other connugate linkers include, but are not limited to, substituted C_1-C_{10} alkyl, substituted or unsubstituted C_2-C_{10} alkenyl or substituted or unsubstituted C_2-C_{10} alkynyl, wherein a nonlimiting list of preferred substituent groups includes hydroxyl, amino, alkoxy, carboxy, benzyl, phenyl, nitro, thiol, thioalkoxy, halogen, alkyl, aryl, alkenyl and alkynyl.

Conjugate groups may be attached to either or both ends of an oligonucleotide (terminal conjugate groups) and/or at any internal position.

In certain embodiments, conjugate groups are at the 3'-end of an oligonucleotide of an oligomeric compound. In certain embodiments, conjugate groups are near the 3'-end. In certain embodiments, conjugates are attached at the 3'end of an oligomeric compound, but before one or more terminal group nucleosides. In certain embodiments, conjugate groups are placed within a terminal group.

Cell culture and antisense compounds treatment

The effects of antisense compounds on the level, activity or expression of TMPRSS6 nucleic acids can be tested in vitro in a variety of cell types. Cell types used for such analyses are available from commercial vendors (e.g., American Type Culture Collection, Manassas, VA; Zen-Bio, Inc., Research Triangle Park, NC; Clonetics Corporation, Walkersville, MD) and cells are cultured according to the vendor's instructions using commercially available reagents (e.g., Invitrogen Life Technologies, Carlsbad, CA). Illustrative cell types include, but are not limited to, HepG2 cells, Hep3B cells, Huh7 (hepatocellular carcinoma) cells, primary hepatocytes, A549 cells, GM04281 fibroblasts and LLC-MK2 cells.

In vitro testing of antisense oligonucleotides

Described herein are methods for treatment of cells with antisense oligonucleotides, which can be modified appropriately for treatment with other antisense compounds.

In general, cells are treated with antisense oligonucleotides when the cells reach approximately 60-80% confluence in culture.

One reagent commonly used to introduce antisense oligonucleotides into cultured cells includes the cationic lipid transfection reagent LIPOFECTIN® (Invitrogen, Carlsbad, CA). Antisense oligonucleotides are mixed with LIPOFECTIN® in OPTI-MEM® 1 (Invitrogen, Carlsbad, CA) to achieve the desired final concentration of antisense oligonucleotide and a LIPOFECTIN® concentration that typically ranges 2 to 12 ug/mL per 100 nM antisense oligonucleotide.

Another reagent used to introduce antisense oligonucleotides into cultured cells includes LIPOFECTAMINE 2000® (Invitrogen, Carlsbad, CA). Antisense oligonucleotide is mixed with LIPOFECTAMINE 2000® in OPTI-MEM® 1 reduced serum medium (Invitrogen, Carlsbad, CA) to achieve the desired concentration of antisense oligonucleotide and a LIPOFECTAMINE® concentration that typically ranges 2 to 12 ug/mL per 100 nM antisense oligonucleotide.

Another reagent used to introduce antisense oligonucleotides into cultured cells includes Cytofectin® (Invitrogen, Carlsbad, CA). Antisense oligonucleotide is mixed with Cytofectin® in OPTI-MEM® 1 reduced serum medium (Invitrogen, Carlsbad, CA) to achieve the desired concentration of antisense oligonucleotide and a Cytofectin® concentration that typically ranges 2 to 12 ug/mL per 100 nM antisense oligonucleotide.

Another reagent used to introduce antisense oligonucleotides into cultured cells includes Oligofectamine™ (Invitrogen Life Technologies, Carlsbad, CA). Antisense oligonucleotide is mixed with Oligofectamine™ in Opti-MEM™-1 reduced serum medium (Invitrogen Life Technologies, Carlsbad, CA) to achieve the desired concentration of oligonucleotide with an Oligofectamine™ to oligonucleotide ratio of approximately 0.2 to 0.8 μL per 100 nM.

Another reagent used to introduce antisense oligonucleotides into cultured cells includes FuGENE 6 (Roche Diagnostics Corp., Indianapolis, IN). Antisense oligomeric compound was mixed with FuGENE 6 in 1 mL of serum-free RPMI to achieve the desired concentration of oligonucleotide with a FuGENE 6 to oligomeric compound ratio of 1 to 4 μL of FuGENE 6 per 100 nM.

Another technique used to introduce antisense oligonucleotides into cultured cells includes electroporation (Sambrook and Russell in *Molecular Cloning. A Laboratory Manual*. Third Edition. Cold Spring Harbor laboratory Press, Cold Spring Harbor, New York. 2001).

Cells are treated with antisense oligonucleotides by routine methods. Cells are typically harvested 16-24 hours after antisense oligonucleotide treatment, at which time RNA or protein levels of target nucleic acids are measured by methods known in the art and described herein (Sambrook and Russell in *Molecular Cloning. A Laboratory Manual*. Third Edition. Cold Spring Harbor laboratory Press, Cold Spring Harbor, New York. 2001). In general, when treatments are performed in multiple replicates, the data are presented as the average of the replicate treatments.

The concentration of antisense oligonucleotide used varies from cell line to cell line. Methods to determine the optimal antisense oligonucleotide concentration for a particular cell line are well known in the art (Sambrook and Russell in *Molecular Cloning. A Laboratory Manual*. Third Edition. Cold Spring Harbor laboratory Press, Cold Spring Harbor, New York. 2001). Antisense oligonucleotides are typically used at concentrations ranging from 1 nM to 300 nM when transfected with LIPOFECTAMINE2000®, Lipofectin or Cytofectin. Antisense oligonucleotides are used at higher concentrations ranging from 625 to 20,000 nM when transfected using electroporation.

RNA Isolation

RNA analysis can be performed on total cellular RNA or poly(A)+ mRNA. Methods of RNA isolation are well known in the art (Sambrook and Russell, Molecular Cloning: A Laboratory Manual, 3rd Ed., 2001). RNA is prepared using methods well known in the art, for example, using the TRIZOL® Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's recommended protocols.

Analysis of inhibition of target levels or expression

Inhibition of levels or expression of a TMPRSS6 nucleic acid can be assayed in a variety of ways known in the art (Sambrook and Russell, Molecular Cloning: A Laboratory Manual, 3rd Ed., 2001). For example, target nucleic acid levels can be quantitated by, e.g., Northern blot analysis, competitive polymerase

chain reaction (PCR), or quantitaive real-time PCR. RNA analysis can be performed on total cellular RNA or poly(A)+ mRNA. Methods of RNA isolation are well known in the art. Northern blot analysis is also routine in the art. Quantitative real-time PCR can be conveniently accomplished using the commercially available ABI PRISM® 7600, 7700, or 7900 Sequence Detection System, available from PE-Applied Biosystems, Foster City, CA and used according to manufacturer's instructions.

Quantitative Real-Time PCR Analysis of Target RNA Levels

Quantitation of target RNA levels may be accomplished by quantitative real-time PCR using the ABI PRISM® 7600, 7700, or 7900 Sequence Detection System (PE-Applied Biosystems, Foster City, CA) according to manufacturer's instructions. Methods of quantitative real-time PCR are well known in the art.

Prior to real-time PCR, the isolated RNA is subjected to a reverse transcriptase (RT) reaction, which produces complementary DNA (cDNA) that is then used as the substrate for the real-time PCR amplification. The RT and real-time PCR reactions are performed sequentially in the same sample well. RT and real-time PCR reagents are obtained from Invitrogen (Carlsbad, CA). RT, real-time-PCR reactions are carried out by methods well known to those skilled in the art.

Gene (or RNA) target quantities obtained by real time PCR are normalized using either the expression level of a gene whose expression is constant, such as cyclophilin A, or by quantifying total RNA using RIBOGREEN® (Invitrogen, Inc. Carlsbad, CA). Cyclophilin A expression is quantified by real time PCR, by being run simultaneously with the target, multiplexing, or separately. Total RNA is quantified using RIBOGREEN® RNA quantification reagent (Invitrogen, Inc. Eugene, OR). Methods of RNA quantification by RIBOGREEN® are taught in Jones, L.J., et al, (Analytical Biochemistry, 1998, 265, 368-374). A CYTOFLUOR® 4000 instrument (PE Applied Biosystems) is used to measure RIBOGREEN® fluorescence.

Probes and primers are designed to hybridize to a TMPRSS6 nucleic acid. Methods for designing real-time PCR probes and primers are well known in the art, and may include the use of software such as PRIMER EXPRESS® Software (Applied Biosystems, Foster City, CA).

Analysis of Protein Levels

Antisense inhibition of TMPRSS6 nucleic acids can be assessed by measuring TMPRSS6 protein levels. Protein levels of TMPRSS6 can be evaluated or quantitated in a variety of ways well known in the art, such as immunoprecipitation, Western blot analysis (immunoblotting), enzyme-linked immunosorbent assay (ELISA), quantitative protein assays, protein activity assays (for example, caspase activity assays), immunohistochemistry, immunocytochemistry or fluorescence-activated cell sorting (FACS) (Sambrook and Russell, Molecular Cloning: A Laboratory Manual, 3rd Ed., 2001). Antibodies directed to a target can be identified and obtained from a variety of sources, such as the MSRS catalog of antibodies (Aerie Corporation, Birmingham, MI), or can be prepared via conventional monoclonal or polyclonal antibody generation methods well known in the art.

In Vivo Testing of Antisense Compounds

Antisense compounds, for example, antisense oligonucleotides, are tested in animals to assess their ability to inhibit expression of TMPRSS6 and produce phenotypic changes, such as, reduced accumulation of iron in the body. Testing can be performed in normal animals, or in experimental disease models. For administration to animals, antisense oligonucleotides are formulated in a pharmaceutically acceptable diluent, such as sterile water-for-injection or phosphate-buffered saline. Administration includes parenteral routes of administration, such as intraperitoneal, intravenous, and subcutaneous. Calculation of antisense oligonucleotide dosage and dosing frequency depends upon factors such as route of administration and animal body weight. In one embodiment, following a period of treatment with antisense oligonucleotides, RNA is isolated from liver tissue and changes in TMPRSS6 nucleic acid expression are measured. Changes in TMPRSS6 protein levels can also be measured. Changes in TMPRSS6 expression can be measured by determining the level of hepcidin expression, plasma levels of iron and percentage saturation of transferrin present in the animal.

Certain Indications

Provided are compositions, compounds and methods for treating an individual comprising administering to the individual one or more compositions or compounds described herein. In certain embodiments, compositions, compounds and methods are provided for reducing TMPRSS6 expression in the individual. In certain embodiments, compositions, compounds and methods are provided for treating the individual by administering to the individual a therapeutically effective amount of a composition or compound comprising an antisense oligonucleotide targeted to a TMPRSS6 nucleic acid. In certain embodiments, the antisense compound targeted to a TMPRSS6 reduces TMPRSS6. In certain embodiments, the individual in need of TMRPSS6 reduction has, or is at risk for, an iron accumulation disease, disorder or condition. In certain embodiments, compositions, compounds and methods described herein are provided herein for use in reducing iron levels in an individual.

In certain embodiments, the iron accumulation is the result of a therapy to treat a disease, disorder or condition in the individual. In certain embodiments, the therapy is transfusion therapy. In certain embodiments, multiple transfusions may lead to polycythemia. In further embodiments, multiple blood transfusions are associated with the animal having anemia. Examples of anemia requiring multiple blood transfusions are hereditary anemia, myelodysplastic syndrome and severe chronic hemolysis. Examples of hereditary anemia include, but are not limited to, sickle cell anemia, thalassemia, Fanconi anemia, Diamond Blackfan anemia, Shwachman Diamond syndrome, red cell membrane disorders, glucose-6-phosphate dehydrogenase deficiency, or hereditary hemorrhagic telangiectasia. In certain embodiments, the thalassemia is β-thalassemia. In certain embodiments, the β-thalassemia is HbE/β-thalassemia, β-thalassemia major, β-thalassemia intermedia or β-thalassemia minor.

In certain embodiments, the iron accumulation is due to a disease, disorder or condition in the individual. In certain embodiments, the disease, disorder or condition is hereditary hemochromatosis or thalassemia. In certain embodiments, the thalassemia is non-transfusion dependent thalassemia (NTDT) or β-thalassemia. In certain embodiments, the β-thalassemia is HbE/β-thalassemia, β-thalassemia major, β-thalassemia intermedia or β-thalassemia minor.

In certain embodiments, the disease, disorder and/or condition is associated with excess parenteral iron supplement intake or excess dietary iron intake.

Provided herein are compositions, compounds and methods for increasing hepcidin levels, such as mRNA or protein expression levels. In certain embodiments, provided are antisense compounds targeting TMPRSS6 as described herein for use in increasing hepcidin levels, such as mRNA or protein expression levels.

Provided herein are compositions, compounds and methods for decreasing the percentage saturation of transferrin in an animal. In certain embodiments, provided are antisense compounds targeting TMPRSS6 as described herein for use in decreasing the percentage saturation of transferrin in an animal. In certain embodiments, decreasing transferrin saturation leads to a decrease in iron supply for erythropoiesis. In certain embodiments, the decrease in erythropoiesis treats, prevents, delays the onset of, ameliorates, and/or reduces polycythemia, or symptom thereof, in the animal. In certain embodiments, provided are antisense compounds targeting TMPRSS6 as described herein for use in treating, preventing, delaying the onset of, ameliorating, and/or reducing polycythemia, or symptom thereof, in the animal. In certain embodiments, the polycythemia is polycythemia vera. In certain embodiments, treatment with the antisense compound targeting TMPRSS6 prevents or delays the polycythemia from progressing into erythroid leukemia.

In certain embodiments, administration of a therapeutically effective amount of an antisense compound targeted to a TMPRSS6 nucleic acid in an individual is accompanied by monitoring of TMPRSS6 levels to determine the individual's response to the antisense compound. In certain embodiments, administration of a therapeutically effective amount of an antisense compound targeted to a TMPRSS6 nucleic acid in an individual is accompanied by monitoring the levels of hepcidin in the individual. In certain embodiments, administration of a therapeutically effective amount of an antisense compound targeted to a TMPRSS6 nucleic acid in an individual is accompanied by monitoring the levels of iron in the individual. In certain embodiments, administration of a therapeutically effective amount of an antisense compound targeted to a TMPRSS6 nucleic acid in an individual is accompanied by evaluating the percentage saturation of transferrin in the individual. An individual's response to administration of the antisense compound is used by a physician to determine the amount and duration of therapeutic intervention.

Provided herein are pharmaceutical compositions comprising an antisense compound targeted to TMPRSS6 for use in the preparation of a medicament for treating a patient suffering from, or susceptible to, an iron accumulation disease, disorder or condition.

In certain embodiments, the methods described herein include administering an antisense compound comprising a modified oligonucleotide having at least an 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 contiguous nucleobase portion complementary to a TMPRSS6 nucleic acid.

Certain Combination Therapies

In certain embodiments, a first agent comprising a composition or compound provided herein is co-administered with one or more secondary agents. In certain embodiments, such second agents are designed to treat the same iron accumulation disease, disorder or condition as the first agent described herein. In certain embodiments, such second agents are designed to treat a different disease, disorder, or condition as the first agent described herein. In certain embodiments, such second agents are designed to treat an undesired side effect of one or more composition or compound as described herein. In certain embodiments, such first agents are designed to treat an undesired side effect of a second agent. In certain embodiments, second agents are co-administered with the first agent to treat an undesired effect of the first agent. In certain embodiments, second agents are co-administered with the first agent to produce a combinational effect. In certain embodiments, second agents are co-administered with the first agent to produce a synergistic effect. In certain embodiments, the co-administration of the first and second agents permits use of lower dosages than would be required to achieve a therapeutic or prophylactic effect if the agents were administered as independent therapy. In certain embodiments, the dose of a co-administered second agent is the same as the dose that would be administered if the second agent was administered alone. In certain embodiments, the dose of a co-administered second agent is lower than the dose that would be administered if the second agent was administered alone. In certain embodiments, the dose of a co-administered second agent is greater than the dose that would be administered if the second agent was administered alone.

In certain embodiments, a first agent and one or more second agents are administered at the same time. In certain embodiments, the first agent and one or more second agents are administered at different times. In certain embodiments, the second agent is administered prior to administration of the first agent. In certain embodiments, the second agent is administered following administration of the first agent. In certain embodiments, the first agent and one or more second agents are prepared together in a single pharmaceutical formulation. In certain embodiments, the first agent and one or more second agents are prepared separately.

In certain embodiments, second agents include, but are not limited to, nucleic acid compounds. Such nucleic acid compounds can include a siRNA, a ribozyme or an antisense compound targeting TMPRSS6 or another target.

In certain embodiments, second agents include, but are not limited to, non-antisense compounds such as iron chelators, transferrin, bone morphogenetic proteins 6 (BMP6), hepcidin agonists, stem cells, antibodies targeting TMPRSS6 or fetal hemoglobin (HbF)-raising agents. In further embodiments, iron chelators are selected from, but not limited to, FBS0701 (FerroKin), Exjade, Desferal, and Deferiprone. In certain embodiments, HBF-raising agents include 5-hydroxyl urea, short chain fatty acid (SCFA) derivatives

(e.g., HQK1001), DNA methyltransferase inhibitors (e.g., decitabine) or histone deacetylase (HDAC) inhibitors (e.g., Zolina, Panobinostat).

In certain embodiments, a second agent includes, but is not limited to, phlebotomy or transfusion therapy. In certain embodiments, the first agent is administered at the same time as phlebotomy or transfusion therapy. In certain embodiments, the first agent is administered prior to phlebotomy or transfusion therapy. In certain embodiments, the first agent is administered following phlebotomy or transfusion therapy. In certain embodiments, administration of a composition or compound provided herein decreases the frequency of phlebotomy or transfusion in an individual. In certain embodiments, administration of a composition or compound provided herein increases the frequency of phlebotomy or transfusion in an individual. In certain embodiments, administration of a composition or compound provided herein decreases the length of time required for phlebotomy or transfusion.

Certain Compounds

Preferred antisense compounds with beneficial properties that enhance their use as therapeutic treatments in humans are demonstrated in the examples herein. For brevity, only the studies that contributed to the selection of the preferred antisense compounds are described. A non-exhaustive summary of the examples is provided below for ease of reference.

About 2200 antisense compounds with a MOE gapmer motif or a cEt containing motif targeting human TMPRSS6 were designed and screened in Hep3B cells for their effect on human TMPRSS6 mRNA after administering a single dose to the cells. Example 1 shows representative single dose screening data for over 100 potent antisense compounds that were selected for further studies.

Of the approximately 2200 antisense compounds tested with a single dose *in vitro*, about 100 antisense compounds were chosen for testing in dose-dependent inhibition studies to determine their half maximal inhibitory concentration (IC_{50}) in Hep3B cells (Example 2).

About 77 antisense compounds were further selected, based on their potency in dose response and/or single dose studies, for study in CD-1 mice to determine tolerability (e.g., plasma chemistry markers, body weight and organ weight) of the antisense compound (Examples 3-4) in mice.

Of the approximately 77 antisense compounds tested in CD-1 mice for tolerability, about 48 antisense compounds were chosen for study in Sprague-Dawley rats to determine tolerability in rats (Example 5).

Base on the rat tolerability study, about 32 antisense compounds were selected for *in vivo* potency testing in human TMPRSS6 transgenic (huTMPRSS6 tg) mice (Example 6).

Antisense compounds identified as potent and tolerable in mice studies were assessed for cross-reactivity to a rhesus monkey TMPRSS6 gene sequence (Example 7). Although the antisense compounds in the studies described herein were tested in cynomolgus monkeys (Example 11), the cynomolgus monkey TMPRSS6 sequence was not available for comparison to the sequences of the antisense compounds, therefore

the sequences of the antisense compounds were compared to that of the closely related rhesus monkey. About seven antisense compounds were found to have no mismatches with the rhesus TMPRSS6 gene sequence.

Based on the results of the mice potency and tolerability studies, and homology to the rhesus monkey sequence, the sequences of seven antisense compounds (585774, 585683, 585775, 630718, 647477, 647449, 647420) from the prior studies were selected for further chemical modification to make them more potent in reducing TMPRSS6 levels. Eight new antisense compounds with a GalNAc conjugate (702843, 705051, 705052, 705053, 706940, 706941, 706942, 706943) were designed based on the seven original antisense compounds (Example 7).

The eight GalNAc conjugated antisense compounds were tested in mice: for tolerability in CD-1 mice (e.g., body weights, organ weights, liver metabolic markers (e.g., ALT, AST and bilirubin), kidney metabolic markers (e.g., BUN and creatinine), histology, hematology parameters (e.g., blood cell counts and hematocrit), and the like were measured (Example 8); and, for potency in human TMPRSS6 transgenic mice (Example 9).

The eight GalNAc conjugated antisense compounds were also assessed for viscosity and seven of the eight were found to have a favorable viscosity level while one was found to have a borderline acceptable viscosity level (Example 10).

Based on the favorable profile seen in the mice and *in vitro* viscosity studies, the eight GalNAc conjugated antisense compounds were further tested for potency in reducing TMPRSS6, tolerability and for their effect on iron parameters (e.g., hepcidin levels, serum iron and transferrin saturation) in cynomolgus monkeys (Example 11). The eight GalNAc conjugated antisense compounds were generally found to be potent and tolerable in cynomolgus monkeys. Antisense compounds 705051, 702843, 706942 and 706943 were found to be especially potent in reducing TMRPSS6, serum iron and transferrin saturation.

Accordingly, provided herein are antisense compounds with any one or more characteristics that are beneficial for their use as a therapeutic agent. In certain embodiments, provided herein are antisense compounds comprising a modified oligonucleotide as described herein targeted to, or specifically hybridizable with, a region of nucleotides selected from any of SEQ ID NOs: 1-6.

In certain embodiments, certain antisense compounds as described herein are efficacious by virtue of their potency in inhibiting TMPRSS6 expression. In certain embodiments, the compounds or compositions inhibit TMPRSS6 by at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90% or at least 95%.

In certain embodiments, certain antisense compounds as described herein are efficacious by virtue of an *in vitro* IC_{50} of less than 20 μM, less than 10 μM, less than 8 μM, less than 5μM, less than 2 μM, less than 1 μM, less than 0.9 μM, less than 0.8 μM, less than 0.7 μM, less than 0.6 μM, or less than 0.5 μM when tested in human cells, for example, in the Hep3B cell line (as described in Example 2).

In certain embodiments, certain antisense compounds as described herein are efficacious by virtue of a median effective dose (ED_{50}) of $\leq$ 5 mpk/wk, $\leq$ 4 mpk/wk, $\leq$ 3 mpk/wk, $\leq$ 2 mpk/wk or $\leq$ 1 mpk/wk *in*

vivo. In certain embodiments, preferred antisense compounds having an $ED_{50} \leqslant 1$ mpk/wk include antisense compounds 702843, 706940, 706942 and 706943 as described in Example 8.

In certain embodiments, certain antisense compounds as described herein are efficacious by virtue of having a viscosity of less than 40 cP, less than 35 cP, less than 30 cP, less than 25 cP, less than 20 cP, less than 15 cP, or less than 10 cP as described in Example 9. Oligonucleotides having a viscosity greater than 40 cP would have less than optimal viscosity.

In certain embodiments, certain antisense compounds as described herein are highly tolerable, as demonstrated by the *in vivo* tolerability measurements described in the examples. In certain embodiments, the certain antisense compounds as described herein are highly tolerable, as demonstrated by having an increase in ALT and/or AST value of no more than 3 fold, 2 fold or 1.5 fold over saline treated animals.

In certain embodiments, certain antisense compounds as described herein are efficacious by virtue of having one or more of an inhibition potency of greater than 50%, an $ED_{50} \leq 1$ mpk/wk, a viscosity of less than 40 cP, and no more than a 3 fold increase in ALT and/or AST in transgenic mice.

In certain embodiments, ISIS 702843 (SEQ ID NO: 36) is preferred. This compound was found to be a potent inhibitor in TMPRSS6 transgenic mice and a very tolerable antisense compound in CD-1 mice. In mice it had less than a 3 fold increase in ALT and/or AST levels over saline treated animals. It had an acceptable viscosity of about 33 cP and an $ED_{50} \leq 1$ mpk/wk in huTMPRSS6 transgenic mice. Also, in monkeys, it was among the most potent compounds in inhibiting TMPRSS6.

In certain embodiments, ISIS 705051 (SEQ ID NO: 36) is preferred. This compound was found to be a potent inhibitor in TMPRSS6 transgenic mice and a very tolerable antisense compound in CD-1 mice. In mice it had less than a 3 fold increase in ALT and/or AST levels over saline treated animals. It had an acceptable viscosity of about 23 cP and an $ED_{50} \leq 3$ mpk/wk in huTMPRSS6 transgenic mice. Also, in monkeys, it was among the most potent compounds in inhibiting TMPRSS6.

In certain embodiments, ISIS 706942 (SEQ ID NO: 77) is preferred. This compound was found to be a potent inhibitor in TMPRSS6 transgenic mice and a very tolerable antisense compound in CD-1 mice. In mice it had less than a 3 fold increase in ALT and/or AST levels over saline treated animals. It had an acceptable viscosity of about 20 cP and an $ED_{50} \leq 1$ mpk/wk in huTMPRSS6 transgenic mice. Also, in monkeys, it was among the most potent compounds in inhibiting TMPRSS6.

In certain embodiments, ISIS 706943 (SEQ ID NO: 77) is preferred. This compound was found to be a potent inhibitor in TMPRSS6 transgenic mice and a very tolerable antisense compound in CD-1 mice. In huTMPRSS6 transgenic mice it had less than a 3 fold increase in ALT and/or AST levels over saline treated animals. It had an acceptable viscosity of about 19 cP and an $ED_{50} \leq 1$ mpk/wk in huTMPRSS6 transgenic mice. Also, in monkeys, it was among the most potent compounds in inhibiting TMPRSS6.

EXAMPLES

Non-limiting disclosure and incorporation by reference

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

Example 1: Antisense oligonucleotides targeting human type II transmembrane serine protease 6 (TMPRSS6)

Approximately 2200 newly designed chimeric antisense oligonucleotides were designed as 5-10-5 MOE gapmers or cET containing gapmers.

The 5-10-5 MOE gapmers were designed as oligonucleotides 20 nucleosides in length, wherein the central gap segment comprises ten 2'-deoxynucleosides and is flanked by wing segments on the 5' direction and the 3' direction comprising five nucleosides each. Each nucleoside in the 5' wing segment and each nucleoside in the 3' wing segment has a 2'-MOE modification. The internucleoside linkages throughout each gapmer are phosphorothioate (P=S) linkages. All cytosine residues throughout each gapmer are 5-methylcytosines.

The cET containing gapmers were designed with varied deoxy, MOE, and (S)-cEt gapmer motifs. The deoxy, MOE and (S)-cEt oligonucleotides are 16 nucleosides in length wherein the nucleosides have either a MOE sugar modification, an (S)-cEt sugar modification, or a deoxyribose. The 'Chemistry' column in Table 3 describes the sugar modifications of each oligonucleotide. 'k' indicates an (S)-cEt sugar modification; 'd' indicates deoxyribose; and 'e' indicates a MOE modification. Unless otherwise specified, the internucleoside linkages throughout each gapmer are phosphorothioate (P=S) linkages. All cytosine residues throughout each gapmer are 5-methylcytosines.

"Start site" indicates the 5'-most nucleoside to which the gapmer is targeted in the human gene sequence. "Stop site" indicates the 3'-most nucleoside to which the gapmer is targeted human gene sequence. Each gapmer listed in the Tables below is targeted to either the human TMPRSS6 mRNA, designated herein as SEQ ID NO: 1 (GENBANK Accession No. NM_153609.2) or the human TMPRSS6 genomic sequence, designated herein as SEQ ID NO: 2 (the complement of GENBANK Accession No. NT_011520.12 truncated from nucleotide 16850000 to 16897000). In the tables below, 'n/a' indicates that the antisense oligonucleotide does not target that particular gene sequence with 100% complementarity.

The 2200 chimeric antisense oligonucleotides were tested for their single dose effects on TMRPSS6 mRNA *in vitro*. Antisense oligonucleotides were tested at least once in a series of experiments that had similar culture conditions.

A representative result for about 110 potent antisense oligonucleotides out of the 2200 tested is presented in Tables 1-3 shown below. These potent antisense oligonucleotides were selected for further studies as described below.

Table 1 shows the percent inhibition of TMPRSS6 mRNA by 5-10-5 MOE gapmers. Cultured Hep3B cells at a density of about 20,000 cells per well were transfected using electroporation with 4,500 nM antisense oligonucleotide. After a treatment period of approximately 24 hours, RNA was isolated from the cells and TMPRSS6 mRNA levels were measured by quantitative real-time PCR. Human primer probe set RTS3840 (forward sequence CAAAGCCCAGAAGATGCTCAA, designated herein as SEQ ID NO: 92; reverse sequence GGAATAGACGGAGCTGGAGTTG, designated herein as SEQ ID NO: 93; probe sequence ACCAGCACCCGCCTGGGAACTT, designated herein as SEQ ID NO: 94) was used to measure mRNA levels. TMPRSS6 mRNA levels were adjusted according to total RNA content, as measured by RIBOGREEN®. Results are presented as percent inhibition of TMPRSS6, relative to untreated control cells.

Table 1

Inhibition of TMPRSS6 mRNA by 5-10-5 MOE gapmers targeting SEQ ID NO: 1 and/or 2

ISIS NO	Sequence	SEQ ID NO: 1 Start Site	SEQ ID NO: 1 Stop Site	SEQ ID NO: 2 Start Site	SEQ ID NO: 2 Stop Site	% Inhibition	SEQ ID NO
585604	CCATCACCTCCGTCCCCCTG	178	197	7011	7030	58	7
585606	TCCGCTTCCTCGCCATCACC	190	209	7023	7042	51	8
585608	TTTTCTCTTGGAGTCCTCAC	233	252	7066	7085	52	9
585609	GCTTTTCTCTTGGAGTCCTC	235	254	7068	7087	79	10
585611	CCGGGCTTTTCTCTTGGAGT	239	258	7072	7091	58	11
585626	GGCTTTGGCGGTTTCACTGC	449	468	11948	11967	79	12
585629	GAGCATCTTCTGGGCTTTGG	461	480	N/A	N/A	80	13
585631	CCTTGAGCATCTTCTGGGCT	465	484	N/A	N/A	84	14
585649	AGTGCCTGCACCACCTCGGG	616	635	14372	14391	79	15
585651	CAGCAGTGCCTGCACCACCT	620	639	14376	14395	70	16
585653	TCCTCCACCAGCAGTGCCTG	628	647	14384	14403	49	17
585654	AGCTCCTCCACCAGCAGTGC	631	650	14387	14406	64	18
585655	CAGCAGCTCCTCCACCAGCA	635	654	14391	14410	66	19
585667	GCTGTGCAGGCCCTTCTTCC	1049	1068	24044	24063	52	20
585668	GTAGTAGCTGTGCAGGCCCT	1055	1074	24050	24069	61	21
585682	ACGGCAAATCATACTTCTGC	1284	1303	26044	26063	60	22
585683	GCACGGCAAATCATACTTCT	1286	1305	26046	26065	58	23
585684	CCCTGGGTGCACGGCAAATC	1294	1313	26054	26073	58	24
585698	CAAACGCAGTTTCTCTCATC	1567	1586	N/A	N/A	52	25
585699	TGCAAACGCAGTTTCTCTCA	1569	1588	N/A	N/A	52	26
585752	GATCACACCTGTGATGCGGG	2504	2523	44266	44285	48	27

585757	CTCCTGCCACCACAGGGCCT	2656	2675	44418	44437	70	28
585758	ACCTCCTGCCACCACAGGGC	2658	2677	44420	44439	69	29
585761	TGCCATCACTGGAGCAGACA	2699	2718	44461	44480	60	30
585762	ATCCTCCTGCCATCACTGGA	2706	2725	44468	44487	38	31
585768	TCCATTCCCAGATCCCAAGT	2978	2997	44740	44759	64	32
585769	CTTCCATTCCCAGATCCCAA	2980	2999	44742	44761	62	33
585770	ACCTTCCATTCCCAGATCCC	2982	3001	44744	44763	52	34
585772	CAAAGGGCAGCTGAGCTCAC	3154	3173	44916	44935	47	35
585774	CTTTATTCCAAAGGGCAGCT	3162	3181	44924	44943	67	36
585775	AGCTTTATTCCAAAGGGCAG	3164	3183	44926	44945	68	37
585776	AGGCAGCTTTATTCCAAAGG	3168	3187	44930	44949	59	38
585777	GATCAGGCAGCTTTATTCCA	3172	3191	44934	44953	65	39
585831	AGGAGCGGCCACCGTCCTGT	N/A	N/A	12340	12359	45	40
				12371	12390		
				12562	12581		
585834	GGCAGGAGCGGCCACCGTCC	N/A	N/A	12343	12362	42	41
				12374	12393		
				12565	12584		
585863	TCCCCCTGAGGCTCTCAGGA	N/A	N/A	16233	16252	32	42
				18737	18756		
585864	TAAGTCCCCCTGAGGCTCTC	N/A	N/A	16237	16256	39	43
				18741	18760		
585906	AAGACTGTTCCTTCTCCTTT	N/A	N/A	27990	28009	44	44
585912	CAGCTTGTGCCTGCCCAGAG	N/A	N/A	29208	29227	45	45
585932	AGTCTATCTGGCCACAGTGA	N/A	N/A	32981	33000	34	46
585937	GGTCCTTCTTTGAGCCTCAC	N/A	N/A	34800	34819	35	47

Table 2 shows the percent inhibition of TMPRSS6 mRNA by additional 5-10-5 MOE gapmers. Cultured Hep3B cells at a density of about 20,000 cells per well were transfected using electroporation with 5,000 nM antisense oligonucleotide. After a treatment period of approximately 24 hours, RNA was isolated from the cells and TMPRSS6 mRNA levels were measured by quantitative real-time PCR. Human primer probe set RTS3840 was used to measure mRNA levels. TMPRSS6 mRNA levels were adjusted according to total RNA content, as measured by RIBOGREEN®. Results are presented as percent inhibition of TMPRSS6, relative to untreated control cells.

Table 2

Inhibition of TMPRSS6 mRNA by 5-10-5 MOE gapmers targeting SEQ ID NO: 1 and/or 2

ISIS NO	Sequence	SEQ ID NO: 1 Start Site	SEQ ID NO: 1 Stop Site	SEQ ID NO: 2 Start Site	SEQ ID NO: 2 Stop Site	% Inhibition	SEQ ID NO
591466	CCTCAGGTCACCACTTGCTG	2533	2552	44295	44314	63	48
591491	GCCACCTCCTGCCACCACAG	2661	2680	44423	44442	72	49
591492	ATGCCACCTCCTGCCACCAC	2663	2682	44425	44444	59	50
591514	CTCCATCCTCCTGCCATCAC	2710	2729	44472	44491	59	51
591536	GCAGCTGAGCTCACCTCCCA	3148	3167	44910	44929	68	52
591537	GGCAGCTGAGCTCACCTCCC	3149	3168	44911	44930	75	53
591549	GGCAGCTTTATTCCAAAGGG	3167	3186	44929	44948	69	54
591550	CAGGCAGCTTTATTCCAAAG	3169	3188	44931	44950	76	55
591552	ATCAGGCAGCTTTATTCCAA	3171	3190	44933	44952	66	56
591578	CCACTGGCCCTGGGTGCACG	1301	1320	26061	26080	65	57
591579	TCCACTGGCCCTGGGTGCAC	1302	1321	26062	26081	68	58

Table 3 shows the percent inhibition of TMPRSS6 mRNA by cEt containing gapmers from a series of experiments. Cultured Hep3B cells at a density of about 20,000 cells per well were transfected using electroporation with 2,000 nM antisense oligonucleotide. After a treatment period of approximately 24 hours, RNA was isolated from the cells and TMPRSS6 mRNA levels were measured by quantitative real-time PCR. Human primer probe set RTS3840 was used to measure mRNA levels. TMPRSS6 mRNA levels were adjusted according to total RNA content, as measured by RIBOGREEN®. Results are presented as percent inhibition of TMPRSS6, relative to untreated control cells.

Table 3

Inhibition of TMPRSS6 mRNA by cEt containing gapmers targeting SEQ ID NO: 1 and/or 2

ISIS NO	Sequence	SEQ ID NO: 1 Start Site	SEQ ID NO: 1 Stop Site	SEQ ID NO: 2 Start Site	SEQ ID NO: 2 Stop Site	Chemistry	% Inhibition	SEQ ID NO
615840	CTTTTGGCTTACAGTG	3057	3072	44819	44834	ekk-d10-kke	59	59
615884	GCTGAGCTCACCTCCC	3149	3164	44911	44926	ekk-d10-kke	70	60
615898	TATTCCAAAGGGCAGC	3163	3178	44925	44940	ekk-d10-kke	69	61
615901	CTTTATTCCAAAGGGC	3166	3181	44928	44943	ekk-d10-kke	68	62
615903	AGCTTTATTCCAAAGG	3168	3183	44930	44945	ekk-d10-kke	70	63
615909	TCAGGCAGCTTTATTC	3174	3189	44936	44951	ekk-d10-kke	69	64
615910	ATCAGGCAGCTTTATT	3175	3190	44937	44952	ekk-d10-kke	69	65
615911	GATCAGGCAGCTTTAT	3176	3191	44938	44953	ekk-d10-kke	69	66

630497	ATTCCAAAGGGCAGCT	3162	3177	44924	44939	kkk-d10-kkk	80	67
630689	CTTACAGTGGCAGCAG	3050	3065	44812	44827	kkk-d10-kkk	71	68
630692	TGGCTTACAGTGGCAG	3053	3068	44815	44830	kkk-d10-kkk	75	69
630693	TTGGCTTACAGTGGCA	3054	3069	44816	44831	kkk-d10-kkk	75	70
630696	CTTTTGGCTTACAGTG	3057	3072	44819	44834	kkk-d10-kkk	66	59
630716	CTTTATTCCAAAGGGC	3166	3181	44928	44943	kkk-d10-kkk	63	62
630717	GCTTTATTCCAAAGGG	3167	3182	44929	44944	kkk-d10-kkk	81	71
630718	AGCTTTATTCCAAAGG	3168	3183	44930	44945	kkk-d10-kkk	84	63
630719	CAGGCAGCTTTATTCC	3173	3188	44935	44950	kkk-d10-kkk	80	72
630722	GATCAGGCAGCTTTAT	3176	3191	44938	44953	kkk-d10-kkk	72	66
630725	TTTGATCAGGCAGCTT	3179	3194	N/A	N/A	kkk-d10-kkk	61	73
630726	TTTTGATCAGGCAGCT	3180	3195	N/A	N/A	kkk-d10-kkk	72	74
630727	TTTTTGATCAGGCAGC	3181	3196	N/A	N/A	kkk-d10-kkk	73	75
630794	ACATCAGGGACGAGAC	2686	2701	44448	44463	kk-d8-kekeke	72	76
647393	TTATTCCAAAGGGCAG	3164	3179	44926	44941	kkk-d10-kkk	78	83
647394	TTTATTCCAAAGGGCA	3165	3180	44927	44942	kkk-d10-kkk	77	84
647395	CAGCTTTATTCCAAAG	3169	3184	44931	44946	kkk-d10-kkk	86	77
647396	GCAGCTTTATTCCAAA	3170	3185	44932	44947	kkk-d10-kkk	86	78
647397	GGCAGCTTTATTCCAA	3171	3186	44933	44948	kkk-d10-kkk	85	82
647398	AGGCAGCTTTATTCCA	3172	3187	44934	44949	kkk-d10-kkk	82	79
647404	GGCAGCTGAGCTCACC	3153	3168	44915	44930	kek-d9-eekk	76	85
647414	TATTCCAAAGGGCAGC	3163	3178	44925	44940	kek-d9-eekk	86	61
647419	AGCTTTATTCCAAAGG	3168	3183	44930	44945	kek-d9-eekk	87	63
647420	CAGCTTTATTCCAAAG	3169	3184	44931	44946	kek-d9-eekk	83	77
647421	GCAGCTTTATTCCAAA	3170	3185	44932	44947	kek-d9-eekk	83	78
647423	AGGCAGCTTTATTCCA	3172	3187	44934	44949	kek-d9-eekk	84	79
647424	CAGGCAGCTTTATTCC	3173	3188	44935	44950	kek-d9-eekk	78	72
647426	ATCAGGCAGCTTTATT	3175	3190	44937	44952	kek-d9-eekk	81	65
647428	TGATCAGGCAGCTTTA	3177	3192	N/A	N/A	kek-d9-eekk	76	80
647429	TTGATCAGGCAGCTTT	3178	3193	N/A	N/A	kek-d9-eekk	78	81
647442	ATTCCAAAGGGCAGCT	3162	3177	44924	44939	kk-d9-eeekk	81	67
647446	CTTTATTCCAAAGGGC	3166	3181	44928	44943	kk-d9-eeekk	79	62
647447	GCTTTATTCCAAAGGG	3167	3182	44929	44944	kk-d9-eeekk	87	71
647448	AGCTTTATTCCAAAGG	3168	3183	44930	44945	kk-d9-eeekk	86	63
647449	CAGCTTTATTCCAAAG	3169	3184	44931	44946	kk-d9-eeekk	89	77
647450	GCAGCTTTATTCCAAA	3170	3185	44932	44947	kk-d9-eeekk	88	78
647451	GGCAGCTTTATTCCAA	3171	3186	44933	44948	kk-d9-eeekk	88	82
647453	CAGGCAGCTTTATTCC	3173	3188	44935	44950	kk-d9-eeekk	77	72
647454	TCAGGCAGCTTTATTC	3174	3189	44936	44951	kk-d9-eeekk	82	64
647457	TGATCAGGCAGCTTTA	3177	3192	N/A	N/A	kk-d9-eeekk	78	80
647475	CTTTATTCCAAAGGGC	3166	3181	44928	44943	kk-d8-eeeekk	77	62
647476	GCTTTATTCCAAAGGG	3167	3182	44929	44944	kk-d8-eeeekk	83	71
647477	AGCTTTATTCCAAAGG	3168	3183	44930	44945	kk-d8-eeeekk	84	63

647478	CAGCTTTATTCCAAAG	3169	3184	44931	44946	kk-d8-eeeekk	79	77
647482	CAGGCAGCTTTATTCC	3173	3188	44935	44950	kk-d8-eeeekk	76	72
647506	AGCTTTATTCCAAAGG	3168	3183	44930	44945	k-d9-kekeke	89	63
647508	GCAGCTTTATTCCAAA	3170	3185	44932	44947	k-d9-kekeke	77	78
647514	GATCAGGCAGCTTTAT	3176	3191	44938	44953	k-d9-kekeke	78	66
647531	CAGCTTTATTCCAAAG	3169	3184	44931	44946	kk-d8-kekeke	88	77
647532	GCAGCTTTATTCCAAA	3170	3185	44932	44947	kk-d8-kekeke	77	78

Example 2: Dose response of antisense oligonucleotides targeting human TMPRSS6 in Hep3B cells

About 100 antisense oligonucleotides selected from the about 2200 antisense oligonucleotides tested in single dose experiments described in Example 1 were also tested at various doses in Hep3B cells in studies of *in vitro* inhibition of human TMPRSS6 mRNA.

For the experiment in Table 4, below, cells were plated at a density of 12,000 cells per well and transfected using electroporation with 0.15 μM, 0.44 μM, 1.33 μM, 4.00 μM and 12.00 μM concentrations of antisense oligonucleotide. After the treatment period of approximately 16 hours, RNA was isolated from the cells and TMPRSS6 mRNA levels were measured by quantitative real-time PCR. Human primer probe set RTS3840 was used to measure mRNA levels. TMPRSS6 mRNA levels were adjusted according to total RNA content, as measured by RIBOGREEN®. Results are presented as percent inhibition of TMPRSS6, relative to untreated control cells. "0" indicate that the antisense oligonucleotide did not reduce TMPRSS6 mRNA levels.

The half maximal inhibitory concentration (IC_{50}) of each oligonucleotide is also presented. TMPRSS6 mRNA levels were significantly reduced in a dose-dependent manner in antisense oligonucleotide treated cells.

Table 4

Dose response assay with 5-10-5 MOE gapmers

ISIS No	0.15 μM	0.44 μM	1.33 μM	4.00 μM	12.00 μM	IC_{50} (μM)
585604	0	0	17	36	63	7
585606	0	0	0	0	35	>12
585608	0	13	6	8	50	>12
585609	0	10	24	44	68	5
585611	0	0	9	33	67	8
585626	3	21	27	55	82	3
585629	37	45	56	71	83	1
585631	29	56	63	70	84	1
585649	0	9	35	46	74	4
585651	0	18	1	39	75	6
585653	10	15	18	42	63	7
585654	0	0	25	33	65	8

585655	0	12	15	34	65	8
585667	0	0	2	30	52	>12
585668	11	6	0	43	70	8
585682	0	0	0	30	63	11
585683	1	9	19	39	77	5
585684	6	1	13	21	57	>12
585698	13	11	37	39	78	4
585699	0	8	25	25	65	8
585752	0	12	37	34	69	5
585757	0	7	16	53	79	4
585758	6	0	25	49	71	5
585761	2	12	13	39	66	7
585762	2	15	26	44	75	4
585768	4	0	20	52	76	4
585769	0	0	0	42	70	7
585770	12	12	42	50	68	3
585772	12	12	23	34	56	12
585774	15	28	58	68	84	1
585775	0	7	28	60	82	3
585776	36	24	56	69	86	1
585777	15	39	63	76	88	1
585831	0	8	3	19	31	>12
585834	0	10	3	6	32	>12
585863	7	7	3	0	51	>12
585864	5	9	19	31	34	>12
585906	13	2	16	11	29	>12
585912	20	0	30	33	32	>12
585932	15	11	25	4	37	>12
585937	20	33	30	30	43	>12
591466	0	14	26	39	71	5
591491	0	11	23	45	68	5
591492	0	0	22	27	64	9
591514	0	0	1	41	75	6
591536	13	22	34	64	81	2
591537	17	44	57	81	88	1
591549	21	26	51	72	87	1
591550	19	34	65	76	89	1
591552	23	49	65	86	90	1
591578	0	17	28	45	55	7
591579	3	13	47	40	58	6

For the experiment in Table 5, below, cells were plated at a density of 5,000 cells per well and transfected using electroporation with 0.19 μM, 0.56 μM, 1.67 μM and 5.0 μM concentrations of antisense

oligonucleotide. After the treatment period of approximately 16 hours, RNA was isolated from the cells and TMPRSS6 mRNA levels were measured by quantitative real-time PCR. Human primer probe set RTS3840 was again used to measure mRNA levels. TMPRSS6 mRNA levels were adjusted according to total RNA content, as measured by RIBOGREEN®. Results are presented as percent inhibition of TMPRSS6, relative to untreated control cells.

The half maximal inhibitory concentration (IC_{50}) of each oligonucleotide is also presented. TMPRSS6 mRNA levels were significantly reduced in a dose-dependent manner in antisense oligonucleotide treated cells.

Table 5

Dose response assay with cEt containing oligonucleotides

ISIS No	0.19 μM	0.56 μM	1.67 μM	5.00 μM	IC_{50} (μM)
630497	28	49	69	86	0.6
647393	28	42	69	84	0.7
647394	43	59	67	83	0.3
647395	11	41	67	83	0.9
647396	25	47	73	79	0.7
647397	27	42	70	83	0.7
647398	27	49	61	84	0.7
647404	23	47	63	79	0.8
647414	38	52	72	87	0.4
647419	45	60	74	84	0.3
647420	28	52	69	82	0.6
647421	23	47	68	85	0.7
647423	23	50	74	81	0.7
647424	20	48	72	83	0.7
647426	26	37	67	76	0.9
647428	25	33	61	83	0.9
647429	20	32	59	83	1
647442	32	51	66	78	0.6
647446	32	48	73	81	0.6
647447	29	52	70	81	0.6
647448	30	56	72	79	0.5
647449	31	45	71	83	0.6
647450	32	54	70	82	0.5
647451	40	62	74	83	0.3
647453	28	52	68	84	0.6
647454	32	45	62	84	0.7
647457	28	46	69	80	0.7
647475	9	52	63	77	1
647476	43	59	70	79	0.3

647477	48	62	77	83	0.2
647478	16	41	68	82	0.9
647482	14	37	73	79	0.9
647506	37	60	75	83	0.4
647508	21	39	52	79	1.1
647514	32	42	63	81	0.7
647531	25	53	73	80	0.6
647532	26	49	61	82	0.7

For the experiment in Table 6, below, cells were plated at a density of 20,000 cells per well and transfected using electroporation with 0.22 μM, 0.67 μM, 2.00 μM and 6.0 μM concentrations of antisense oligonucleotide. After the treatment period of approximately 16 hours, RNA was isolated from the cells and TMPRSS6 mRNA levels were measured by quantitative real-time PCR. Human primer probe set RTS3840 was used to measure mRNA levels. TMPRSS6 mRNA levels were adjusted according to total RNA content, as measured by RIBOGREEN®. Results are presented as percent inhibition of TMPRSS6, relative to untreated control cells.

The half maximal inhibitory concentration (IC_{50}) of each oligonucleotide is also presented. TMPRSS6 mRNA levels were significantly reduced in a dose-dependent manner in antisense oligonucleotide treated cells.

Table 6

Dose response assay with cEt containing oligonucleotides

ISIS No	0.22 μM	0.67 μM	2.00 μM	6.00 μM	IC_{50} (μM)
630497	34	54	81	89	0.5
630689	43	61	77	87	0.3
630692	54	64	85	95	0.2
630693	42	66	75	86	0.3
630696	20	37	66	82	1.1
630717	48	73	84	83	0.1
630718	49	81	88	89	0.1
630719	42	69	83	95	0.3
630722	40	56	70	90	0.4
630726	24	45	64	82	0.9
630727	36	57	73	82	0.5
630794	25	46	71	84	0.8

Example 3: Tolerability of 5-10-5 MOE gapmers targeting human TMPRSS6 in CD1 mice

CD1® mice (Charles River, MA) are a multipurpose mice model, frequently utilized for safety and efficacy testing. The mice were treated with about 26 ISIS 5-10-5 MOE gapmer antisense oligonucleotides selected from the tables above and evaluated for changes in the levels of various plasma chemistry markers.

Treatment

Groups of six week old male CD1 mice were injected subcutaneously twice a week for six weeks with 50 mg/kg of ISIS oligonucleotides (100 mg/kg/week dose). One group of male CD1 mice was injected subcutaneously twice a week for 6 weeks with PBS. Mice were euthanized 48 hours after the last dose, and organs and plasma were harvested for further analysis.

Plasma chemistry markers

To evaluate the effect of ISIS oligonucleotides on liver and kidney function, plasma levels of transaminases (ALT and AST), total bilirubin (Tbil), albumin (Alb), creatinine (Creat), and BUN were measured using an automated clinical chemistry analyzer (Hitachi Olympus AU400e, Melville, NY). The results are presented in Table 7. ISIS oligonucleotides that caused changes in the levels of any of the liver or kidney function markers outside the expected range for antisense oligonucleotides were excluded in further studies.

Table 7

Plasma chemistry markers in CD1 mice at week six

ISIS No.	ALT (U/L)	AST (U/L)	BUN (mg/dL)	Creat (mg/dL)	Tbil (mg/dL)	Alb (g/dL)
PBS	24	51	27	0.17	0.17	2.9
585626	167	155	30	0.18	0.15	2.9
585649	263	157	28	0.17	0.15	3.0
585653	147	89	28	0.18	0.39	3.4
585654	778	300	26	0.15	0.17	3.0
585655	1709	1353	29	0.16	0.35	3.0
585683	45	63	31	0.18	0.20	3.0
585698	53	73	34	0.21	0.19	3.0
585752	90	99	29	0.16	0.17	2.9
585757	246	180	30	0.16	0.15	2.8
585758	212	305	28	0.18	0.28	2.9
585761	659	439	28	0.16	0.43	2.7
585762	597	551	27	0.17	0.64	3.0
585768	483	387	26	0.18	0.19	2.7
585774	109	126	31	0.16	0.14	2.6

585775	60	70	28	0.17	0.15	2.9
585776	654	388	27	0.17	0.13	2.9
585777	159	200	24	0.16	0.17	2.7
591466	46	53	27	0.15	0.12	3.0
591491	761	729	28	0.18	0.25	3.2
591514	230	215	33	0.15	0.14	2.5
591536	540	416	26	0.16	0.13	3.0
591537	552	346	27	0.17	0.16	3.0
591549	708	488	30	0.14	0.14	2.7
591550	294	225	31	0.17	0.12	2.9
591552	1098	680	24	0.17	0.17	3.0
591579	135	85	25	0.16	0.12	2.8

Body and organ weights

Body weights of all the groups of mice were measured at the start of the experiment, and every week until the end of the study. Liver, spleen and kidney weights were also measured at the end of the study, and the change in body weight and organ weights relative to the PBS control group at baseline are presented in Table 8. ISIS oligonucleotides that caused any changes in organ weights outside the expected range for antisense oligonucleotides were excluded from further studies.

Table 8

Body weight and relative organ weights of CD1 mice (in grams) at week six

ISIS No.	BW change (g)	Relative liver weight (g)	Relative kidney weight (g)	Relative spleen weight (g)
PBS	1.4	1.0	1.0	1.0
585626	1.4	1.2	0.9	1.1
585649	1.3	1.2	1.0	1.1
585653	1.4	1.1	1.0	0.9
585654	1.2	1.2	1.0	1.1
585655	1.3	1.4	1.0	1.3
585683	1.4	1.0	0.9	1.1
585698	1.5	1.2	1.0	1.4
585752	1.3	1.1	1.0	1.3
585757	1.4	1.5	1.0	1.1
585758	1.4	1.4	0.9	1.0
585761	1.1	1.4	1.0	1.3
585762	1.2	2.1	1.0	0.8
585768	1.5	1.1	1.1	1.3
585774	1.5	1.1	1.0	1.1
585775	1.5	0.9	1.0	1.2
585776	1.4	1.3	1.1	1.5

585777	1.4	1.2	1.1	1.5
591466	1.5	1.0	1.0	1.0
591491	1.3	1.2	1.0	1.1
591514	1.4	1.1	0.9	1.5
591536	1.4	1.3	1.0	1.1
591537	1.3	1.3	0.9	1.3
591549	1.4	1.2	1.0	1.5
591550	1.4	1.1	0.9	1.5
591552	1.4	1.5	1.1	1.5
591579	1.5	1.0	0.9	1.1

From these tolerability studies, it was observed that most of the 5-10-5 MOE gapmer antisense oligonucleotides were well-tolerated after six weeks of dosing.

Example 4: Tolerability of cEt containing oligonucleotides targeting human TMPRSS6 in CD1 mice

CD1® mice (Charles River, MA) are a multipurpose mice model, frequently utilized for safety and efficacy testing. The mice were treated with about 51 cEt containing antisense oligonucleotides selected from the tables described above, and evaluated for changes in the levels of various plasma chemistry markers.

Treatment

Groups of five- to six-week-old male CD1 mice (n=4 per treatment group) were injected subcutaneously twice a week for six weeks with 25 mg/kg of ISIS oligonucleotides (50 mg/kg/week dose). One group of male CD1 mice was injected subcutaneously twice a week for 6 weeks with PBS. Mice were euthanized 48 hours after the last dose, and organs and plasma were harvested for further analysis. Liver, kidney and spleen were collected for histology, and plasma was collected to measure levels of certain plasma chemistry markers.

The oligonucleotides were split into two test groups with the same conditions and the results are presented to in the tables below.

Plasma chemistry markers

To evaluate the effect of ISIS oligonucleotides on liver and kidney function, plasma levels of transaminases, bilirubin, albumin, creatinine, and BUN were measured using an automated clinical chemistry analyzer (Hitachi Olympus AU400e, Melville, NY). The results are presented in Tables 9-10. ISIS oligonucleotides causing changes in the levels of any of the liver or kidney function markers outside the expected range for antisense oligonucleotides were excluded from further studies.

Table 9

Plasma chemistry markers in CD1 mice at week six

ISIS No.	ALT (U/L)	AST (U/L)	BUN (mg/dL)	Creat (mg/dL)	Tbil (mg/dL)	Alb (g/dL)
PBS	55	53	24	0.1	0.2	2.7
615840	752	636	26	0.15	0.23	2.5
615884	1039	664	25	0.17	0.17	2.8
615898	754	420	25	0.17	0.14	2.5
615901	118	120	22	0.11	0.18	2.5
615903	33	46	22	0.12	0.18	2.5
615909	2042	2464	49	0.16	1.19	2.7
615910	978	1058	22	0.15	1.24	2.4
615911	474	366	23	0.14	0.34	2.4
630696	1117	853	26	0.15	0.21	2.3
630716	41	67	25	0.13	0.14	2.4
630717	1005	483	23	0.13	0.19	2.3
630718	57	86	25	0.13	0.13	2.4
630722	207	168	21	0.13	0.16	2.2
630725	1729	897	20	0.12	0.15	2.2
630726	1330	774	22	0.10	0.10	2.1
630727	614	653	23	0.10	0.13	1.6
630794	39	78	24	0.12	0.16	2.6

Table 10

Plasma chemistry markers in CD1 mice at week six

ISIS No.	ALT (U/L)	AST (U/L)	BUN (mg/dL)	Creat (mg/dL)	Tbil (mg/dL)	Alb (g/dL)
PBS	31.3	54.8	32.3	0.14	0.19	3.0
630497	429.0	297.5	31.0	0.18	0.11	2.8
630689	2088.3	1306.0	34.7	0.10	0.22	2.2
630692	1634.8	1402.5	30.9	0.16	0.25	3.4
630693	1247.5	1193.8	33.6	0.19	0.68	2.8
630719	2553.0	2594.7	28.6	0.12	2.55	3.8
647414	718.5	444.0	32.7	0.13	0.12	3.0
647419	39.3	66.5	27.0	0.13	0.15	2.9
647420	90.3	100.8	30.8	0.13	0.19	3.1
647421	613.3	607.3	15.5	0.09	1.61	2.6
647423	1290.3	807.5	29.8	0.28	0.30	3.7
647424	1451.0	1198.3	25.2	0.16	0.37	3.7
647426	548.5	393.0	23.7	0.12	0.16	2.7
647428	2658.8	2232.8	24.8	0.21	0.52	3.0
647429	1306.3	725.3	23.2	0.12	0.21	2.8

647442	564.8	371.5	29.7	0.08	0.13	3.0
647446	69.0	91.3	27.6	0.10	0.14	2.9
647447	61.5	76.3	27.2	0.11	0.13	2.8
647448	100.8	110.5	24.4	0.10	0.14	2.9
647449	61.3	88.0	27.7	0.10	0.13	3.1
647450	1850.8	1512.0	18.3	0.09	0.47	2.9
647451	1376.3	588.3	26.0	0.15	0.29	3.7
647453	1774.3	1674.5	28.8	0.16	1.24	3.7
647454	324.3	409.3	27.0	0.11	0.15	2.7
647457	1609.0	1194.8	25.6	0.12	0.21	2.6
647475	40.0	80.5	25.1	0.10	0.12	2.6
647476	62.0	81.0	26.1	0.11	0.14	2.8
647477	74.8	94.0	26.5	0.11	0.15	2.9
647478	62.0	88.0	28.2	0.11	0.13	3.1
647482	959.8	975.8	25.8	0.11	0.19	2.9
647506	36.3	65.3	25.8	0.10	0.14	2.9
647508	49.8	93.3	26.3	0.11	0.14	3.1
647514	276.0	221.8	28.3	0.11	0.17	2.9
647531	248.5	175.0	28.7	0.11	0.16	3.2
647532	156.8	180.0	21.3	0.09	0.10	3.0

Body and organ weights

Body weights of all the groups of mice were measured at the start of the experiment, and every week until the end of the study. Liver, spleen and kidney weights were also measured at the end of the study, and the change in body weight and organ weights relative to the PBS control group at baseline are presented in Tables 11-12. ISIS oligonucleotides that caused any changes in organ weights outside the expected range for antisense oligonucleotides were excluded from further studies.

Table 11

Body weight and relative organ weights of CD1 mice (in grams) at week six

ISIS No.	BW change (g)	Relative liver weight (g)	Relative kidney weight (g)	Relative spleen weight (g)
PBS	1.5	1	1	1
615840	1.2	1.1	1.0	0.8
615884	1.4	1.5	1.1	1.2
615898	1.5	1.3	1.1	1.4
615901	1.5	1.3	1.1	2.0
615903	1.4	1.1	1.1	1.2
615909	0.8	1.6	1.2	0.7
615910	1.2	1.9	1.0	2.3
615911	1.5	1.4	1.1	1.6
630696	1.1	1.2	0.9	1.2

630716	1.4	1.2	1.2	1.2
630717	1.2	1.4	1.0	1.7
630718	1.4	1.2	1.1	1.4
630722	1.6	1.2	1.1	1.6
630725	1.3	1.2	1.1	1.8
630726	1.4	1.1	1.2	1.9
630727	1.3	1.2	1.2	3.5
630794	1.4	1.0	1.1	1.1

Table 12

Body weight and relative organ weights of CD1 mice (in grams) at week six

ISIS No.	BW change (g)	Relative liver weight (g)	Relative kidney weight (g)	Relative spleen weight (g)
PBS	1.5	1	1	1
630497	1.3	1.2	1.0	1.1
630689	1.6	1.3	1.0	1.4
630692	1.5	1.9	0.9	1.2
630693	1.2	1.3	0.8	0.9
630719	0.8	1.4	1.1	0.4
647414	1.4	1.2	1.1	1.0
647419	1.5	1.0	1.1	1.2
647420	1.4	1.1	1.0	1.4
647421	1.2	1.1	1.1	1.3
647423	1.4	1.7	1.1	1.3
647424	1.1	1.8	1.2	0.6
647426	1.4	1.5	1.1	1.8
647428	1.3	1.4	1.1	1.9
647429	1.4	1.2	1.0	1.6
647442	1.3	1.1	1.1	1.1
647446	1.4	1.2	1.2	1.4
647447	1.5	1.3	1.2	1.4
647448	1.5	1.1	1.1	1.5
647449	1.5	1.1	1.1	1.6
647450	1.4	1.3	1.1	1.9
647451	1.4	1.6	1.0	1.8
647453	1.2	1.8	1.4	1.5
647454	1.5	1.6	1.0	2.2
647457	1.4	1.3	1.0	1.8
647475	1.4	1.2	1.1	1.5
647476	1.5	1.1	1.2	1.8
647477	1.5	1.2	1.0	1.5
647478	1.6	1.1	1.0	1.2
647482	1.4	1.7	1.2	1.5

647506	1.5	1.1	1.0	1.2
647508	1.6	1.0	1.0	1.2
647514	1.5	1.0	1.0	1.5
647531	1.4	1.0	1.0	1.4
647532	1.5	1.3	1.1	1.4

Example 5: Tolerability of oligonucleotides targeting human TMPRSS6 in Sprague-Dawley rats

Sprague-Dawley rats are a multipurpose model used for safety and efficacy evaluations. The rats were treated with about 48 antisense oligonucleotides, found potent *in vitro* and tolerable in mice from the studies described in the Examples above, and evaluated for changes in the levels of various plasma chemistry markers.

Treatment

Male Sprague-Dawley rats (roughly eight weeks old) were maintained on a 12-hour light/dark cycle and fed ad libitum with Purina normal rat chow, diet 5001. Groups of four Sprague-Dawley rats each were injected subcutaneously once a week for 6 weeks with 100 mg/kg of MOE gapmer; or 50 mg/kg of cEt containing antisense oligonucleotides. One to two days after the final dose, urine protein/creatinine (P/C) ratio was assayed and blood was drawn 3 days after the last dose for hematologic assessments described below. Three days after the last dose, rats were euthanized and organs and plasma were harvested for further analysis.

Plasma chemistry markers

To evaluate the effect of ISIS oligonucleotides on liver and kidney function, plasma levels of transaminases (alanine transaminase (ALT) and aspartate transaminase (AST), total bilirubin (Tbil), albumin (Alb), creatinine (Creat), and BUN were measured using an automated clinical chemistry analyzer (Hitachi Olympus AU400e, Melville, NY). The results are presented in Table 13. ISIS oligonucleotides that caused changes in the levels of any of the liver or kidney function markers outside the expected range for antisense oligonucleotides were excluded in further studies.

Table 13

Plasma chemistry markers in Sprague-Dawley rats

ISIS No.	ALT (IU/L)	AST (IU/L)	BUN (mg/dL)	Creat (mg/dL)	Tbil (mg/dL)	Alb (g/dL)
PBS	60	92	18	0.3	0.1	3.7
585626	66	139	25	0.4	0.1	3.2
585653	92	154	26	0.4	0.1	3.9
585683	73	109	19	0.4	0.1	3.3
585698	66	104	22	0.4	0.1	3.4
585752	64	145	21	0.4	0.1	3.0

585758	113	669	21	0.3	0.2	2.8
585774	125	220	25	0.4	0.2	3.2
585775	66	117	24	0.4	0.1	3.2
585777	302	321	25	0.4	0.2	3.4
591466	368	444	22	0.4	0.2	3.1
591514	91	218	22	0.3	0.2	3.3
591579	484	655	20	0.4	0.2	3.8
614954	146	132	26	0.1	0.2	2.8
615895	291	383	26	0.4	0.2	3.4
615897	1946	1467	26	0.5	0.2	4.0
615899	70	113	25	0.4	0.1	3.4
615900	93	131	26	0.4	0.1	3.1
615903	59	70	22	0.4	0.1	3.5
630716	57	86	26	0.5	0.1	3.1
630718	61	72	23	0.4	0.1	3.4
630722	117	153	24	0.4	0.1	3.2
630794	90	113	29	0.5	0.1	3.4
630800	92	133	25	0.4	0.1	3.6
630948	48	77	21	0.4	0.1	3.3
630950	79	83	25	0.4	0.1	3.3
630952	208	243	31	0.4	0.2	2.9
630953	87	135	22	0.4	0.1	3.0
630957	110	115	26	0.4	0.1	3.6
637749	63	102	25	0.1	0.2	3.2
647384	135	158	24	0.4	0.1	3.7
647389	243	272	25	0.2	0.2	3.6
647391	205	520	27	0.0	1.1	2.1
647393	142	172	27	0.2	0.1	3.4
647394	391	340	29	0.1	0.2	2.8
647395	68	95	24	0.1	0.1	3.2
647419	53	66	23	0.4	0.1	3.5
647420	56	80	23	0.1	0.1	3.3
647446	66	110	23	0.2	0.1	3.4
647447	54	67	22	0.1	0.1	3.1
647448	55	73	26	0.4	0.1	3.3
647449	46	81	24	0.4	0.1	3.2
647475	45	78	26	0.4	0.1	3.5
647476	52	85	20	0.4	0.1	3.2
647477	58	89	24	0.5	0.1	3.5
647478	50	82.8	22.8	0.4	0.1	3.2
647506	45	95.3	22.9	0.4	0.1	3.2

647508	73	183.3	33.3	0.3	0.1	2.5
647532	108	179.5	47.8	0.5	0.1	1.8

Table 14

P/C ratio in urine of Sprague-Dawley rats

PBS	1.0
585626	6.7
585653	9.4
585683	7.0
585698	6.2
585752	13.4
585758	11.5
585774	7.5
585775	6.7
585777	7.6
591466	8.0
591514	8.0
591579	7.3
614954	5.2
615895	2.9
615897	4.7
615899	4.2
615900	4.5
615903	5.7
630716	3.9
630718	4.5
630722	4.3
630794	2.3
630800	5.1
630948	2.4
630950	6.3
630952	6.6
630953	4.4
630957	3.8
637749	3.0
647384	2.2
647389	2.4
647391	3.4
647393	3.7
647394	9.9
647395	5.2
647419	5.0

647420	4.9
647446	3.8
647447	3.9
647448	5.6
647449	5.0
647475	4.1
647476	4.6
647477	5.8
647478	4.6
647506	4.7
647508	9.2
647532	49.4

Hematology assays

Blood samples of approximately 1.3 mL of blood were collected from each of the available study animals in tubes containing K_2-EDTA and sent to IDEXX Laboratories, Inc. (Fremont, CA) for measurement and analysis of red blood cell (RBC) count, white blood cells (WBC) count, individual white blood cell counts -- such as that of monocytes, neutrophils, lymphocytes -- as well as for platelet count, total hemoglobin content and hematocrit (HCT). The results are presented in Table 15. ISIS oligonucleotides that caused changes in the levels of any of the hematology markers outside the expected range for antisense oligonucleotides were excluded in further studies.

Table 15

Hematology markers in Sprague-Dawley rats

ISIS No.	WBC (x 10^3/μL)	RBC (x 10^6/μL)	HCT (%)	Lymphocytes (/mm^3)	Monocytes (/mm^3)	Platelets (x 10^3/μL)
PBS	4.8	8.5	52.7	3567	93	812
585626	10.1	8.3	46.9	8969	252	1237
585653	13.8	8.2	48.3	11190	359	1305
585683	17.8	7.9	45.7	15773	557	826
585698	16.9	7.9	46.0	15380	344	761
585752	15.3	8.0	46.0	11396	585	1158
585758	18.4	7.9	44.0	6369	61	1548
585774	14.7	8.5	48.6	12818	552	873
585775	7.3	8.4	48.4	6218	219	1161
585777	11.2	8.1	47.1	9548	175	982
591466	14.3	8.1	45.6	12519	226	812
591514	14.9	8.5	48.2	10993	169	1157
591579	12.5	9.1	51.1	8540	222	1080
614954	13.6	5.2	29.9	12186	441	511
615895	15.2	8.0	45.9	11868	603	926
615897	14.5	7.5	43.3	10920	786	902

615899	19.8	7.8	43.7	17319	525	566
615900	14.0	7.1	41.0	12167	267	770
615903	9.4	8.5	51.3	7113	268	687
630716	21.1	7.8	45.3	18994	449	601
630718	8.9	8.9	52.5	7071	269	657
630722	17.0	9.1	51.6	13397	721	693
630794	8.8	8.7	50.5	7098	137	529
630800	16.6	8.0	45.3	13210	478	695
630948	7.2	8.5	50.2	5359	158	670
630950	11.0	8.8	52.4	8833	307	544
630952	24.2	7.7	42.8	17991	798	958
630953	25.0	6.9	42.4	20205	713	662
630957	11.7	8.7	50.5	8913	340	684
637749	12.8	7.5	44.7	10837	765	661
647384	14.8	9.0	54.5	11682	354	642
647389	12.8	8.2	51.0	10621	534	1075
647391	16.8	2.3	20.3	13574	807	240
647393	14.5	6.9	40.8	12467	423	1112
647394	24.9	6.5	39.6	21847	1070	990
647395	10.4	7.4	45.2	8685	515	1092
647419	13.8	8.3	48.5	11866	257	939
647420	11.1	8.0	47.3	9350	521	1079
647446	5.9	7.5	44.8	4805	258	1076
647447	10.2	7.8	47.3	8542	260	1019
647448	10.7	7.9	45.3	9050	260	933
647449	21.1	7.7	45.5	18809	479	630
647475	17.4	8.3	49.0	14951	562	776
647476	14.2	8.3	47.7	12336	339	979
647477	16.8	8.3	46.3	14089	726	697
647478	23.7	7.4	42.9	22039	440	762
647506	12.9	7.9	45.4	11679	268	711
647508	12.2	6.8	38.8	9800	431	647
647532	33.1	5.3	31.0	27732	963	844

Body and organ weights

Body weights of all the groups of rats were measured at the start of the experiment, and every week until the end of the study. Liver, spleen and kidney weights were also measured at the end of the study, and the change in body weight and organ weights relative to the PBS control group at baseline are presented in Table 16. ISIS oligonucleotides that caused any changes in organ weights outside the expected range for antisense oligonucleotides were excluded from further studies.

Table 16

Body weight and relative organ weights of Sprague-Dawley rats (in grams) at week six

ISIS No.	Liver (g)	Kidney (g)	Spleen (g)	Body weight (g)
PBS	1.0	1.0	1.0	1.8
585626	1.1	0.9	2.3	1.4
585653	1.1	1.0	2.1	1.5
585683	1.1	0.9	3.3	1.4
585698	1.1	0.9	2.8	1.4
585752	1.1	0.9	2.5	1.3
585758	1.5	0.9	2.3	1.2
585774	1.1	0.9	2.2	1.4
585775	1.0	0.9	1.7	1.3
585777	1.0	0.9	2.3	1.4
591466	1.0	0.9	2.7	1.3
591514	1.1	1.0	2.4	1.1
591579	1.0	0.8	1.9	1.3
614954	1.4	1.3	4.1	1.4
615895	1.0	1.1	1.7	1.5
615897	1.3	1.1	2.1	1.7
615899	1.1	1.1	2.0	1.6
615900	1.2	1.2	2.1	1.8
615903	1.2	1.0	1.5	1.9
630716	1.1	1.1	2.8	1.6
630718	1.1	1.0	2.1	1.8
630722	1.2	1.2	1.6	1.5
630794	0.9	1.0	1.6	1.8
630800	1.3	1.3	2.4	1.6
630948	1.0	1.1	1.7	1.9
630950	1.2	1.0	2.3	1.8
630952	1.4	1.3	2.6	1.2
630953	1.4	1.2	4.2	1.6
630957	1.2	1.0	1.7	1.6
637749	1.4	1.3	4.4	1.4
647384	1.0	1.0	1.1	1.7
647389	1.0	1.1	1.8	1.7
647391	1.8	1.5	13.1	1.4
647393	1.3	1.1	1.8	1.6
647394	1.2	1.2	2.8	1.6
647395	1.3	1.3	1.8	1.7
647419	1.3	1.1	1.6	1.8
647420	1.2	1.1	2.1	1.6

647446	1.3	1.2	2.3	1.8
647447	1.1	1.1	1.9	1.7
647448	1.2	1.2	1.6	1.7
647449	1.2	1.2	1.7	1.7
647475	1.2	1.1	1.5	1.7
647476	1.1	1.1	1.5	1.5
647477	1.2	1.1	1.7	1.6
647478	1.2	1.3	1.8	1.7
647506	1.2	1.3	2.0	1.6
647508	1.7	2.1	2.9	1.3
647532	2.0	1.7	3.7	1.3

Example 6: Effect of antisense inhibition of TMPRSS6 in transgenic mouse model

About 32 antisense oligonucleotides found tolerable in the rat studies above were further evaluated for their ability to reduce human TMPRSS6 mRNA transcript in mice with the human TMPRSS6 transgene ("huTMPRSS6" or "Tg" mice).

Treatment

Eight to sixteen week old male and female huTMPRSS6 transgenic mice were injected subcutaneously with five doses of 6 mg/kg per dose of ISIS antisense oligonucleotides targeting TMPRSS6, administered over a period of two weeks (30 mg/kg total), or with PBS as a control. Each treatment group consisted of 4 animals. Forty-eight hours after the administration of the last dose, blood was drawn from each mouse and the mice were sacrificed and tissues were collected.

RNA analysis

At the end of the study, RNA was extracted from liver for real-time PCR analysis of liver TMPRSS6 mRNA expression. Results are presented in Table 17 as percent inhbition with respect to PBS treated animals. Human primer probe set RTS4586 (forward sequence TGATAACAGCTGCCCACTG, designated herein as SEQ ID NO: 86; reverse sequence TCACCTTGAAGGACACCTCT, designated herein as SEQ ID NO: 87; probe sequence AGTTCTGCCACACCTTGCCCA, designated herein as SEQ ID NO: 88) was used to measure mRNA levels. The mRNA levels were normalized with levels of cyclophilin A, a housekeeping gene,which were determined using primer probe set mCYCLO_24 (forward primer TCGCCGCTTGCTGCA, designated herein as SEQ ID NO: 89; reverse primer ATCGGCCGTGATGTCGA, designated herein as SEQ ID NO: 90; probe CCATGGTCAACCCCACCGTGTTC, designated herein as SEQ ID NO: 91).

Table 17

% inhibition of TMPRSS6 mRNA in transgenic mice liver normalized to PBS expression

ISIS No	% inhibition
585626	57
585653	74
585683	81
585698	59
585698	59
585774	69
585775	81
591514	73
615899	88
615900	88
615903	97
630716	82
630718	99
630722	92
630794	71
630800	81
630948	65
630950	81
630957	70
647384	66
647393	95
647395	100
647419	99
647420	96
647446	84
647447	89
647448	96
647449	88
647475	84
647476	84
647477	96
647478	91
647506	91

Example 7: Antisense compounds conjugated to $GalNAc_3$ targeting TMPRSS6

The sequences of selected antisense oligonucleotides targeting TMPRSS6 found potent and tolerable in the examples above were chosen as parent sequences to design new $GalNAc_3$ conjugated antisense compounds targeting human TMPRSS6.

As summarized in Table 18, below, each of the newly designed antisense compounds described in this example had a 5'-Trishexylamino-(THA)-C6 $GalNAc_3$ endcap. ISIS 702843 was a 5-10-5 MOE gapmer having a mixed (phosphorothioate and phosphodiester) backbone ("MBB") with a 5'-Trishexylamino-(THA)-C6 $GalNAc_3$ endcap. ISIS 705051, 705052 and 705053 were 5-10-5 MOE gapmers having a phosphorothioate backbone with a 5'-Trishexylamino-(THA)-C6 $GalNAc_3$ endcap. ISIS 706940 was a 3-10-3 cEt gapmer with all phosphorothioate internucleoside linkages and a 5'-Trishexylamino-(THA)-C6 $GalNAc_3$ endcap; ISIS 706941, 706942 and 706943 are deoxy, MOE, and (S)-cEt containing gapmers having a phosphorothioate backbone with a 5'-Trishexylamino-(THA)-C6 $GalNAc_3$ endcap.

Table 18

Eight unconjugated antisense compounds targeting TMPRSS6 mRNA and corresponding $GalNAc_3$ conjugate antisense compounds

Parent Sequence ISIS#	GalNAc Conjugated ISIS#	Backbone	Length	Sequence	Chemistry	SEQ ID NO
585774	702843	MBB	20	CTTTATTCCAAAGGGCAGCT	5'-THA $GalNAc_3$ 5-10-5 MOE	36
585774	705051	PS	20	CTTTATTCCAAAGGGCAGCT	5'-THA $GalNAc_3$ 5-10-5 MOE	36
585683	705052	PS	20	GCACGGCAAATCATACTTCT	5'-THA $GalNAc_3$ 5-10-5 MOE	23
585775	705053	PS	20	AGCTTTATTCCAAAGGGCAG	5'-THA $GalNAc_3$ 5-10-5 MOE	37
630718	706940	PS	16	AGCTTTATTCCAAAGG	5'-THA $GalNAc_3$ kkk-d10-kkk	63
647477	706941	PS	16	AGCTTTATTCCAAAGG	5'-THA $GalNAc_3$ kk-d8-eeeekk	63
647449	706942	PS	16	CAGCTTTATTCCAAAG	5'-THA $GalNAc_3$ kk-d9-eeekk	77
647420	706943	PS	16	CAGCTTTATTCCAAAG	5'-THA $GalNAc_3$ kek-d9-eekk	77

All of the oligonucleotides sequences described in Table 18 were complementary to both human and Rhesus monkey sequences. At the time the studies described herein were undertaken, the cynomolgus monkey genomic sequence for TMPRSS6 was not available in the National Center for Biotechnology Information (NCBI) database; therefore, cross-reactivity of antisense oligonucleotides targeting human

TMPRSS6 with the cynomolgus monkey gene sequence could not be confirmed. Instead, the sequences of antisense oligonucleotides were compared to a rhesus monkey sequence for homology. It is expected that ISIS oligonucleotides with homology to the rhesus monkey sequence are fully cross-reactive with the cynomolgus monkey sequence as well.

The antisense oligonucleotides selected for GalNAc conjugation are fully complementary to the rhesus genomic sequence (the complement of GENBANK Accession No. NW_001095180.1, truncated from nucleotides 380000 to 422000, designated herein as SEQ ID NO: 95). The start and stop sites of each oligonucleotide to the rhesus sequence is presented in Table 19 while the start and stop sites of each oligonucleotide to the human sequence is presented in Table 20. "Start site" indicates the 5'-most nucleotide to which the gapmer is targeted in the rhesus monkey or human sequences.

Table 19

ASOs complementary to the rhesus TMPRSS6 genomic sequence (SEQ ID NO: 95)

ISIS No	rhesus Start Site	rhesus Stop Site	Chemistry	Sequence	SEQ ID NO
585774	40518	40537	5-10-5 MOE	CTTTATTCCAAAGGGCAGCT	36
702843	40518	40537	5'-THA GalNAc3 5-10-5 MOE	CTTTATTCCAAAGGGCAGCT	36
705051	40518	40537	5'-THA GalNAc3 5-10-5 MOE	CTTTATTCCAAAGGGCAGCT	36
705052	22499	22518	5'-THA GalNAc3 5-10-5 MOE	GCACGGCAAATCATACTTCT	23
705053	40520	40539	5'-THA GalNAc3 5-10-5 MOE	AGCTTTATTCCAAAGGGCAG	37
630718	40524	40539	kkk-10-kkk	AGCTTTATTCCAAAGG	63
706940	40524	40539	5'-THA GalNAc3 kkk-10-kkk	AGCTTTATTCCAAAGG	63
706941	40524	40539	5'-THA GalNAc3 kk-8-eeeekk	AGCTTTATTCCAAAGG	63
706942	40525	40540	5'-THA GalNAc3 kk-9-eeekk	CAGCTTTATTCCAAAG	77
706943	40525	40540	5'-THA GalNAc3 kk-9-eeekk	CAGCTTTATTCCAAAG	77

Table 20

Sites on TMPRSS6 mRNA (SEQ ID NO: 1) and/or genomic (SEQ ID NO: 2) sequences targeted by $GalNAc_3$-modified antisense oligonucleotides

ISIS NO	SEQ ID NO: 1 Start Site	SEQ ID NO: 1 Stop Site	SEQ ID NO: 2 Start Site	SEQ ID NO: 2 Stop Site	SEQ ID NO
702843	3162	3181	44924	44943	36
705051	3162	3181	44924	44943	36
705052	1286	1305	26046	26065	23
705053	3164	3183	44926	44945	37
706940	3168	3183	44930	44945	63

706941	3168	3183	44930	44945	63
706942	3169	3184	44931	44946	77
706943	3169	3184	44931	44946	77

Example 8: Tolerability of GalNAc3-modified antisense oligonucleotides targeted to human TMPRSS6 in CD-1 mice

CD1® mice (Charles River, MA) were treated with ISIS $GalNAc_3$-modified antisense oligonucleotides described in Table 18 above, and evaluated for changes in the levels of various plasma chemistry markers.

Treatment

Groups of six-week-old male CD1 mice (n=4 per treatment group) were injected subcutaneously twice a week for six weeks with 40 mg/kg of ISIS MOE gapmer GalNAc3-modified antisense oligonucleotides (80 mg/kg/week dose) or with 20 mg/kg of ISIS (S)-cEt containing gapmer GalNAc3-modified antisense oligonucleotides described in Table 14 above (40 mg/kg/week dose). One group of male CD1 mice was injected subcutaneously twice a week for 6 weeks with PBS. Mice were euthanized 48 hours after the last dose, and organs and plasma were harvested for further analysis. Liver, kidney, spleen, heart and lung were collected for histology, and plasma was collected to measure levels of certain plasma chemistry markers.

Plasma chemistry markers

To evaluate the effect of ISIS $GalNAc_3$-modified antisense oligonucleotides on liver and kidney function, plasma levels of transaminases, bilirubin, albumin, creatinine, and BUN were measured using an automated clinical chemistry analyzer (Hitachi Olympus AU400e, Melville, NY). The results are presented in Table 21. ISIS oligonucleotides causing changes in the levels of any of the liver or kidney function markers outside the expected range for antisense oligonucleotides were excluded from further studies.

Table 21

Plasma chemistry markers in CD1 mice at week six

ISIS No.	ALT (U/L)	AST (U/L)	BUN (mg/dL)	Creat (mg/dL)	Tbil (mg/dL)	Alb (g/dL)
PBS	32	70	27.3	0.12	0.17	2.8
702843	59	72	28	0.17	0.16	2.9
705051	47	73	26.6	0.16	0.17	2.8
705052	81	94	26.3	0.16	0.17	2.8
705053	139	129	28.2	0.17	0.18	2.9
706940	46	66	28.1	0.18	0.14	3.0
706941	40	57	25.5	0.18	0.16	2.9

706942	195	145	27	0.16	0.14	3.0
706943	178	144	26.1	0.16	0.16	3.9

Body and organ weights

Body weights of all groups of mice were measured at the start of the experiment, and every week until the end of the study. Liver, kidney and spleen weights were also measured at the end of the study, and the change in body weight and organ weights relative to the PBS control group at baseline are presented in Table 22. ISIS oligonucleotides that caused any changes in organ weights outside the expected range for antisense oligonucleotides were excluded from further studies.

Table 22

Change in body weight and relative organ weights of CD1 mice (in grams) at week six

ISIS No.	BW change (g)	Relative liver weight (g)	Relative kidney weight (g)	Relative spleen weight (g)
PBS	1.41	1.00	1.00	1.00
702843	1.39	1.05	1.00	1.08
705051	1.38	0.98	1.00	1.05
705052	1.39	1.02	0.96	1.32
705053	1.37	1.03	0.98	1.22
706940	1.31	0.97	1.01	1.16
706941	1.39	0.90	0.98	1.12
706942	1.39	1.09	1.09	1.40
706943	1.44	1.06	1.02	1.08

Hematology

To evaluate any effect of ISIS $GalNAc_3$-modified antisense oligonucleotides in CD1 mice on hematologic parameters, blood samples of approximately 1.3 mL of blood was collected from each of the available study animals in tubes containing K_2-EDTA. Samples were analyzed for red blood cell (RBC) count, white blood cells (WBC) count, individual white blood cell counts, such as that of monocytes, neutrophils, lymphocytes, as well as for platelet count, hemoglobin content and hematocrit, using an ADVIA120 hematology analyzer (Bayer, USA). The data is presented in Table 23.

The data indicate the oligonucleotides did not cause significant changes in hematologic parameters outside the expected range for antisense oligonucleotides at this dose. Generally, ISIS GalNAc-conjugated antisense oligonucleotides were well tolerated in terms of the hematologic parameters of the mice.

Table 23

Blood cell counts in CD1 mice

ISIS No.	WBC (x 10^3/μL)	RBC (x 10^6/μL)	HCT (%)	Lymphocytes (/mm^3)	Monocytes (/mm^3)	Platelets (x 10^3/μL)
PBS	2.9	8.9	49.9	1916.5	38.8	659.0
702843	4.9	8.9	48.5	3630.0	90.3	700.5

705051	4.0	8.5	47.8	2961.0	80.7	781.3
705052	3.2	9.3	50.7	2553.7	146.0	750.7
705053	5.3	9.1	49.8	3856.0	179.5	913.3
706940	3.7	8.5	46.7	2591.3	154.0	935.3
706941	5.5	8.8	49.9	3940.3	177.5	911.8
706942	5.7	9.4	51.8	4126.3	155.3	955.7
706943	3.4	8.9	48.2	3067.0	0.0	1021.3

Histological assessment of the GalNAc-conjugated TMPRSS6 antisense compounds in liver, spleen, kidney, heart and lung from the CD-1 Mice was performed. Overall, despite dosing $GalNAc_3$-conjugated antisense oligonucleotides at doses having approximately 8-times more activity in liver than unconjugated oligonucleotides, they were well tolerated and useful compounds for inhibiting TMPRSS6 and are important candidates for the treatment of an iron accumulation disease, disorder or condition.

Example 9: Dose-response of antisense oligonucleotides targeting TMPRSS6 in huTMPRSS6 transgenic mice

The eight ISIS $GalNAc_3$-modified antisense oligonucleotides targeting TMPRSS6 (ISIS Nos. 702843, 705051, 705052, 705053, 706940, 706941, 706942 and 706943) as well as two parent compounds (ISIS 585774 and ISIS 630718) were tested and evaluated in a dose-response study for their ability to inhibit human TMPRSS6 mRNA expression in huTMPRSS6 transgenic mice.

Treatment

huTMPRSS6 Tg mice were maintained on a 12-hour light/dark cycle and were fed *ad libitum* normal mouse chow. Animals were acclimated for at least 7 days in the research facility before initiation of the experiment. Antisense oligonucleotides (ASOs) were prepared in buffered saline (PBS) and sterilized by filtering through a 0.2 micron filter. Oligonucleotides were dissolved in 0.9% PBS for injection.

Male and female huTMPRSS6 mice, roughly 3.5 to 4.5 months old, were divided into 44 groups of four mice each (two males and two females in each group). The mice received subcutaneous injections of ISIS oligonucleotide, twice per week for three weeks. One group of mice received subcutaneous injections of PBS twice per week for three weeks. Forty-eight hours after the administration of the last dose, blood was drawn from each mouse and the mice were sacrificed and tissues were collected.

RNA analysis

At the end of the treatment period, total RNA was extracted from the livers of transgenic mice for quantitative real-time PCR analysis and measurement of human TMPRSS6 mRNA expression. TMPRSS6 mRNA levels were normalized with levels of cyclophilin A, a housekeeping gene, which were determined using mCYCLO_24 primer probe set according to standard protocols. The results below are presented in

Table 24 as the average percent of TMPRSS6 mRNA levels for each treatment group, normalized to PBS-treated control and are denoted as "% PBS". Values above 100 were simply noted as "100". Negative values were simply noted as "0".

Human primer probe set RTS4586 (forward sequence TGATAACAGCTGCCCACTG, designated herein as SEQ ID NO: 86; reverse sequence TCACCTTGAAGGACACCTCT, designated herein as SEQ ID NO: 87; probe sequence AGTTCTGCCACACCTTGCCCA, designated herein as SEQ ID NO: 88) was used to measure mRNA levels.

Table 24

Response to eight ISIS $GalNAc_3$-conjugated and two unconjugated compounds targeting TMPRSS6 in Tg mice

Treatment	Dose (mpk/wk)	TMPRSS6 % PBS	TMPRSS6 % Inhibition
585774	100	4	96
	30	35	65
	10	99	1
	3	100	0
702843	10	0	100
	3	16	84
	1	55	45
	0.3	100	0
705051	10	1	99
	3	68	32
	1	72	28
	0.3	100	0
705052	10	28	72
	3	23	77
	1	100	0
	0.3	100	0
705053	10	7	93
	3	30	70
	1	100	0
	0.3	100	0
630718	30	0	100
	10	37	63
	3	100	0
	1	100	0
706940	3	0	100
	1	4	96
	0.3	52	48
	0.1	100	0

706941	3	8	92
	1	71	29
	0.3	100	0
	0.1	100	0
706942	3	2	98
	1	47	53
	0.3	82	18
	0.1	100	0
706943	3	2	98
	1	15	85
	0.3	100	0
	0.1	100	0

Plasma chemistry markers

To evaluate the effect of ISIS oligonucleotides on liver and kidney function, serum levels of transaminases, bilirubin and BUN were measured using an automated clinical chemistry analyzer (Hitachi Olympus AU400e, Melville, NY) and presented in Table 25 below. ISIS oligonucleotides causing changes in the levels of any of the liver or kidney function markers outside the expected range for antisense oligonucleotides were excluded from further studies.

Table 25

Serum chemistries of eight ISIS $GalNAc_3$-modified ASOs and two unconjugated compounds targeting TMPRSS6 in transgenic mice

	Dose (mg/kg/wk)	**ALT**	**AST**	**BUN**
PBS	n/a	39.5	64.8	40.4
585774	100	40.8	68.5	42.5
	30	36.5	70.8	37.2
	10	38.5	59.0	38.9
	3	39.8	59.5	41.6
702843	10	38.3	57.3	35.6
	3	41.8	65.5	38.9
	1	41.8	100.3	34.7
	0.3	43.3	65.3	38.8
705051	10	47.3	79.8	35.4
	3	37.0	58.5	34.9
	1	33.0	57.0	35.7
	0.3	42.0	67.5	34.6
705052	10	34.8	61.5	33.9
	3	37.0	62.5	32.8

	1	35.8	57.8	35.1
	0.3	35.0	65.0	34.1
705053	10	39.0	55.8	32.4
	3	35.3	62.8	38.6
	1	39.8	73.5	36.6
	0.3	39.5	73.3	37.9
630718	30	58.8	160.8	37.7
	10	38.3	73.0	33.8
	3	39.3	92.3	32.8
	1	38.0	67.8	35.0
706940	3	36.3	54.8	33.7
	1	39.8	65.0	35.7
	0.3	38.3	66.8	34.9
	0.1	36.8	52.8	31.8
706941	3	37.5	59.0	31.6
	1	34.3	75.8	32.3
	0.3	40.5	72.8	34.9
	0.1	45.3	63.8	31.3
706942	3	34.3	90.5	35.8
	1	36.8	58.3	32.8
	0.3	46.8	270.0	39.8
	0.1	35.5	76.5	31.0
706943	3	35.5	81.3	34.6
	1	33.3	71.8	31.0
	0.3	35.0	54.5	32.2
	0.1	42.3	60.0	33.1

All GalNAc conjugated ASOs were well-tolerated with no major changes in organ and body weights nor serum transaminase levels..

The half maximal effective dosage (ED_{50}) of each ASO was calculated and is presented in Table 26, below.

Table 26

Potencies of eight ISIS $GalNAc_3$-modified ASOs and two unconjugated compounds targeting TMPRSS6

ISIS #	ED_{50} (mpk/wk)
585774	26.0
702843	~ 1.0
705051	3.7

705052	~ 2.7
705053	~ 2.8
630718	~ 9.7
706940	~ 0.3
706941	1.3
706942	0.9
706943	~ 0.9

ED_{50} calculations showed that GalNAc-conjugated ASOs are approximately 10-fold more potent than unconjugated ASOs. ISIS 702843 was the most potent GalNAc conjugated 5-10-5 MOE gapmer compound.

Example 10: Viscosity assessment of antisense oligonucleotides targeting TMPRSS6

The viscosity of the antisense oligonucleotides was measured with the aim of screening out antisense oligonucleotides which have a viscosity more than 40 cP. Oligonucleotides having a viscosity greater than 40 cP would not be optimal for administration to a subject.

ISIS oligonucleotides (32-35 mg) were weighed into a glass vial, 120 μL of water was added and the antisense oligonucleotide was dissolved into solution by heating the vial at 50^0C. Part of (75 μL) the pre-heated sample was pipetted to a micro-viscometer (Cambridge). The temperature of the micro-viscometter was set to 25^0C and the viscosity of the sample was measured. Another part (20 μL) of the pre-heated sample was pipetted into 10 mL of water for UV reading at 260 nM at 85^0C (Cary UV instrument). The results are presented in Table 27 and indicate that most of the GalNAc antisense oligonucleotides solutions are optimal in their viscosity under the criterion stated above. Antisense oligonucleotide 706941 was the only antisense oligonucleotide tested that had a viscosity level above 40 cP.

Table 27

Viscosity Data for GalNAc-Conjugated ASOs

ISIS #	Chemistry	cP
702843	5'-THA $GalNAc_3$ 5-10-5 MOE (MBB)	33
705051	5'-THA $GalNAc_3$ 5-10-5 MOE (PS)	23
705052	5'-THA $GalNAc_3$ 5-10-5 MOE (PS)	16
705053	5'-THA $GalNAc_3$ 5-10-5 MOE (PS)	26
706940	5'-THA $GalNAc_3$ kkk-10-kkk (PS)	39

706941	5'-THA $GalNAc_3$ kk-8-eeeekk (PS)	54
706942	5'-THA $GalNAc_3$ kk-9-eeekk (PS)	20
706943	5'-THA $GalNAc_3$ kek-9-eekk (PS)	19

Example 11: Antisense inhibition *in vivo* by oligonucleotides targeting TMPRSS6 comprising a $GalNAc_3$ conjugate in cynomolgus monkeys

At the time this study was undertaken, the cynomolgus monkey genomic sequence for TMPRSS6 was not available in the National Center for Biotechnology Information (NCBI) database; therefore, cross-reactivity of antisense oligonucleotides targeting human TMPRSS6 with the cynomolgus monkey gene sequence could not be confirmed. Instead, the sequences of antisense oligonucleotides were compared to a rhesus monkey sequence for homology as described in Example 6, above. It is expected that ISIS oligonucleotides with homology to the rhesus monkey sequence are fully cross-reactive with the cynomolgus monkey sequence as well.

The ten human TMPRSS6 antisense oligonucleotides selected for testing in cynomolgus monkey had 0 mismatches with the rhesus genomic sequence (SEQ ID NO: 95) as described in Example 6, above.

Study design

Ten antisense oligonucleotides were evaluated for efficacy and tolerability, and for their pharmacokinetic profile in the liver and kidney in a 13-week study of antisense inhibition of TMPRSS6 mRNA in male cynomolgus monkeys. The monkeys were treated by subcutaneous administration with the eight ISIS $GalNAc_3$-modified ASOs and two unconjugated parent antisense oligonucleotides antisense oligonucleotides targeting TMPRSS6 as shown in Table 28.

Table 28

ASOs compared in cynomolgus monkey studies

Group	ISIS#	Dose
1	PBS Control	n/a
2	585774	25 mpk
3	705051	30 mpk
4	705052	30 mpk
5	705053	30 mpk
6	702843	30 mpk
7	705051	5 mpk
8	702843	5 mpk

9	630718	23 mpk
10	706940	30 mpk
11	706941	30 mpk
12	706942	30 mpk
13	706943	30 mpk
14	706940	5 mpk

High-dose (30mpk) groups for the GalNAc-conjugated ASOs assessed toxicity. Low-dose (5 mpk) groups for GalNAc-conjugated ASOs were compared to a corresponding unconjugated parent sequence to assess activity. Groups 2, 3, 6, 7 and 8 are the same sequence, and the mixed backbone (MBB) compound ISIS No.702843 is tested at both low and high doses, as well as compared to the full phosphorothioate compound ISIS No. 705051 (also tested at both low and high doses). Groups 9, 10, 11 and 14 are the same sequence, and ISIS No.706940 is tested at both low and high doses.

Treatment

Prior to the study, the monkeys were kept in quarantine during which the animals were observed daily for general health. The monkeys were two to four years old and weighed two to four kg. 56 male cynomolgus monkeys were randomly assigned to 14 treatment groups with four monkeys per group. Monkeys were each injected subcutaneously every other day for the first week, and then once weekly for 11 weeks for a total of 15 doses with ISIS oligonucleotide or PBS using a stainless steel dosing needle and syringe of appropriate size. Tail bleeds were conducted at 1 week prior to the first administration, then again at days 9, 16, 30, 44, 58, 72 and 86.

During the study period, the monkeys were observed twice daily for signs of illness or distress. Any animal experiencing more than momentary or slight pain or distress due to the treatment, injury or illness was treated by the veterinary staff with approved analgesics or agents to relieve the pain after consultation with the Study Director. Any animal in poor health or in a possible moribund condition was identified for further monitoring and possible euthanasia. Scheduled euthanasia of the animals was conducted on day 86. The protocols described in the Example were approved by the Institutional Animal Care and Use Committee (IACUC).

Prior to the first dose and at various time points thereafter, blood draws were performed for clinical pathology endpoints (hematology, clinical chemistry, coagulation, Complement Bb and C3, cytokine and chemokine analyses), and urine chemistry was also measured. At baseline and at the end of the experimental period, certain pharmacology endpoints were measured, such as liver TMPRSS6 mRNA expression, serum hepcidin (Intrinsic LifeSciences, San Diego, CA), serum iron and serum transferrin saturation. At the end of

the study, body and organ weights, histopathology of tissues and PK analysis of liver and kidney were measured. No significant changes in body weight, cytokine or albumin levels were observed.

TMPRSS6 RNA analysis

At the end of the study, RNA was extracted from liver for real-time PCR analysis of measurement of mRNA expression of TMPRSS6 using various primer-probe sets. Representative data using the primer probe set RTS3840 is presented in the table below. Results in Table 29 are presented as percent inhibition of TMPRSS6 mRNA relative to saline control, normalized with cyclophilin (mCYCLO_24 primer probe set).

Table 29

Reduction of monkey liver TMPRSS6 mRNA after 12-weeks ASO administration

Treatment	Dose (mg/kg)	% inhibition	Group
585774	25	76	2
705051	30	90	3
705052	30	64	4
705053	30	49	5
702843	30	89	6
705051	5	77	7
702843	5	82	8
630718	23	65	9
706940	30	71	10
706941	30	72	11
706942	30	93	12
706943	30	91	13
706940	5	61	14

ISIS Nos. 705051, 702843, 706942 and 706943 were quite efficacious, demonstrating ≥ 89% target reduction at 30 mpk after 13-weeks of dosing.

Hepcidin analysis

Serum hepcidin levels were measured at the time points shown in Table 30 below. Results are presented as percent saline control. "Day -7" indicates one week before the first dose was administered.

Table 30

Monkey serum hepcidin levels

	Dose (mg/kg)	Day -7	Day 9	Day 16	Day 44	Day 86
Saline	n/a	1.0	1.0	1.0	1.0	1.0
585774	25	0.9	1.3	1.4	1.1	1.4
705051	30	0.9	1.1	1.5	1.5	1.8

702843	30	0.9	1.2	1.2	1.3	1.9
706942	30	0.7	1.0	1.5	1.3	1.9
706943	30	0.8	0.9	1.5	1.2	1.6

The table shows that serum hepcidin levels increased over the course of the study.

Serum iron and transferrin saturation analysis

The averages of the four subjects from each of the 14 treatment groups are presented in Table 31, below. As is shown in Table 31, serum iron levels and transferrin saturation ("Tf sat") were reduced at day 86 in treated groups compared to control.

Table 31

Monkey serum iron and transferrin saturation levels at day 86

Group #	Treatment	Dose (mg/kg)	iron	Tf sat
1	Saline	n/a	125.7	38.8
2	585774	25	55.2	15.7
3	705051	30	36.6	10.0
4	705052	30	61.9	15.8
5	705053	30	96.0	27.0
6	702843	30	42.3	13.3
7	705051	5	63.7	20.0
8	702843	5	51.7	16.5
9	630718	23	61.4	17.7
10	706940	30	71.6	20.5
11	706941	30	55.7	15.8
12	706942	30	25.9	6.9
13	706943	30	30.3	7.4
14	706940	5	82.8	23.7

CLAIMS

What is claimed is:

1. A compound comprising a modified oligonucleotide consisting of 12 to 30 linked nucleosides having a nucleobase sequence comprising a portion of at least 8 contiguous nucleobases complementary to an equal length portion of nucleobases 3162 to 3184 of SEQ ID NO: 1, wherein the nucleobase sequence of the modified oligonucleotide is at least 80% complementary to SEQ ID NO: 1.

2. The compound of claim 1, wherein the modified oligonucleotide consists of 15 to 30, 15 to 25, 15 to 24, 16 to 24, 17 to 24, 18 to 24, 19 to 24, 20 to 24, 19 to 22, 20 to 22, 16 to 20, 16 or 20 linked nucleosides.

3. The compound of claim 1, wherein the modified oligonucleotide comprises a nucleobase sequence comprising a portion of at least 10, least 11, at least 12, least 13, at least 14, least 15, at least 16, least 17, at least 18, at least 19, or at least 20 contiguous nucleobases complementary to an equal length portion of SEQ ID NO: 1

4. The compound of any preceding claim, wherein the nucleobase sequence of the modified oligonucleotide is at least 85%, at least 90%, at least 95%, or 100% complementary to SEQ ID NO: 1

5. . A compound comprising a modified oligonucleotide consisting of 12 to 30 linked nucleosides having a nucleobase sequence comprising at least 8, at least 9, at least 10, at least 11, at least 12, at least 13, at least 14, at least 15, or 16 contiguous nucleobases of any of the nucleobase sequences of SEQ ID NOs: 63, 77.

6. A compound comprising a modified oligonucleotide consisting of 12 to 30 linked nucleosides having a nucleobase sequence comprising at least 8, at least 9, at least 10, at least 11, at least 12, at least 13, at least 14, at least 15, at least 16, at least 17, at least 18, at least 19, or 20 contiguous nucleobases of any of the nucleobase sequences of SEQ ID NOs: 23, 36, 37.

7. A compound comprising a modified oligonucleotide consisting of 12 to 30 linked nucleosides and having a nucleobase sequence comprising at least 8, at least 9, at least 10, at least 11, at least 12, at least 13, at least 14, at least 15, at least 16, at least 17, at least 18, at least 19, or 20 contiguous nucleobases of the nucleobase sequence of SEQ ID NO: 36.

8. The compound of any preceding claim, wherein the modified oligonucleotide is single-stranded.

9. The compound of any preceding claim, wherein at least one internucleoside linkage is a modified internucleoside linkage.

10. The compound of claim 9, wherein at least one modified internucleoside linkage is a phosphorothioate internucleoside linkage.

11. The compound of claim 9, wherein each modified internucleoside linkage is a phosphorothioate internucleoside linkage.

12. The compound of any preceding claim, wherein the modified oligonucleotide comprises at least one modified sugar.

13. The compound of claim 12, wherein at least one modified sugar is a bicyclic sugar.

14. The compound of claim 12, wherein at least one modified sugar comprises a 2'-O-methoxyethyl, a constrained ethyl, a 3'-fluoro-HNA or a 4'- $(CH_2)_n$-O-2' bridge, wherein n is 1 or 2.

15. The compound of any preceding claim, wherein at least one nucleoside comprises a modified nucleobase.

16. The compound of claim 15, wherein the modified nucleobase is a 5-methylcytosine.

17. The compound of any preceding claim, wherein the modified oligonucleotide consists of 12 to 30 linked nucleosides and comprises:

a. a gap segment consisting of linked deoxynucleosides;
b. a 5' wing segment consisting of linked nucleosides; and
c. a 3' wing segment consisting of linked nucleosides;

wherein the gap segment is positioned between the 5' wing segment and the 3' wing segment and wherein each nucleoside of each wing segment comprises a modified sugar.

18. The compound of claim 17, wherein the modified oligonucleotide consists of 16 to 20 linked nucleobases.

19. The compound of claim 17, wherein the modified oligonucleotide consists of 20 linked nucleosides comprising:

a. a gap segment consisting of ten linked deoxynucleosides;

b. a 5' wing segment consisting of five linked nucleosides; and

c. a 3' wing segment consisting of five linked nucleosides;

wherein the gap segment is positioned between the 5' wing segment and the 3' wing segment, wherein each nucleoside of each wing segment comprises a 2'-O-methoxyethyl sugar, wherein at least one internucleoside linkage is a phosphorothioate linkage and wherein each cytosine residue is a 5-methylcytosine.

20. A compound comprising a modified oligonucleotide consisting of 20 linked nucleosides having a nucleobase sequence comprising at least 8 contiguous nucleobases of SEQ ID NO: 36, wherein the modified oligonucleotide comprises:

a. a gap segment consisting of ten linked deoxynucleosides;

b. a 5' wing segment consisting of five linked nucleosides;

c. a 3' wing segment consisting of five linked nucleosides; and

d. a GalNAc conjugate;

wherein the gap segment is positioned between the 5' wing segment and the 3' wing segment, wherein each nucleoside of each wing segment comprises a 2'-O-methoxyethyl sugar, wherein at least one internucleoside linkage is a phosphorothioate linkage and wherein each cytosine residue is a 5-methylcytosine.

21. A compound comprising a modified oligonucleotide consisting of 16 linked nucleosides having a nucleobase sequence comprising at least 8 contiguous nucleobases of SEQ ID NO: 77, wherein the modified oligonucleotide comprises:

a. a gap segment consisting of nine linked deoxynucleosides;

b. a 5' wing segment consisting of three linked nucleosides;

c. a 3' wing segment consisting of four linked nucleosides; and

d. a GalNAc conjugate;

wherein the gap segment is positioned between the 5' wing segment and the 3' wing segment, wherein each nucleoside of each wing segment comprises a modified sugar, wherein each internucleoside linkage is a phosphorothioate linkage and wherein each cytosine residue is a 5-methylcytosine.

22. A compound comprising a modified oligonucleotide according to the following formula: mCes Teo Teo Teo Aeo Tds Tds mCds mCds Ads Ads Ads Gds Gds Gds mCeo Aeo Ges mCes Te (SEQ ID NO: 36); wherein,

A = an adenine,

mC = a 5-methylcytosine

G = a guanine,

T = a thymine,

e = a 2'-O-methoxyethyl modified nucleoside,

d = a 2'-deoxynucleoside, and

s = a phosphorothioate internucleoside linkage.

23. A compound comprising a modified oligonucleotide according to the following formula: mCks Aes Gks mCds Tds Tds Tds Ads Tds Tds mCds mCds Aes Aes Aks Gk (SEQ ID NO: 77); wherein,

A = an adenine,

mC = a 5-methylcytosine

G = a guanine,

T = a thymine,

e = a 2'-O-methoxyethyl modified nucleoside,

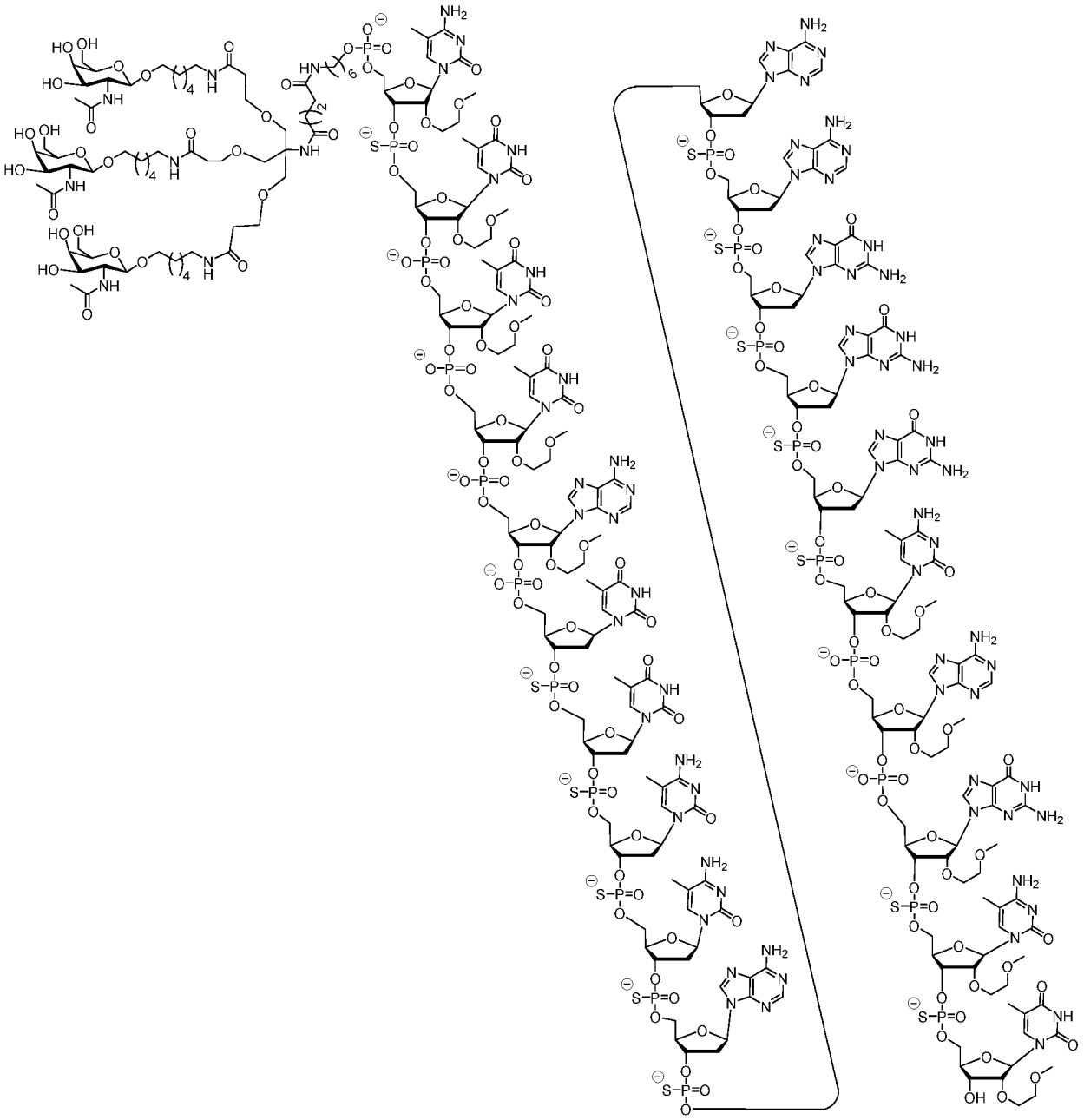
d = a 2'-deoxynucleoside,

s = a phosphorothioate internucleoside linkage, and

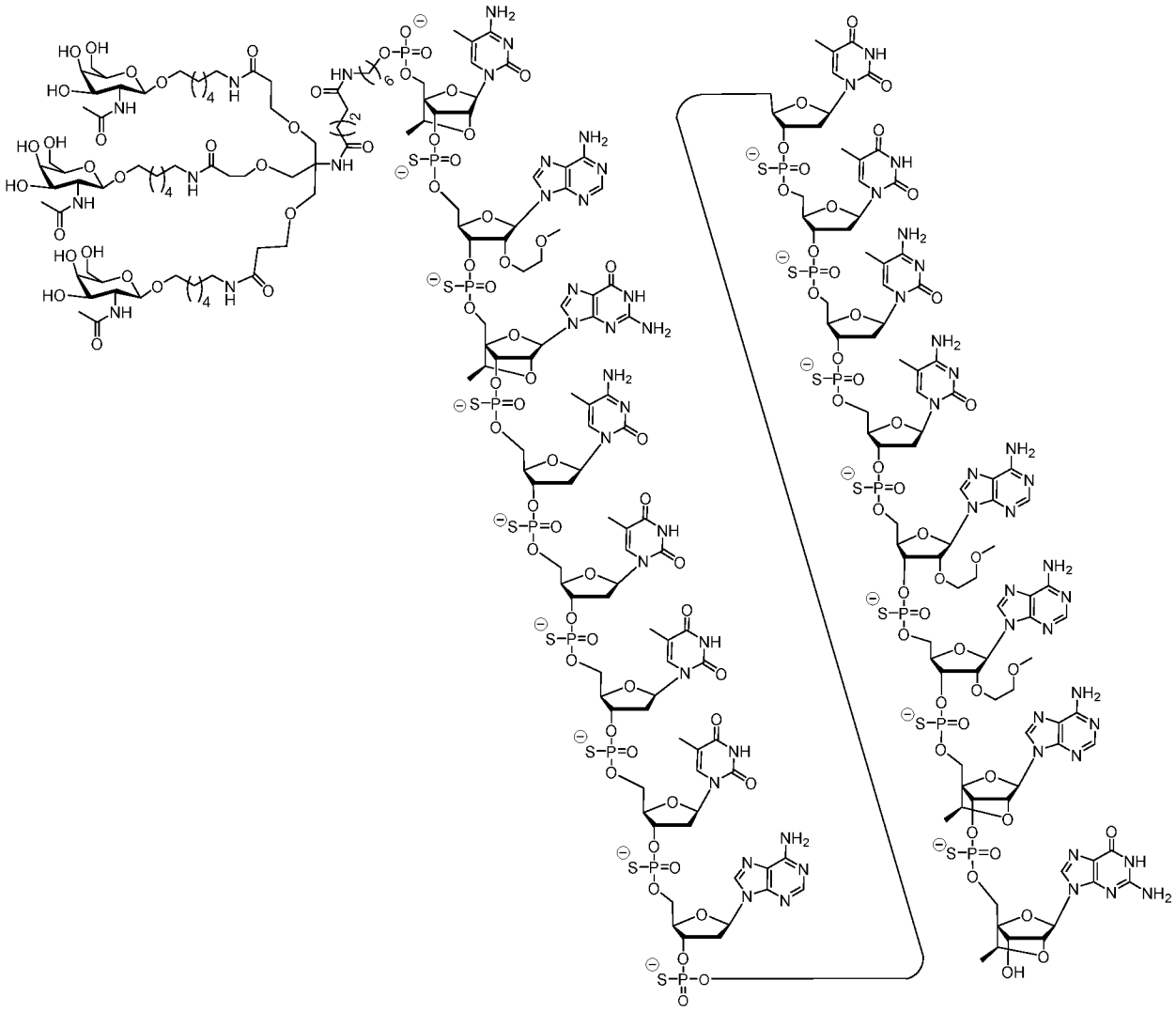
k = a constrained ethyl.

24. The compound of any preceding claim, further comprising a conjugated GalNAc moiety.

25. A modified oligonucleotide according to the following formula:



26. A modified oligonucleotide according to the following formula:



27. A composition comprising a compound or modified oligonucleotide of any preceding claim, or a salt thereof, and a pharmaceutically acceptable carrier or diluent.

28. A composition comprising a compound or modified oligonucleotide of any preceding claim, for use in therapy.

29. A composition comprising a compound or modified oligonucleotide of any preceding claim, for use in the preparation of a medicament.

30. The compound or modified oligonucleotide of any preceding claim, for use in reducing TMPRSS6 in a cell, tissue, organ or animal.

31. The compound or modified oligonucleotide of any preceding claim, for use in reducing iron accumulation, increasing hepcidin expression levels, and/or decreasing the percentage saturation of transferrin in an animal.

32. The compound or modified oligonucleotide of any preceding claim, for use in treating, preventing, or slowing progression of a disease, disorder or condition related to excess iron accumulation in an animal.

33. The compound or modified oligonucleotide of claim 32, wherein the disease, disorder or condition is polycythemia, hemochromatosis or anemia.

34. The compound or modified oligonucleotide of claim 33, wherein the anemia is hereditary anemia, myelodysplastic syndrome or severe chronic hemolysis.

35. The compound or modified oligonucleotide claim 34, wherein the hereditary anemia is sickle cell anemia, thalassemia, Fanconi anemia, Diamond Blackfan anemia, Shwachman Diamond syndrome, red cell membrane disorders, glucose-6-phosphate dehydrogenase deficiency, or hereditary hemorrhagic telangiectasia.

36. The compound or modified oligonucleotide claim 35, wherein the thalassemia is β-thalassemia.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 16/25883

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - C12N 15/113; A61K 45/00, 31/713 (2016.01)
CPC - C12N 15/1137; A61K 45/00, 31/713
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC(8): C12N 15/113; A61K 45/00, 31/713 (2016.01)
CPC: C12N 15/1137; A61K 45/00, 31/713

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
CPC: C12N 15/1137; A61K 45/00, 31/713 (text search)
USPC: 514/44A, 536/23.1, 24.1(text search)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Electronic data bases: PatBase; Google Patents; Google Scholar
Search terms: RNAi, antisense oligonucleotide (ASO), modified oligonucleotides, TMPRSS6 (Transmembrane Protease, Serine 6), GalNac conjugation, 5' wing segment, 3' wing segment, 2'-O-methoxyethyl sugar, methylcytosine, phosphorothioate

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2014/0194489 A1 (BUMCROT et al.) 10 July 2014 (10.07.2014). Especially para [0373], pg 52 Tab;e 2; claims 1, 2, 5; SEQ ID NOs: 1, 374, 376	1-5
A	US 2014/0309286 A1 (Isis Pharmaceuticals, Inc.) 16 October 2014 (16.10.2014). Especially para [0145], [0274], SEQ ID NO: 2	1-5
A	US 2007/0134655 A1 (BENTWICH) 14 June 2007 (14.06.2014). Especially para [0002]; [0012]; SEQ ID NO: 395460	1-5
A	WO 2014/179625 A1 (Isis Pharmaceuticals, Inc.) 6 November 2014 (06.11.2014). Especially pg 22 ln 25-27, pg 46 ln 33 continued to pg 47 ln 4.	1-5

☐ 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
19 August 2016	07 SEP 2016
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. 571-273-8300	Authorized officer: Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 16/25883

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

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:

   a. ☒ forming part of the international application as filed:

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

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

   b. ☐ furnished together with the international application under PCT Rule 13*ter*.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.

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

      ☐ in the form of an Annex C/ST.25 text file (Rule 13*ter*.1(a)).

      ☐ on paper or in the form of an image file (Rule 13*ter*.1(b) and Administrative Instructions, Section 713).

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

3. Additional comments:
GenCore ver 6.4.1 SEQ ID NOs: 1, 63

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 16/25883

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.: 8-19, 24, 27-36
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:
This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I+: Claims 1-7, 20-23, 25, 26, drawn to a composition comprising a modified oligonucleotide consisting of 12 to 30 linked nucleosides having a nucleobase sequence comprising a portion of at least 8 contiguous nucleobases complementary to an equal length portion of nucleobases 3162 to 3184 of SEQ ID NO: 1, wherein the nucleobase sequence of the modified oligonucleotide is at least 80% complementary to SEQ ID NO: 1.

-----Go to Extra Sheet for continuation------

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-7, 20-23, 25, 26, limited to SEQ ID NO: 63 (claims 1-5)

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.

----continuation of Box III (Lack of Unity of Invention)------

The modified oligonucleotide will be searched to the extent to encompass the first named oligonculeotide SEQ ID NO: 63 [nt 3168-3183 of SEQ ID NO: 1, according to instant application Table 3, pg 77]. It is believed that claims 1-5 read on this first named invention and thus these claims will be searched without fee to the extent that it encompasses SEQ ID NO: 63. [Note: claims 25 and 26 are excluded from the first invention because these modified oligonucleotides are conjugated versions of SEQ ID NOs: 36 and 77, respectively and because SEQ ID NO: 63 does not comprise either SEQ ID NOs: 36 or 77]. Additional modified oligonucleotides will be searched upon payment of additional fees. Applicant must specify the claims that encompass any additional elected modified oligonucleotides. Applicants must further indicate, if applicable, the claims which read on the first named invention if different than what was indicated above for this group. Failure to clearly identify how any paid additional invention fees are to be applied to the "+" group(s) will result in only the first claimed invention to be searched/examined. An exemplary election would be: SEQ ID NO: 36 [nucleotides 3162-3181 of SEQ ID NO: 1, see Instant Application Table 1, pg 76] (claims 1-4, 6, 7, 20, 22, 25).

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

Special Technical Features:

Among the inventions listed as Groups I+ is the specific oligonucleotide sequences recited therein. Each invention requires an oligonucleotide sequence not required by any of the other inventions.

Common Technical Feature:

The inventions of Group I+ share the common technical feature of a modified oligonucleotide consisting of 12 to 30 linked nucleosides having a nucleobase sequence comprising a portion of at least 8 contiguous nucleobases complementary to SEQ ID NO: 1, TMPRSS6 (Transmembrane Protease, Serine 6). The inventions of Group I+ further share the common technical feature of an oligonucleotide comprising 5' wing-gap-3' wing, conjugated to a GalNac; wherein the gap segment is positioned between the 5' wing segment and the 3' wing segment, wherein each nucleoside of each wing segment comprises a 2'-O-methoxyethyl sugar, wherein at least one internucleoside linkage is a phosphorothioate linkage and wherein each cytosine residue is a 5-methylcytosine.

However, said common technical feature does not represent a contribution over the prior art, and is obvious over US 2014/0309286 A1 (Isis Pharmaceuticals, Inc.) (hereinafter "ISIS'286"), in view of WO 2014/179625 A1 (Isis Pharmaceuticals, Inc.) (hereinafter "ISIS'625").

ISIS'286 teaches a modified oligonucleotide consisting of 12 to 30 linked nucleosides having a nucleobase sequence comprising a portion of at least 8 contiguous nucleobases complementary to SEQ ID NO: 1, TMPRSS6 (Transmembrane Protease, Serine 6) (Claims 2, 17-20; 2. A method of treating, preventing, delaying the onset of, ameliorating, and/or reducing a disease, disorder and/or condition, or a symptom thereof, associated with excess accumulation of iron in an animal, comprising administering to the animal a therapeutically effective amount of an antisense compound targeting TMPRSS6, wherein the disease, disorder and/or condition, or the symptom thereof, associated with excess accumulation of iron is treated, prevented, the onset delayed, ameliorated, and/or reduced in the animal. 17. The method of claim 2, wherein the antisense compound is a modified oligonucleotide. 18. The method of claim 17, wherein the modified oligonucleotide consists of 12 to 30 linked nucleosides. 19. The method of claim 17, wherein the modified oligonucleotide is single-stranded. 20. The method of claim 17, wherein the modified oligonucleotide has a nucleobase sequence that is at least 90%, at least 95%, at least 99%, or 100% complementary to a human TMPRSS6 nucleic acid; SEQ ID NO 1 (SEQ ID NO: 2, nt 1-3212, 100% match). ISIS'286 further teaches conjugation of the oligonucleotide to a moiety; (para [0274]; In certain embodiments, the compounds of the invention can be covalently linked to one or more moieties or conjugates which enhance the activity, cellular distribution or cellular uptake of the resulting antisense oligonucleotides") and further teaches at least one internucleoside linkage is a phosphorothioate linkage (para [0145]; the modified oligonucleotide further comprises at least one phosphorothioate internucleoside linkage" and wherein each cytosine residue is a 5-methylcytosine (para [0256]; In certain embodiments, the modified nucleobase is 5-methylcytosine. In certain embodiments, each cytosine is a 5-methylcytosine"). ISIS'286 does not specifically teach conjugating to a GalNac; wherein the gap segment is positioned between the 5' wing segment and the 3' wing segment, wherein each nucleoside of each wing segment comprises a 2'-O-methoxyethyl sugar. However, such properties were well known in the art, as for example, as taught by ISIS'625 (pg 22 ln 25-27; "Accordingly "GalNac3-I" refers to a specific carbohydrate cluster portion of a conjugate group having 3 GalNac groups and specifically identified tether, branching and linking groups. Such carbohydrate cluster fragment is attached to an oligomeric compound"; pg 46 ln 33 continued to pg 47 ln 4; Certain embodiments provide a compound comprising a modified oligonucleotide targeting apo(a) and a conjugate group, wherein the modified oligonucleotide consists of 20 linked nucleosides and comprises: ?.....(c) a 3' wing segment consisting of five linked nucleosides; and wherein the gap segment is positioned between the 5' wing segment and the 3' wing segment, wherein each nucleoside of each wing segment comprises a 2'-O-methoxyethyl sugar, wherein at least one internucleoside linkage is a phosphorothioate linkage and wherein each cytosine residue is a 5-methylcytosine").

-------continued on next sheet-----

continuation from prior sheet

These features of said modified oligonculeotides, as taught by ISIS'625, could have been incorporated into the modified oligonucleotide taught by ISIS'286, without undue experimentation, and with a high probability of success.

As the common technical feature was known in the art at the time of the invention, this cannot be considered a common special technical feature that would otherwise unify the groups. The inventions lack unity with one another.

Therefore, Group I+ lack unity of invention under PCT Rule 13 because they do not share a same or corresponding special technical feature

Note concerning item 4: Claims 8-19, 24, 27-36 are dependent claims and are not drafted according to the second and third sentences of PCT Rule 6.4(a).