DOUBLE-STRANDED RNA (DSRNA) FOR INHIBITING EXPRESSION OF TMPRSS6

Cross-Reference to Related Applications

This application of United States provisional application 61/468,830, filed March 29, 2011, and United States provisional application 61/568,942, filed December 9, 2011. These prior applications are incorporated by reference herein in their entirety.

Field of the Invention

The invention relates to the specific inhibition of the expression of the TMPRSS6 gene.

Background of the Invention

TMPRSS6 (Transmembrane Protease, Serine 6) encodes a type II serine protease and is expressed mainly in the liver. TMPRSS6 influences iron levels in the liver by binding and proteolytically degrading the hepcidin activator and BMP co-receptor HJV (hemojuvelin), which causes down-regulation of hepcidin levels.

TMPRSS6 consists of a short N-terminal intracytoplasmic tail, a type II transmembrane domain, a stem region composed of two extracellular CUB (complement factor C1s/C1r, urchin embryonic growth factor and BMP (bone morphogenetic protein)) domains, three LDLR (low-density-lipoprotein receptor class A) domains, and a C-terminal trypsin-like serine protease domain. There are also consensus sites for N-glycosylation in the extracellular domain, and a potential phosphorylation site in the intracytoplasmic tail region.

Summary of the Invention

Described herein are compositions and methods that effect the RNA-induced silencing complex (RISC)-mediated cleavage of RNA transcripts of the TMPRSS6 gene, such as in a cell or mammal. Also described are compositions and methods for treating pathological conditions and diseases caused by the expression of a TMPRSS6 gene, such as a disorder characterized by iron overabundance (*e.g.*, thalassemia, *e.g.*, a β-thalassemia intermedia or an α-thalassemia). Also described are compositions and methods for

decreasing or preventing iron absorption or mobilization, thereby ameliorating iron overload in certain pathological conditions. The methods and compositions described herein are generally useful for the treatment of hemochromatosis (iron build-up in the body).

As used herein, the term "iRNA" refers to an agent that contains RNA as that term is defined herein, and which mediates the targeted cleavage of an RNA transcript via an RNA-induced silencing complex (RISC) pathway. In one embodiment, an iRNA as described herein inhibits the expression of TMPRSS6 in a cell or mammal. Alternatively, in another embodiment, the iRNA up-regulates the expression of TMPRSS6 in a cell or mammal.

The iRNAs included in the compositions featured herein encompass a double-stranded RNA (dsRNA) having an RNA strand (the antisense strand) having a region that is 30 nucleotides or less, generally 19-24 nucleotides in length, that is substantially complementary to at least part of an mRNA transcript of a TMPRSS6 gene. In one embodiment, the dsRNA comprises a region of at least 15 contiguous nucleotides.

In one embodiment, an iRNA for inhibiting expression of a TMPRSS6 gene includes at least two sequences that are complementary to each other. The iRNA includes a sense strand having a first sequence and an antisense strand having a second sequence. The antisense strand includes a nucleotide sequence that is substantially complementary to at least part of an mRNA encoding TMPRSS6, and the region of complementarity is 30 nucleotides or less, and at least 15 nucleotides in length. Generally, the iRNA is 19 to 24, *e.g.*, 19 to 21 nucleotides in length. In some embodiments the iRNA is from about 15 to about 25 nucleotides in length, and in other embodiments the iRNA is from about 25 to about 30 nucleotides in length. The iRNA, upon contacting with a cell expressing TMPRSS6, inhibits the expression of a TMPRSS6 gene by at least 10%, at least 20%, at least 25%, at least 30%, at least 35% or at least 40% or more, such as when assayed by a method as described herein. In one embodiment, the TMPRSS6 iRNA is formulated in a stable nucleic acid lipid particle (SNALP).

In one embodiment, an iRNA featured herein includes a first sequence of a dsRNA that is selected from the group consisting of the sense sequences of Tables 2, 3 or 4 and a second sequence that is selected from the group consisting of the corresponding antisense sequences of Tables 2, 3 or 4. The iRNA molecules featured herein can include naturally

occurring nucleotides or can include at least one modified nucleotide, including, but not limited to a 2'-O-methyl modified nucleotide, a nucleotide having a 5'-phosphorothioate group, and a terminal nucleotide linked to a cholesteryl derivative. Alternatively, the modified nucleotide may be chosen from the group of: a 2'-deoxy-2'-fluoro modified nucleotide, a 2'-deoxy-modified nucleotide, a locked nucleotide, an abasic nucleotide, 2'-amino-modified nucleotide, 2'-alkyl-modified nucleotide, morpholino nucleotide, a phosphoramidate, and a non-natural base comprising nucleotide. Generally, such a modified sequence will be based on a first sequence of said iRNA selected from the group consisting of the sense sequences of Tables 2, 3 or 4 and a second sequence selected from the group consisting of the antisense sequences of Tables 2, 3 or 4.

In one embodiment, an iRNA featured herein includes a sense strand of a TMPRSS6 dsRNA having a sequence selected from the group consisting of SEQ ID NO:111, SEQ ID NO:455, SEQ ID NO:109, SEQ ID NO:524, SEQ ID NO:89, SEQ ID NO:494, SEQ ID NO:445, SEQ ID NO:592, SEQ ID NO:47, and SEQ ID NO:540; and an antisense strand consisting of a sequence selected from the group consisting of SEQ ID NO:112, SEQ ID NO:456, SEQ ID NO:110, SEQ ID NO:525, SEQ ID NO:90, SEQ ID NO:495, SEQ ID NO:446, SEQ ID NO:593, SEQ ID NO:48 and SEQ ID NO:541.

In another embodiment, a composition containing a dsRNA targeting TMPRSS6 is administered to a subject who has elevated iron levels, *e.g.*, elevated levels of iron in the liver. A subject who has elevated iron levels can be identified as a subject who has elevated serum iron levels (*e.g.*, over 350 μg/dL, over 500 μg/dL or over 1000 μg/dL or more), elevated serum ferritin levels, or a transferrin saturation level greater than 40%, greater than 45%, greater than 50%, greater than 60% or more.

Mild-to-moderate iron overload is indicated by serum ferritin levels of 300-2500 μg/L, while levels >2500 μg/L are associated with an increased risk of cardiac disease. Serum ferritin >1000 μg/L has been shown to be associated with adverse outcomes in both primary and secondary iron overload. Serum ferritin levels higher than 200 μg/L in premenopausal women, and 300 μg/L in men and postmenopausal women indicate primary iron overload due to hemochromatosis, and ferritin levels higher than 1000 μg/L typically suggest liver damage due to iron overload. A subject having a serum ferritin level higher than 300 μg/L, 500 μg/L, 1000 μg/L, 1500 μg/L, 2000 μg/L, or 2500 μg/L or more is a candidate for treatment with a dsRNA targeting TMPRSS6.

In another embodiment, a composition containing a dsRNA targeting TMPRSS6 is administered to a subject who has elevated transferrin levels, *e.g.*, transferrin levels greater than 400 mg/dL, greater than 500 mg/dL, greater than 1000 mg/dL or more)

Iron levels can also be measured by a TIBC (Total Iron Binding Capacity) test. The TIBC test measures the amount of iron that the blood would carry if the transferrin were fully saturated. Since transferrin is produced by the liver, the TIBC can be used to monitor liver function and nutrition. A subject having TIBC values greater than 400μg/dL, greater than 500μg/dL, or greater than 1000 μg/dL or more is a candidate for treatment with a dsRNA targeting TMPRSS6.

In one embodiment, administration of the dsRNA lowers iron levels, *e.g.*, in the liver, or in serum, by at least 5%, *e.g.*, by at least 10%, by at least 15%, at least 20%, at least 25%, at least 30%, at least 40%, at least 50%, or at least 60%, or more. In some embodiments, one or more of serum ferritin levels, serum transferrin levels, transferrin saturation levels or TIBC values are decreased by at least 5%, at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 40%, at least 50%, or at least 60%, or more, as compared to pretreatment levels. In another embodiment, the decrease in iron levels, decrease in serum ferritin levels, decrease in transferrin or transferrin saturation levels, or decrease in TIBC values is maintained for at least 5, 10, 20, 30, or 40 days or longer.

In one embodiment, the subject is selected, at least in part, on the basis of needing (as opposed to merely selecting a patient on the grounds of who happens to be in need of) lower iron levels.

In one embodiment, an iRNA as described herein targets a wildtype TMPRSS6 RNA transcript, and in another embodiment, the iRNA targets a mutant transcript (*e.g.*, a TMPRSS6 RNA carrying an allelic variant). For example, an iRNA featured in the invention can target a polymorphic variant, such as a single nucleotide polymorphism (SNP), of TMPRSS6. In another embodiment, the iRNA targets both a wildtype and a mutant TMPRSS6 transcript. In yet another embodiment, the iRNA targets a transcript variant of TMPRSS6.

In one embodiment, an iRNA featured in the invention targets a non-coding region of a TMPRSS6 RNA transcript, such as the 5' or 3' untranslated region.

In one embodiment, an iRNA featured in the invention is delivered to the liver, *e.g.*, hepatocytes of the liver or Kupffer cells, *e.g.*, hypertrophic Kupffer cells.

In one aspect, embodiments featured in the invention provide a cell containing at least one of the iRNAs featured in the invention. The cell is generally a mammalian cell, such as a human cell.

In another aspect, embodiments featured in the invention provide a pharmaceutical composition for inhibiting the expression of a TMPRSS6 gene in an organism, generally a human subject. The composition typically includes one or more of the iRNAs described herein and a pharmaceutically acceptable carrier or delivery vehicle. In one embodiment, the composition is used for treating a disorder that causes increased iron levels, *e.g.*, hemochromatosis. For example, the composition is useful for treating a thalassemia, such as β-thalassemia intermedia.

In another embodiment, the pharmaceutical composition is formulated for administration of a dosage regimen described herein, *e.g.*, not more than once every two months, not more than once per month, not more than twice per month, not more than once every four weeks, not more than once every three weeks, not more than once every two weeks, or not more than once every week. In another embodiment, administration of the pharmaceutical composition can be maintained for a month or longer, *e.g.*, one, two, three, or six months, or one year, or five years, or ten years, or longer, including the remaining lifetime of a subject.

In another embodiment, a composition containing an iRNA described herein, *e.g.*, a dsRNA targeting TMPRSS6, is administered with a non-iRNA therapeutic agent, such as an agent known to treat hemochromatosis, or a disorder that causes hemochromatosis, such as a thalassemia. For example, an iRNA featured in the invention can be administered with an agent for treatment of a β thalassemia, *e.g.*, β-thalassemia intermedia, or another disorder associated with increased iron levels.

In another embodiment, a TMPRSS6 iRNA is administered to a patient, and then the non-iRNA agent is administered to the patient (or vice versa). In another embodiment, a TMPRSS6 iRNA and the non-iRNA therapeutic agent are administered at the same time. In one embodiment, the agent is, for example, an agent that affects iron levels, such as an iron chelator (*e.g.*, desferrioxamine), or folic acid.

In another aspect, provided herein is a method for inhibiting the expression of a TMPRSS6 gene in a cell by performing the following steps:

(a) introducing into the cell a double-stranded ribonucleic acid (dsRNA), wherein the dsRNA includes at least two sequences that are complementary to each other. The dsRNA has a sense strand having a first sequence and an antisense strand having a second sequence; the antisense strand has a region of complementarity that is substantially complementary to at least a part of an mRNA encoding TMPRSS6, and where the region of complementarity is 30 nucleotides or less, *i.e.*, 15-30 nucleotides in length, and generally 19-24 nucleotides in length, and where the dsRNA, upon contact with a cell expressing TMPRSS6, inhibits expression of a TMPRSS6 gene by at least 10%, preferably at least 20%, at least 30%, at least 40% or more; and

(b) maintaining the cell produced in step (a) for a time sufficient to obtain degradation of the mRNA transcript of the TMPRSS6 gene, thereby inhibiting expression of a TMPRSS6 gene in the cell.

In another aspect, the invention provides methods and compositions useful for activating expression of a TMPRSS6 gene in a cell or mammal.

In another aspect, the invention provides a method for modulating the expression of a TMPRSS6 gene in a cell by performing the following steps:

(a) introducing into the cell a double-stranded ribonucleic acid (dsRNA), wherein the dsRNA includes at least two sequences that are complementary to each other. The dsRNA has a sense strand having a first sequence and an antisense strand having a second sequence; the antisense strand has a region of complementarity that is substantially complementary to at least a part of an mRNA encoding TMPRSS6, and where the region of complementarity is 30 nucleotides or less, *i.e.*, 15-30 nucleotides in length, and generally 19-24 nucleotides in length, and where the dsRNA, upon contact with a cell expressing TMPRSS6, modulates expression of a TMPRSS6 gene by at least 10%, preferably at least 20%, at least 30%, at least 40% or more; and

(b) maintaining the cell produced in step (a) for a time sufficient to obtain degradation or protection of the mRNA transcript of the TMPRSS6 gene, thereby modulating expression of a TMPRSS6 gene in the cell.

In one embodiment, the method is for inhibiting gene expression in a liver cell, such as a hepatocyte, or a Kupffer cell. In another embodiment, the method is for activating gene expression in a liver cell.

In other aspects, the invention provides methods for treating, preventing, reversing, or managing pathological processes mediated by TMPRSS6 expression, such as a disorder associated with hemochromatosis. In one embodiment, the method includes administering to a patient in need of such treatment, prevention, reversal, or management, a therapeutically or prophylactically effective amount of one or more of the iRNAs featured in the invention. In one embodiment the patient has a thalassemia, such as β-thalassemia intermedia. In another embodiment, administration of the iRNA targeting TMPRSS6 alleviates or relieves the severity of at least one symptom of a TMPRSS6-mediated disorder in the patient, such as a symptom associated with iron overload, *e.g.*, joint pain, abdominal pain, or weakness.

In one aspect, the invention provides a vector for inhibiting the expression of a TMPRSS6 gene in a cell. In one embodiment, the vector includes at least one regulatory sequence operably linked to a nucleotide sequence that encodes at least one strand of an iRNA as described herein.

In another aspect, the invention provides a cell containing a vector for inhibiting the expression of a TMPRSS6 gene in a cell. The vector includes a regulatory sequence operably linked to a nucleotide sequence that encodes at least one strand of one of the iRNAs as described herein.

In yet another aspect, the invention provides a composition containing a TMPRSS6 iRNA, in combination with a second iRNA targeting a second gene involved in a pathological disease, and useful for treating the disease, *e.g.*, a β-thalassemia. For example, a second iRNA can target a negative regulator of hepcidin, such as a hypoxia inducible factor, *e.g.*, a HIF-1a or HIF-2a; GDF15; or TWSG1. In one embodiment, the second iRNA targets a gene involved in a second disorder that results from the β-thalassemia. For example, the second iRNA can target a gene involved in diabetes mellitus, thrombosis or osteopenia.

The details of various embodiments of the invention are set forth in the description below. Other features, objects, and advantages of the invention will be apparent from the description and the drawings, and from the claims.

Description of the Drawings

FIG. 1 is the sequence of human TMPRSS6 mRNA (Ref. Seq. NM_153609.2, GI:56682967, Record dated January 23, 2011, SEQ ID NO:1).

FIGs. 2A and 2B depict the potency of two chemically modified TMPRSS6 targeting siRNAs in the reduction of TMPRSS6 gene expression in primary mouse hepatocytes.

FIGs. 3A and 3B depict the effect of LNP-TMPRSS6 siRNA-1 (AD-46273) and LNP-TMPRSS6 siRNA-2 (AD-46286), on TMPRSS6 and HAMP1 gene expression, respectively, in WT C57BL/6 mice.

FIG. 4 depicts the duration of the TMPRSS6 siRNA mediated effects on TMPRSS6 gene expression, HAMP1 gene expression, and serum iron levels in WT C57BL/6 mice.

FIG. 5 depicts the level of TMPRSS6 siRNA mediated silencing of TMPRSS6 necessary to maintain the TMPRSS6 siRNA mediated effects on HAMP1 gene expression and serum iron levels in WT C57BL/6 mice.

FIGs. 6A and 6B depict the effect of TMPRSS6 siRNA mediated silencing of TMPRSS6 on hematological parameters in WT C57BL/6 mice. FIG. 6A depicts the effect of TMPRSS6 siRNA mediated silencing of TMPRSS6 on hemoglobin (HBG) in WT C57BL/6 mice 6 hours, 24 hours, 48 hours, 72 hours, 7 days, and 14 days post administration. FIG. 6B depicts the effect of TMPRSS6 siRNA mediated silencing of TMPRSS6 on hematocrit in WT C57BL/6 mice 6 hours, 24 hours, 48 hours, 72 hours, 7 days, and 14 days post administration.

FIG. 7 depicts the effect of TMPRSS6 siRNA mediated silencing of TMPRSS6 on serum iron parameters in thalassemic mice (Th3/+), including serum iron levels, unsaturated iron binding capacity (UIBC) levels, and transferrin saturation levels.

FIGs. 8A to 8C depict the effects of TMPRSS6 siRNA mediated silencing of TMPRSS6 on reticulocyte and erythrocyte parameters in thalassemic mice (Th3/+). FIG. 8A depicts the effect on the number of reticulocytes (%), FIG. 8B depicts the effect on the hemoglobin content of reticulocytes (CHr), and FIG. 8C depicts the effect on the number of mature red blood cells.

FIGs. 9A to 9D depicts the effect of TMPRSS6 siRNA mediated silencing of TMPRSS6 on hematological parameters in thalassemic mice (Th3/+). FIG. 9A depicts the effect on hematocrit (HCT) levels, FIG. 9B depicts the effect on hemoglobin (HGB), FIG. 9C depicts the effect on red blood cell (RBC) distribution width (RDW), and FIG. 9D depicts the effect on mean corpuscle value (MCV).

FIGs. 10A to 10C depict the effect of TMPRSS6 siRNA mediated silencing of TMPRSS6 on spleen and liver iron content in thalassemic mice (Th3/+). FIG. 10A depicts the effect on total spleen iron content, FIG. 10B depicts the effect on spleen weight, and FIG. 10C depicts the effect of TMPRSS6 siRNA mediated silencing of TMPRSS6 on the concentration of iron in the liver.

Detailed Description of the Invention

Described herein are iRNAs and methods of using them for inhibiting the expression of a TMPRSS6 gene in a cell or a mammal where the iRNA targets a TMPRSS6 gene. Also provided are compositions and methods for treating pathological conditions and diseases caused by TMPRSS6 gene expression, such as conditions associated with elevated levels of iron. iRNA directs the sequence-specific degradation of mRNA through a process known as RNA interference (RNAi). In an alternative embodiment, the iRNA activates the expression of a TMPRSS6 gene in a cell or mammal, where the iRNA targets a TMPRSS6 gene.

TMPRSS6 plays an important role in iron homeostasis as an inhibitor of HAMP gene expression. The HAMP gene encodes for the liver hormone hepcidin, which is a central regulator of iron homeostasis. Hepcidin binds to the iron exporter protein ferroportin (FPN1), which is localized mainly on absorptive enterocytes, hepatocytes and macrophages. Hepcidin binding to the extracellular domain of ferroportin leads to the internalization and degradation of ferroportin, thus decreasing the absorption of dietary iron from the intestine, and the release of iron from macrophages and hepatocytes. HAMP gene expression can be stimulated in response to iron through Bone Morphogenetic Protein (BMP)/Sons of Mothers Against Decapentaplegic (SMAD)-dependent signal transduction cascade mediated by the BMP-co-receptor hemojuvelin (HJV). The key role of TMPRSS6 in HAMP regulation is in the inhibition of BMP-mediated HAMP upregulation. TMPRSS6 inhibits BMP-mediated HAMP upregulation by cleaving the BMP co-receptor HJV, which is essential for BMP

mediated HAMP upregulation; thus preventing BMP signaling, SMAD translocation to the nucleus, and HAMP transcriptional activation.

Several human and mouse studies have confirmed the role of TMPRSS6 in HAMP regulation and iron homeostasis (Du *et al.* Science 2008, Vol. 320, pp1088-1092; Folgueras *et al.* Blood 2008, Vol. 112, pp2539-45). Studies have shown that loss of function mutations in TMPRSS6 can lead to the upregulation of hepcidin expression, causing an inherited iron deficiency anemia called iron refractory iron deficiency anemia (IRIDA) (Finberg. Seminars in Hematology 2009, Vol. 46, pp378-86), which is characterized by elevated hepcidin levels, hypochromic microcytic anemia, low mean corpuscular volume (MCV), low transferrin saturation, poor absorption of oral iron, and incomplete response to parenteral iron. However, loss of function mutations in positive regulators of HAMP (*e.g.*, BMP1, BMP4, and HFE) have been shown to downregulate hepcidin expression and cause iron overload disorders (Milet *et al.* Am J Hum Gen 2007, Vol. 81, pp799-807; Finberg *et al.* Blood 2011, Vol. 117, pp4590-9). In the primary iron overload disorders, collectively called hereditary hemochromatosis (HH), in anemias characterized by massive ineffective hematopoiesis, and in iron overload (secondary hemochromatosis), such as β-thalassemia intermedia (TI), hepcidin levels are low despite elevated serum iron concentrations and iron stores. A mouse model of β-thalassemia intermedia has demonstrated that the loss of TMPRSS6 expression leads to elevated levels of hepcidin (Finberg 2010 Oral Presentation: "TMPRSS6, an inhibitor of Hepatic BMP/Smad Signaling, is required for Hepcidin Suppression and Iron Loading in a Mouse Model of β-Thalassemia. American Society of Hematology Annual Meeting 2010, Abstract No.:164).

The present invention describes methods and iRNA compositions for modulating the expression of a TMPRSS6 gene. In certain embodiments, expression of TMPRSS6 is reduced or inhibited using a TMPRSS6-specific iRNA, thereby leading to increase HAMP expression, and decreased serum iron levels. Thus, inhibition of TMPRSS6 gene expression or activity using the iRNA compositions featured in the invention can be a useful approach to therapies aimed at reducing the iron levels in a subject. Such inhibition can be useful for treating disorders associated with elevated iron levels, such as hemochromatosis or thalassemia, *e.g.*, β-thalassemia.

The iRNAs of the compositions described herein include an RNA strand (the antisense strand) having a region which is 30 nucleotides or less in length, *i.e.*, 15-30

nucleotides in length, generally 19-24 nucleotides in length, which region is substantially complementary to at least part of an mRNA transcript of a TMPRSS6 gene. The use of these iRNAs enables the targeted degradation of mRNAs of genes that are implicated in pathologies associated with TMPRSS6 expression in mammals. Very low dosages of TMPRSS6 iRNAs in particular can specifically and efficiently mediate RNAi, resulting in significant inhibition of expression of a TMPRSS6 gene. Using cell-based assays, the present inventors have demonstrated that iRNAs targeting TMPRSS6 can specifically and efficiently mediate RNAi, resulting in significant inhibition of expression of a TMPRSS6 gene. Thus, methods and compositions including these iRNAs are useful for treating pathological processes that can be mediated by down regulating TMPRSS6, such as in the treatment of a disorder that causes elevated iron levels, *e.g.*, a hemochromatosis, or a β-thalassemia, *e.g.*, β-thalassemia intermedia. The following detailed description discloses how to make and use compositions containing iRNAs to inhibit the expression of a TMPRSS6 gene, as well as compositions and methods for treating diseases and disorders caused by the expression of this gene.

Embodiments of the pharmaceutical compositions featured herein also include an iRNA having an antisense strand comprising a region which is 30 nucleotides or less in length, generally 19-24 nucleotides in length, which region is substantially complementary to at least part of an RNA transcript of a TMPRSS6 gene, together with a pharmaceutically acceptable carrier. Embodiments of compositions featured in the invention also include an iRNA having an antisense strand having a region of complementarity which is 30 nucleotides or less in length, generally 19-24 nucleotides in length, and is substantially complementary to at least part of an RNA transcript of a TMPRSS6 gene.

Accordingly, in some aspects, pharmaceutical compositions containing a TMPRSS6 iRNA and a pharmaceutically acceptable carrier, methods of using the compositions to inhibit expression of a TMPRSS6 gene, and methods of using the pharmaceutical compositions to treat diseases caused by expression of a TMPRSS6 gene are featured in the invention.

I. Definitions

For convenience, the meaning of certain terms and phrases used in the specification, examples, and appended claims, are provided below. If there is an apparent discrepancy

between the usage of a term in other parts of this specification and its definition provided in this section, the definition in this section shall prevail.

"G," "C," "A," "T" and "U" each generally stand for a nucleotide that contains guanine, cytosine, adenine, thymidine and uracil as a base, respectively. However, it will be understood that the term "ribonucleotide" or "nucleotide" can also refer to a modified nucleotide, as further detailed below, or a surrogate replacement moiety. The skilled person is well aware that guanine, cytosine, adenine, and uracil may be replaced by other moieties without substantially altering the base pairing properties of an oligonucleotide comprising a nucleotide bearing such replacement moiety. For example, without limitation, a nucleotide comprising inosine as its base may base pair with nucleotides containing adenine, cytosine, or uracil. Hence, nucleotides containing uracil, guanine, or adenine can be replaced in the nucleotide sequences of dsRNA featured herein by a nucleotide containing, for example, inosine. In another example, adenine and cytosine anywhere in the oligonucleotide can be replaced with guanine and uracil, respectively to form G-U Wobble base pairing with the target mRNA. Sequences containing such replacement moieties are suitable for the compositions and methods described herein.

As used herein, "Transmembrane Protease, Serine 6" ("TMPSSR6") refers to a particular polypeptide expressed in a cell. TMPRSS6 is also known as matriptase-2, IRIDA (iron refractory iron-deficiency anemia), transmembrane protease serine 6, type II transmembrane serine protease 6, and membrane-bound mosaic serine proteinase matriptase-2. TMPRSS6 is a serine protease Type II transmembrane protein of approximately 899 amino acids in length. TMPRSS6 contains multiple domains, *e.g.*, a short endo domain, a transmembrane domain, a sea urchin sperm protein/enteropeptidase domain/agrin (SEA) domain, two complement factor/urchin embryonic growth factor/BMP domains (CUB), three LDL-R class a domains (LDLa), and a trypsin-like serine protease domain with conserved His-Asp-Ser triad (HDS). The sequence of a human TMPRSS6 mRNA transcript can be found at NM_153609.2 (SEQ ID NO:1) (FIG. 1).

As used herein, the term "iRNA" refers to an agent that contains RNA as that term is defined herein, and which mediates the targeted cleavage of an RNA transcript via an RNA-induced silencing complex (RISC) pathway. In one embodiment, an iRNA as described herein effects inhibition of TMPRSS6 expression. Alternatively, in another embodiment, an iRNA as described herein activates TMPRSS6 expression.

As used herein, "target sequence" refers to a contiguous portion of the nucleotide sequence of an mRNA molecule formed during the transcription of a TMPRSS6 gene, including messenger RNA (mRNA) that is a product of RNA processing of a primary transcription product. The target portion of the sequence will be at least long enough to serve as a substrate for iRNA-directed cleavage at or near that portion. For example, the target sequence will generally be from 9-36 nucleotides in length, *e.g.*, 15-30 nucleotides in length, including all sub-ranges there between. As non-limiting examples, the target sequence can be from 15-30 nucleotides, 15-26 nucleotides, 15-23 nucleotides, 15-22 nucleotides, 15-21 nucleotides, 15-20 nucleotides, 15-19 nucleotides, 15-18 nucleotides, 15-17 nucleotides, 18-30 nucleotides, 18-26 nucleotides, 18-23 nucleotides, 18-22 nucleotides, 18-21 nucleotides, 18-20 nucleotides, 19-30 nucleotides, 19-26 nucleotides, 19-23 nucleotides, 19-22 nucleotides, 19-21 nucleotides, 19-20 nucleotides, 20-30 nucleotides, 20-26 nucleotides, 20-25 nucleotides, 20-24 nucleotides, 20-23 nucleotides, 20-22 nucleotides, 20-21 nucleotides, 21-30 nucleotides, 21-26 nucleotides, 21-25 nucleotides, 21-24 nucleotides, 21-23 nucleotides, or 21-22 nucleotides.

As used herein, the term "strand comprising a sequence" refers to an oligonucleotide comprising a chain of nucleotides that is described by the sequence referred to using the standard nucleotide nomenclature.

As used herein, and unless otherwise indicated, the term "complementary," when used to describe a first nucleotide sequence in relation to a second nucleotide sequence, refers to the ability of an oligonucleotide or polynucleotide comprising the first nucleotide sequence to hybridize and form a duplex structure under certain conditions with an oligonucleotide or polynucleotide comprising the second nucleotide sequence, as will be understood by the skilled person. Such conditions can, for example, be stringent conditions, where stringent conditions may include: 400 mM NaCl, 40 mM PIPES pH 6.4, 1 mM EDTA, 50°C or 70°C for 12-16 hours followed by washing. Other conditions, such as physiologically relevant conditions as can be encountered inside an organism, can apply. The skilled person will be able to determine the set of conditions most appropriate for a test of complementarity of two sequences in accordance with the ultimate application of the hybridized nucleotides.

Complementary sequences within an iRNA, *e.g.*, within a dsRNA as described herein, include base-pairing of the oligonucleotide or polynucleotide comprising a first nucleotide sequence to an oligonucleotide or polynucleotide comprising a second nucleotide sequence

over the entire length of one or both nucleotide sequences. Such sequences can be referred to as "fully complementary" with respect to each other herein. However, where a first sequence is referred to as "substantially complementary" with respect to a second sequence herein, the two sequences can be fully complementary, or they can form one or more, but generally not more than 5, 4, 3 or 2 mismatched base pairs upon hybridization for a duplex up to 30 base pairs (bp), while retaining the ability to hybridize under the conditions most relevant to their ultimate application, *e.g.*, inhibition of gene expression via a RISC pathway. However, where two oligonucleotides are designed to form, upon hybridization, one or more single stranded overhangs, such overhangs shall not be regarded as mismatches with regard to the determination of complementarity. For example, a dsRNA comprising one oligonucleotide 21 nucleotides in length and another oligonucleotide 23 nucleotides in length, wherein the longer oligonucleotide comprises a sequence of 21 nucleotides that is fully complementary to the shorter oligonucleotide, may yet be referred to as "fully complementary" for the purposes described herein.

"Complementary" sequences, as used herein, can also include, or be formed entirely from, non-Watson-Crick base pairs and/or base pairs formed from non-natural and modified nucleotides, in as far as the above requirements with respect to their ability to hybridize are fulfilled. Such non-Watson-Crick base pairs include, but are not limited to, G:U Wobble or Hoogstein base pairing.

The terms "complementary," "fully complementary" and "substantially complementary" herein can be used with respect to the base matching between the sense strand and the antisense strand of a dsRNA, or between the antisense strand of an iRNA agent and a target sequence, as will be understood from the context of their use.

As used herein, a polynucleotide that is "substantially complementary to at least part of" a messenger RNA (an mRNA) refers to a polynucleotide that is substantially complementary to a contiguous portion of the mRNA of interest (*e.g.*, an mRNA encoding TMPRSS6). For example, a polynucleotide is complementary to at least a part of a TMPRSS6 mRNA if the sequence is substantially complementary to a non-interrupted portion of an mRNA encoding TMPRSS6.

The term "double-stranded RNA" or "dsRNA," as used herein, refers to an iRNA that includes an RNA molecule or complex of molecules having a hybridized duplex region that

comprises two anti-parallel and substantially complementary nucleic acid strands, which will be referred to as having "sense" and "antisense" orientations with respect to a target RNA. The duplex region can be of any length that permits specific degradation of a desired target RNA through a RISC pathway, but will typically range from 9 to 36 base pairs in length, *e.g.*, 15-30 base pairs in length. Considering a duplex between 9 and 36 base pairs, the duplex can be any length in this range, for example, 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, or 36 and any sub-range therein between, including, but not limited to 15-30 base pairs, 15-26 base pairs, 15-23 base pairs, 15-22 base pairs, 15-21 base pairs, 15-20 base pairs, 15-19 base pairs, 15-18 base pairs, 15-17 base pairs, 18-30 base pairs, 18-26 base pairs, 18-23 base pairs, 18-22 base pairs, 18-21 base pairs, 18-20 base pairs, 19-30 base pairs, 19-26 base pairs, 19-23 base pairs, 19-22 base pairs, 19-21 base pairs, 19-20 base pairs, 20-30 base pairs, 20-26 base pairs, 20-25 base pairs, 20-24 base pairs, 20-23 base pairs, 20-22 base pairs, 20-21 base pairs, 21-30 base pairs, 21-26 base pairs, 21-25 base pairs, 21-24 base pairs, 21-23 base pairs, or 21-22 base pairs. dsRNAs generated in the cell by processing with Dicer and similar enzymes are generally in the range of 19-22 base pairs in length. One strand of the duplex region of a dsDNA comprises a sequence that is substantially complementary to a region of a target RNA. The two strands forming the duplex structure can be from a single RNA molecule having at least one self-complementary region, or can be formed from two or more separate RNA molecules. Where the duplex region is formed from two strands of a single molecule, the molecule can have a duplex region separated by a single stranded chain of nucleotides (herein referred to as a "hairpin loop") between the 3'-end of one strand and the 5'-end of the respective other strand forming the duplex structure. The hairpin loop can comprise at least one unpaired nucleotide; in some embodiments the hairpin loop can comprise at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, at least 10, at least 20, at least 23 or more unpaired nucleotides. Where the two substantially complementary strands of a dsRNA are comprised by separate RNA molecules, those molecules need not, but can be covalently connected. Where the two strands are connected covalently by means other than a hairpin loop, the connecting structure is referred to as a "linker." The term "siRNA" is also used herein to refer to a dsRNA as described above.

The skilled artisan will recognize that the term "RNA molecule" or "ribonucleic acid molecule" encompasses not only RNA molecules as expressed or found in nature, but also analogs and derivatives of RNA comprising one or more ribonucleotide/ribonucleoside

analogs or derivatives as described herein or as known in the art. Strictly speaking, a "ribonucleoside" includes a nucleoside base and a ribose sugar, and a "ribonucleotide" is a ribonucleoside with one, two or three phosphate moieties. However, the terms "ribonucleoside" and "ribonucleotide" can be considered to be equivalent as used herein. The RNA can be modified in the nucleobase structure or in the ribose-phosphate backbone structure, *e.g.*, as described herein below. However, the molecules comprising ribonucleoside analogs or derivatives must retain the ability to form a duplex. As non-limiting examples, an RNA molecule can also include at least one modified ribonucleoside including but not limited to a 2'-O-methyl modified nucleoside, a nucleoside comprising a 5' phosphorothioate group, a terminal nucleoside linked to a cholesteryl derivative or dodecanoic acid bisdecylamide group, a locked nucleoside, an abasic nucleoside, a 2'-deoxy-2'-fluoro modified nucleoside, a 2'-amino-modified nucleoside, 2'-alkyl-modified nucleoside, morpholino nucleoside, a phosphoramidate or a non-natural base comprising nucleoside, or any combination thereof. Alternatively, an RNA molecule can comprise at least two modified ribonucleosides, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 9, at least 10, at least 15, at least 20 or more, up to the entire length of the dsRNA molecule. The modifications need not be the same for each of such a plurality of modified ribonucleosides in an RNA molecule. In one embodiment, modified RNAs contemplated for use in methods and compositions described herein are peptide nucleic acids (PNAs) that have the ability to form the required duplex structure and that permit or mediate the specific degradation of a target RNA via a RISC pathway.

In one aspect, a modified ribonucleoside includes a deoxyribonucleoside. In such an instance, an iRNA agent can comprise one or more deoxynucleosides, including, for example, a deoxynucleoside overhang(s), or one or more deoxynucleosides within the double stranded portion of a dsRNA. However, it is self evident that under no circumstances is a double stranded DNA molecule encompassed by the term "iRNA."

In one aspect, an RNA interference agent includes a single stranded RNA that interacts with a target RNA sequence to direct the cleavage of the target RNA. Without wishing to be bound by theory, long double stranded RNA introduced into plants and invertebrate cells is broken down into siRNA by a Type III endonuclease known as Dicer (Sharp *et al.*, Genes Dev. 2001, 15:485). Dicer, a ribonuclease-III-like enzyme, processes the dsRNA into 19-23 base pair short interfering RNAs with characteristic two base 3' overhangs

(Bernstein, *et al.*, (2001) Nature 409:363). The siRNAs are then incorporated into an RNA-induced silencing complex (RISC) where one or more helicases unwind the siRNA duplex, enabling the complementary antisense strand to guide target recognition (Nykanen, *et al.*, (2001) Cell 107:309). Upon binding to the appropriate target mRNA, one or more endonucleases within the RISC cleaves the target to induce silencing (Elbashir, *et al.*, (2001) Genes Dev. 15:188). Thus, in one aspect the invention relates to a single stranded RNA that promotes the formation of a RISC complex to effect silencing of the target gene.

As used herein, the term "nucleotide overhang" refers to at least one unpaired nucleotide that protrudes from the duplex structure of an iRNA, *e.g.*, a dsRNA. For example, when a 3'-end of one strand of a dsRNA extends beyond the 5'-end of the other strand, or vice versa, there is a nucleotide overhang. A dsRNA can comprise an overhang of at least one nucleotide; alternatively the overhang can comprise at least two nucleotides, at least three nucleotides, at least four nucleotides, at least five nucleotides or more. A nucleotide overhang can comprise or consist of a nucleotide/nucleoside analog, including a deoxynucleotide/nucleoside. The overhang(s) may be on the sense strand, the antisense strand or any combination thereof. Furthermore, the nucleotide(s) of an overhang can be present on the 5' end, 3' end or both ends of either an antisense or sense strand of a dsRNA.

In one embodiment, the antisense strand of a dsRNA has a 1-10 nucleotide overhang at the 3' end and/or the 5' end. In one embodiment, the sense strand of a dsRNA has a 1-10 nucleotide overhang at the 3' end and/or the 5' end. In another embodiment, one or more of the nucleotides in the overhang is replaced with a nucleoside thiophosphate.

The terms "blunt" or "blunt ended" as used herein in reference to a dsRNA mean that there are no unpaired nucleotides or nucleotide analogs at a given terminal end of a dsRNA, *i.e.*, no nucleotide overhang. One or both ends of a dsRNA can be blunt. Where both ends of a dsRNA are blunt, the dsRNA is said to be blunt ended. To be clear, a "blunt ended" dsRNA is a dsRNA that is blunt at both ends, *i.e.*, no nucleotide overhang at either end of the molecule. Most often such a molecule will be double-stranded over its entire length.

The term "antisense strand" or "guide strand" refers to the strand of an iRNA, *e.g.*, a dsRNA, which includes a region that is substantially complementary to a target sequence. As used herein, the term "region of complementarity" refers to the region on the antisense strand

that is substantially complementary to a sequence, for example a target sequence, as defined herein. Where the region of complementarity is not fully complementary to the target sequence, the mismatches may be in the internal or terminal regions of the molecule. Generally, the most tolerated mismatches are in the terminal regions, *e.g.*, within 5, 4, 3, or 2 nucleotides of the 5' and/or 3' terminus.

The term "sense strand," or "passenger strand" as used herein, refers to the strand of an iRNA that includes a region that is substantially complementary to a region of the antisense strand as that term is defined herein.

As used herein, in one embodiment, the term "SNALP" refers to a stable nucleic acid-lipid particle. A SNALP represents a vesicle of lipids coating a reduced aqueous interior comprising a nucleic acid such as an iRNA or a plasmid from which an iRNA is transcribed. SNALPs are described, *e.g.*, in U.S. Patent Application Publication Nos. 20060240093, 20070135372, and in International Application No. WO 2009082817. Examples of "SNALP" formulations are described elsewhere herein.

"Introducing into a cell," when referring to an iRNA, means facilitating or effecting uptake or absorption into the cell, as is understood by those skilled in the art. Absorption or uptake of an iRNA can occur through unaided diffusive or active cellular processes, or by auxiliary agents or devices. The meaning of this term is not limited to cells *in vitro*; an iRNA can also be "introduced into a cell," wherein the cell is part of a living organism. In such an instance, introduction into the cell will include the delivery to the organism. For example, for *in vivo* delivery, iRNA can be injected into a tissue site or administered systemically. *In vivo* delivery can also be by a β-glucan delivery system, such as those described in U.S. Patent Nos. 5,032,401 and 5,607,677, and U.S. Publication No. 2005/0281781 which are hereby incorporated by reference in their entirety. *In vitro* introduction into a cell includes methods known in the art such as electroporation and lipofection. Further approaches are described herein below or are known in the art.

As used herein, the term "modulate the expression of," refers to at an least partial "inhibition" or partial "activation" of TMPRSS6 gene expression in a cell treated with an iRNA composition as described herein compared to the expression of TMPRSS6 in an untreated cell.

The terms "activate," "enhance," "up-regulate the expression of," "increase the expression of," and the like, in so far as they refer to a TMPRSS6 gene, herein refer to the at least partial activation of the expression of a TMPRSS6 gene, as manifested by an increase in the amount of TMPRSS6 mRNA, which can be isolated from or detected in a first cell or group of cells in which a TMPRSS6 gene is transcribed and which has or have been treated such that the expression of a TMPRSS6 gene is increased, as compared to a second cell or group of cells substantially identical to the first cell or group of cells but which has or have not been so treated (control cells).

In one embodiment, expression of a TMPRSS6 gene is activated by at least about 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, or 50% by administration of an iRNA as described herein. In some embodiments, a TMPRSS6 gene is activated by at least about 60%, 70%, or 80% by administration of an iRNA featured in the invention. In some embodiments, expression of a TMPRSS6 gene is activated by at least about 85%, 90%, or 95% or more by administration of an iRNA as described herein. In some embodiments, the TMPRSS6 gene expression is increased by at least 1-fold, at least 2-fold, at least 5-fold, at least 10-fold, at least 50-fold, at least 100-fold, at least 500-fold, at least 1000 fold or more in cells treated with an iRNA as described herein compared to the expression in an untreated cell. Activation of expression by small dsRNAs is described, for example, in Li *et al.*, 2006 *Proc. Natl. Acad. Sci. U.S.A.* 103:17337-42, and in US20070111963 and US2005226848, each of which is incorporated herein by reference.

The terms "silence," "inhibit the expression of," "down-regulate the expression of," "suppress the expression of," and the like, in so far as they refer to a TMPRSS6 gene, herein refer to the at least partial suppression of the expression of a TMPRSS6 gene, as manifested by a reduction of the amount of TMPRSS6 mRNA which can be isolated from or detected in a first cell or group of cells in which a TMPRSS6 gene is transcribed and which has or have been treated such that the expression of a TMPRSS6 gene is inhibited, as compared to a second cell or group of cells substantially identical to the first cell or group of cells but which has or have not been so treated (control cells). The degree of inhibition is usually expressed in terms of

$$\frac{(\text{mRNA in control cells}) - (\text{mRNA in treated cells})}{(\text{mRNA in control cells})} \bullet 100\%$$

Alternatively, the degree of inhibition can be given in terms of a reduction of a parameter that is functionally linked to TMPRSS6 gene expression, *e.g.*, the amount of protein encoded by a TMPRSS6 gene, or the number of cells displaying a certain phenotype, *e.g.*, a decrease in iron levels, or in iron absorption. In principle, TMPRSS6 gene silencing can be determined in any cell expressing TMPRSS6, either constitutively or by genomic engineering, and by any appropriate assay.

For example, in certain instances, expression of a TMPRSS6 gene is suppressed by at least about 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, or 50% by administration of an iRNA featured in the invention. In some embodiments, a TMPRSS6 gene is suppressed by at least about 60%, 70%, or 80% by administration of an iRNA described herein. In some embodiments, a TMPRSS6 gene is suppressed by at least about 85%, 90%, 95%, 98%, 99%, or more, by administration of an iRNA as described herein.

As used herein in the context of TMPRSS6 expression, the terms "treat," "treatment," and the like, refer to relief from or alleviation of pathological processes mediated by TMPRSS6 expression. In the context of the present invention insofar as it relates to any of the other conditions recited herein below (other than pathological processes mediated by TMPRSS6 expression), the terms "treat," "treatment," and the like mean to relieve or alleviate at least one symptom associated with such condition, or to slow or reverse the progression or anticipated progression of such condition, such as slowing the progression of a hemochromatosis, such as a β-thalassemia.

By "lower" in the context of a disease marker or symptom is meant a statistically significant decrease in such level. The decrease can be, for example, at least 10%, at least 20%, at least 30%, at least 40% or more, and is preferably down to a level accepted as within the range of normal for an individual without such disorder.

As used herein, the phrases "therapeutically effective amount" and "prophylactically effective amount" refer to an amount that provides a therapeutic benefit in the treatment, prevention, or management of pathological processes mediated by TMPRSS6 expression or an overt symptom of pathological processes mediated by TMPRSS6 expression. The specific amount that is therapeutically effective can be readily determined by an ordinary medical practitioner, and can vary depending on factors known in the art, such as, for example, the type of pathological processes mediated by TMPRSS6 expression, the patient's history and

age, the stage of pathological processes mediated by TMPRSS6 expression, and the administration of other agents that inhibit pathological processes mediated by TMPRSS6 expression.

As used herein, a "pharmaceutical composition" comprises a pharmacologically effective amount of an iRNA and a pharmaceutically acceptable carrier. As used herein, "pharmacologically effective amount," "therapeutically effective amount" or simply "effective amount" refers to that amount of an iRNA effective to produce the intended pharmacological, therapeutic or preventive result. For example, if a given clinical treatment is considered effective when there is at least a 10% reduction in a measurable parameter associated with a disease or disorder, a therapeutically effective amount of a drug for the treatment of that disease or disorder is the amount necessary to effect at least a 10% reduction in that parameter. For example, a therapeutically effective amount of an iRNA targeting TMPRSS6 can reduce TMPRSS6 protein levels by at least 10%.

As used herein, the term "thalassemia" refers to an inherited recessive blood disorder. A loss-of-function mutation results in reduced rate of synthesis or no synthesis of one of the globin chains that makes up hemoglobin, and causes a deficiency in normal globin proteins. Thalassemia patients produce a deficiency of either α globin (called α-thalassemia), β globin (called β-thalassemia) or, in rare cases, δ globin. In α-thalassemia, an excess of β chains form unstable tetramers, which have abnormal oxygen dissociation curves. β-thalassemias can be minor, major or intermedia.

β globin chains are encoded by a single gene called the HBB (hemoglobin, β) gene. β-thalassemia minor occurs in patients carrying one mutant β-thalassemia allele, and one wildtype allele. This condition has no effect on blood iron levels, and patients do not require treatment. β-thalassemia major results when a patient carries two knock-out mutant β-thalassemia alleles. Excess iron accumulates in these patients, and the excess iron is stored primarily in hypertrophic Kupffer cells. Patients with β-thalassemia major are typically treated with chronic blood transfusion therapy, iron chelation, splenectomy and allogeneic hematopoietic transplantation. β-thalassemia intermedia results when a patient carries one knock-out allele of the β-thalassemia gene and one partial loss-of-function allele. Excess iron accumulates in these patients, and the excess iron is stored primarily in hepatocytes. Patients with thalassemia major and thalassemia intermedia have anemia (hypoxia), which leads to an increase in EPO (erythropoietin) and consequently, dramatic compensatory and ineffective

erythropoiesis (the production of red blood cells by stem cells in bone marrow). Patients with thalassemia intermedia sometimes develop hepatosplenomegaly, jaundice, osteopenia, thrombotic events, leg ulcers, pulmonary hypotension, congestive heart failure, diabetes mellitus, growth hormone deficiency, hypothyroidism, hypoparathyroidism, hypogonadism, and facial deformities.

As used herein, the term "hemochromatosis" refers to a disorder, which results in too much iron being absorbed from the gastrointestinal tract. Hemochromatosis occurs in two forms: primary and secondary. Primary hemochromatosis, the most common genetic disorder in the United States (affecting an estimated 1 of every 200 to 300 Americans), is usually caused by a specific genetic problem that causes too much iron to be absorbed. Secondary, or acquired, hemochromatosis, can be caused by diseases such as thalassemia or sideroblastic anemia. Secondary hemochromatosis sometimes develops in patients with hemolytic anemia and chronic alcoholism. Symptoms of hemochromatosis include abdominal pain, joint pain, fatigue, lack of energy, weakness, darkening of the skin (often referred to as "bronzing"), and loss of body hair.

The term "pharmaceutically acceptable carrier" refers to a carrier for administration of a therapeutic agent. Such carriers include, but are not limited to, saline, buffered saline, dextrose, water, glycerol, ethanol, and combinations thereof. The term specifically excludes cell culture medium. For drugs administered orally, pharmaceutically acceptable carriers include, but are not limited to pharmaceutically acceptable excipients such as inert diluents, disintegrating agents, binding agents, lubricating agents, sweetening agents, flavoring agents, coloring agents and preservatives. Suitable inert diluents include sodium and calcium carbonate, sodium and calcium phosphate, and lactose, while corn starch and alginic acid are suitable disintegrating agents. Binding agents may include starch and gelatin, while the lubricating agent, if present, will generally be magnesium stearate, stearic acid or talc. If desired, the tablets may be coated with a material such as glyceryl monostearate or glyceryl distearate, to delay absorption in the gastrointestinal tract. Agents included in drug formulations are described further herein below.

As used herein, a "subject" is a mammal, *e.g.* a dog, horse, cat, and other non-human primates. In a preferred embodiment, a subject is a human.

As used herein, the term "LNPXX", wherein the "XX" are numerals, is also referred to as "AFXX" herein. For example, LNP09 is also referred to AF09 and LNP12 is also known as or referred to as AF12.

As used herein, the term "comprising" or "comprises" is used in reference to compositions, methods, and respective component(s) thereof, that are essential to the invention, yet open to the inclusion of unspecified elements, whether essential or not.

As used herein, the term "consisting essentially of" refers to those elements required for a given embodiment. The term permits the presence of elements that do not materially affect the basic and novel or functional characteristic(s) of that embodiment featured in the invention.

The term "consisting of" refers to compositions, methods, and respective components thereof as described herein, which are exclusive of any element not recited in that description of the embodiment.

II. Double-stranded ribonucleic acid (dsRNA)

Described herein are iRNA agents that modulate the expression of the TMPRSS6 gene. In one embodiment, the iRNA agent includes double-stranded ribonucleic acid (dsRNA) molecules for inhibiting the expression of a TMPRSS6 gene in a cell or mammal, *e.g.*, in a human having elevated iron levels, such as in a patient with a β-thalassemia, or a hemachromatosis. The dsRNA includes an antisense strand having a region of complementarity which is complementary to at least a part of an mRNA formed in the expression of a TMPRSS6 gene. The region of complementarity is 30 nucleotides or less in length, generally 19-24 nucleotides in length, and where the dsRNA, upon contact with a cell expressing the TMPRSS6 gene, inhibits the expression of the TMPRSS6 gene by at least 10% as assayed by, for example, a PCR or branched DNA (bDNA)-based method, or by a protein-based method, such as by Western blot. In one embodiment, the iRNA agent activates the expression of a TMPRSS6 gene in a cell or mammal. Expression of a TMPRSS6 gene in cell culture, such as in COS cells, HeLa cells, primary hepatocytes, HepG2 cells, primary cultured cells or in a biological sample from a subject, can be assayed by measuring TMPRSS6 mRNA levels, such as by bDNA or TaqMan® assay, or by measuring protein levels, such as by immunofluorescence analysis, using, for example, Western blotting or flow cytometric techniques.

A dsRNA includes two RNA strands that are complementary to hybridize to form a duplex structure under conditions in which the dsRNA will be used. One strand of a dsRNA (the antisense strand) includes a region of complementarity that is substantially complementary, and generally fully complementary, to a target sequence. The target sequence can be derived from the sequence of an mRNA formed during the expression of a TMPRSS6 gene. The other strand (the sense strand) includes a region that is complementary to the antisense strand, such that the two strands hybridize and form a duplex structure when combined under suitable conditions. Generally, the duplex structure is between 15 and 30 inclusive, more generally between 18 and 25 inclusive, yet more generally between 19 and 24 inclusive, and most generally between 19 and 21 base pairs in length, inclusive. Similarly, the region of complementarity to the target sequence is between 15 and 30 inclusive, more generally between 18 and 25 inclusive, yet more generally between 19 and 24 inclusive, and most generally between 19 and 21 nucleotides in length, inclusive. In some embodiments, the dsRNA is between 15 and 20 nucleotides in length, inclusive, and in other embodiments, the dsRNA is between 25 and 30 nucleotides in length, inclusive. As the ordinarily skilled person will recognize, the targeted region of an RNA targeted for cleavage will most often be part of a larger RNA molecule, often an mRNA molecule. Where relevant, a "part" of an mRNA target is a contiguous sequence of an mRNA target of sufficient length to be a substrate for RNAi-directed cleavage (*i.e.*, cleavage through a RISC pathway). dsRNAs having duplexes as short as 9 base pairs can, under some circumstances, mediate RNAi-directed RNA cleavage. Most often a target will be at least 15 nucleotides in length, preferably 15-30 nucleotides in length.

One of skill in the art will also recognize that the duplex region is a primary functional portion of a dsRNA, *e.g.*, a duplex region of 9 to 36, *e.g.*, 15-30 base pairs. Thus, in one embodiment, to the extent that it becomes processed to a functional duplex of, *e.g.*, 15-30 base pairs that targets a desired RNA for cleavage, an RNA molecule or complex of RNA molecules having a duplex region greater than 30 base pairs is a dsRNA. Thus, an ordinarily skilled artisan will recognize that in one embodiment, an miRNA is a dsRNA. In another embodiment, a dsRNA is not a naturally occurring miRNA. In another embodiment, an iRNA agent useful to target TMPRSS6 expression is not generated in the target cell by cleavage of a larger dsRNA.

A dsRNA as described herein can further include one or more single-stranded nucleotide overhangs. The dsRNA can be synthesized by standard methods known in the art as further discussed below, *e.g.*, by use of an automated DNA synthesizer, such as are commercially available from, for example, Biosearch, Applied Biosystems, Inc. In one embodiment, a TMPRSS6 gene is a human TMPRSS6 gene. In another embodiment the TMPRSS6 gene is a mouse or a rat TMPRSS6 gene. The sequence of mouse TMPRSS6 mRNA can be found at GenBank Accession No. NM_027902 (GI:125656151, Record dated December 28, 2010). The sequence of rat TMPRSS6 mRNA can be found at GenBank Accession No. NM_001130556.1 (GI:194474097, Record dated January 17, 2011). In specific embodiments, the first sequence is a sense strand of a dsRNA that includes a sense sequence of one of Tables 2, 3 or 4 and the second sequence is an antisense strand of a dsRNA that includes an antisense sequence of one of Tables 2, 3 or 4. Alternative dsRNA agents that target elsewhere in the target sequence provided in Tables 2, 3 or 4 can readily be determined using the target sequence and the flanking TMPRSS6 sequence.

In one aspect, a dsRNA will include at least two nucleotide sequences, a sense and an antisense sequence, whereby the sense strand is selected from the groups of sequences provided in Tables 2, 3 or 4. In this aspect, one of the two sequences is complementary to the other of the two sequences, with one of the sequences being substantially complementary to a sequence of an mRNA generated in the expression of a TMPRSS6 gene. As such, in this aspect, a dsRNA will include two oligonucleotides, where one oligonucleotide is described as the sense strand in Tables 2, 3 or 4 and the second oligonucleotide is described as the corresponding antisense strand of the sense strand from Tables 2, 3 or 4. As described elsewhere herein and as known in the art, the complementary sequences of a dsRNA can also be contained as self-complementary regions of a single nucleic acid molecule, as opposed to being on separate oligonucleotides.

The skilled person is well aware that dsRNAs having a duplex structure of between 20 and 23, but specifically 21, base pairs have been hailed as particularly effective in inducing RNA interference (Elbashir *et al.*, EMBO 2001, 20:6877-6888). However, others have found that shorter or longer RNA duplex structures can be effective as well. In the embodiments described above, by virtue of the nature of the oligonucleotide sequences provided in Tables 2, 3 or 4 dsRNAs described herein can include at least one strand of a length of minimally 21 nt. It can be reasonably expected that shorter duplexes having one of the sequences of Tables

2, 3 or 4 minus only a few nucleotides on one or both ends may be similarly effective as compared to the dsRNAs described above. Hence, dsRNAs having a partial sequence of at least 15, 16, 17, 18, 19, 20, or more contiguous nucleotides from one of the sequences of Tables 2, 3 or 4 and differing in their ability to inhibit the expression of a TMPRSS6 gene by not more than 5, 10, 15, 20, 25, or 30 % inhibition from a dsRNA comprising the full sequence, are contemplated according to the invention.

In addition, the RNAs provided in Tables 2, 3 or 4 identify a site in a TMPRSS6 transcript that is susceptible to RISC-mediated cleavage. As such, the present invention further features iRNAs that target within one of such sequences. As used herein, an iRNA is said to target within a particular site of an RNA transcript if the iRNA promotes cleavage of the transcript anywhere within that particular site. Such an iRNA will generally include at least 15 contiguous nucleotides from one of the sequences provided in Tables 2, 3 or 4 coupled to additional nucleotide sequences taken from the region contiguous to the selected sequence in a TMPRSS6 gene.

While a target sequence is generally 15-30 nucleotides in length, there is wide variation in the suitability of particular sequences in this range for directing cleavage of any given target RNA. Various software packages and the guidelines set out herein provide guidance for the identification of optimal target sequences for any given gene target, but an empirical approach can also be taken in which a "window" or "mask" of a given size (as a non-limiting example, 21 nucleotides) is literally or figuratively (including, *e.g.*, in silico) placed on the target RNA sequence to identify sequences in the size range that may serve as target sequences. By moving the sequence "window" progressively one nucleotide upstream or downstream of an initial target sequence location, the next potential target sequence can be identified, until the complete set of possible sequences is identified for any given target size selected. This process, coupled with systematic synthesis and testing of the identified sequences (using assays as described herein or as known in the art) to identify those sequences that perform optimally can identify those RNA sequences that, when targeted with an iRNA agent, mediate the best inhibition of target gene expression. Thus, while the sequences identified, for example, in Tables 2, 3 or 4 represent effective target sequences, it is contemplated that further optimization of inhibition efficiency can be achieved by progressively "walking the window" one nucleotide upstream or downstream of the given sequences to identify sequences with equal or better inhibition characteristics.

Further, it is contemplated that for any sequence identified, *e.g.*, in Tables 2, 3 or 4 further optimization could be achieved by systematically either adding or removing nucleotides to generate longer or shorter sequences and testing those sequences generated by walking a window of the longer or shorter size up or down the target RNA from that point. Again, coupling this approach to generating new candidate targets with testing for effectiveness of iRNAs based on those target sequences in an inhibition assay as known in the art or as described herein can lead to further improvements in the efficiency of inhibition. Further still, such optimized sequences can be adjusted by, *e.g.*, the introduction of modified nucleotides as described herein or as known in the art, addition or changes in overhang, or other modifications as known in the art and/or discussed herein to further optimize the molecule (*e.g.*, increasing serum stability or circulating half-life, increasing thermal stability, enhancing transmembrane delivery, targeting to a particular location or cell type, increasing interaction with silencing pathway enzymes, increasing release from endosomes, *etc.*) as an expression inhibitor.

An iRNA as described herein can contain one or more mismatches to the target sequence. In one embodiment, an iRNA as described herein contains no more than 3 mismatches. If the antisense strand of the iRNA contains mismatches to a target sequence, it is preferable that the area of mismatch not be located in the center of the region of complementarity. If the antisense strand of the iRNA contains mismatches to the target sequence, it is preferable that the mismatch be restricted to be within the last 5 nucleotides from either the 5' or 3' end of the region of complementarity. For example, for a 23 nucleotide iRNA agent RNA strand which is complementary to a region of a TMPRSS6 gene, the RNA strand generally does not contain any mismatch within the central 13 nucleotides. The methods described herein or methods known in the art can be used to determine whether an iRNA containing a mismatch to a target sequence is effective in inhibiting the expression of a TMPRSS6 gene. Consideration of the efficacy of iRNAs with mismatches in inhibiting expression of a TMPRSS6 gene is important, especially if the particular region of complementarity in a TMPRSS6 gene is known to have polymorphic sequence variation within the population.

In one embodiment, at least one end of a dsRNA has a single-stranded nucleotide overhang of 1 to 4, generally 1 or 2 nucleotides. Such dsRNAs having at least one nucleotide overhang have unexpectedly superior inhibitory properties relative to their blunt-ended

counterparts. In yet another embodiment, the RNA of an iRNA, *e.g.*, a dsRNA, is chemically modified to enhance stability or other beneficial characteristics. The nucleic acids featured in the invention may be synthesized and/or modified by methods well established in the art, such as those described in "Current protocols in nucleic acid chemistry," Beaucage, S.L. *et al.* (Edrs.), John Wiley & Sons, Inc., New York, NY, USA, which is hereby incorporated herein by reference. Modifications include, for example, (a) end modifications, *e.g.*, 5' end modifications (phosphorylation, conjugation, inverted linkages, *etc.*) 3' end modifications (conjugation, DNA nucleotides, inverted linkages, *etc.*), (b) base modifications, *e.g.*, replacement with stabilizing bases, destabilizing bases, or bases that base pair with an expanded repertoire of partners, removal of bases (abasic nucleotides), or conjugated bases, (c) sugar modifications (*e.g.*, at the 2' position or 4' position) or replacement of the sugar, as well as (d) backbone modifications, including modification or replacement of the phosphodiester linkages. Specific examples of RNA compounds useful in the embodiments described herein include, but are not limited to, RNAs containing modified backbones or no natural internucleoside linkages. RNAs having modified backbones include, among others, those that do not have a phosphorus atom in the backbone. For the purposes of this specification, and as sometimes referenced in the art, modified RNAs that do not have a phosphorus atom in their internucleoside backbone can also be considered to be oligonucleosides. In particular embodiments, the modified RNA will have a phosphorus atom in its internucleoside backbone.

Modified RNA backbones include, for example, phosphorothioates, chiral phosphorothioates, phosphorodithioates, phosphotriesters, aminoalkylphosphotriesters, methyl and other alkyl phosphonates including 3'-alkylene phosphonates and chiral phosphonates, phosphinates, phosphoramidates including 3'-amino phosphoramidate and aminoalkylphosphoramidates, thionophosphoramidates, thionoalkylphosphonates, thionoalkylphosphotriesters, and boranophosphates having normal 3'-5' linkages, 2'-5' linked analogs of these, and those) having inverted polarity wherein the adjacent pairs of nucleoside units are linked 3'-5' to 5'-3' or 2'-5' to 5'-2'. Various salts, mixed salts and free acid forms are also included.

Representative U.S. patents that teach the preparation of the above phosphorus-containing linkages include, but are not limited to, U.S. Pat. Nos. 3,687,808; 4,469,863; 4,476,301; 5,023,243; 5,177,195; 5,188,897; 5,264,423; 5,276,019; 5,278,302;

5,286,717; 5,321,131; 5,399,676; 5,405,939; 5,453,496; 5,455,233; 5,466,677; 5,476,925; 5,519,126; 5,536,821; 5,541,316; 5,550,111; 5,563,253; 5,571,799; 5,587,361; 5,625,050; 6,028,188; 6,124,445; 6,160,109; 6,169,170; 6,172,209; 6, 239,265; 6,277,603; 6,326,199; 6,346,614; 6,444,423; 6,531,590; 6,534,639; 6,608,035; 6,683,167; 6,858,715; 6,867,294; 6,878,805; 7,015,315; 7,041,816; 7,273,933; 7,321,029; and U.S. Pat. RE39464, each of which is herein incorporated by reference.

Modified RNA backbones that do not include a phosphorus atom therein have backbones that are formed by short chain alkyl or cycloalkyl internucleoside linkages, mixed heteroatoms and alkyl or cycloalkyl internucleoside linkages, or one or more short chain heteroatomic or heterocyclic internucleoside linkages. These include those having morpholino linkages (formed in part from the sugar portion of a nucleoside); siloxane backbones; sulfide, sulfoxide and sulfone backbones; formacetyl and thioformacetyl backbones; methylene formacetyl and thioformacetyl backbones; alkene containing backbones; sulfamate backbones; methyleneimino and methylenehydrazino backbones; sulfonate and sulfonamide backbones; amide backbones; and others having mixed N, O, S and CH_2 component parts.

Representative U.S. patents that teach the preparation of the above oligonucleosides include, but are not limited to, U.S. Pat. Nos. 5,034,506; 5,166,315; 5,185,444; 5,214,134; 5,216,141; 5,235,033; 5,64,562; 5,264,564; 5,405,938; 5,434,257; 5,466,677; 5,470,967; 5,489,677; 5,541,307; 5,561,225; 5,596,086; 5,602,240; 5,608,046; 5,610,289; 5,618,704; 5,623,070; 5,663,312; 5,633,360; 5,677,437; and, 5,677,439, each of which is herein incorporated by reference.

In other RNA mimetics suitable or contemplated for use in iRNAs, both the sugar and the internucleoside linkage, *i.e.*, the backbone, of the nucleotide units are replaced with novel groups. The base units are maintained for hybridization with an appropriate nucleic acid target compound. One such oligomeric compound, an RNA mimetic that has been shown to have excellent hybridization properties, is referred to as a peptide nucleic acid (PNA). In PNA compounds, the sugar backbone of an RNA is replaced with an amide containing backbone, in particular an aminoethylglycine backbone. The nucleobases are retained and are bound directly or indirectly to aza nitrogen atoms of the amide portion of the backbone. Representative U.S. patents that teach the preparation of PNA compounds include, but are not

limited to, U.S. Pat. Nos. 5,539,082; 5,714,331; and 5,719,262, each of which is herein incorporated by reference. Further teaching of PNA compounds can be found, for example, in Nielsen *et al.*, Science, 1991, 254, 1497-1500.

Some embodiments featured in the invention include RNAs with phosphorothioate backbones and oligonucleosides with heteroatom backbones, and in particular --CH_2--NH--CH_2--, --CH_2--N(CH_3)--O--CH_2--[known as a methylene (methylimino) or MMI backbone], --CH_2--O--N(CH_3)--CH_2--, --CH_2--N(CH_3)--N(CH_3)--CH_2-- and --N(CH_3)--CH_2--CH_2--[wherein the native phosphodiester backbone is represented as --O--P--O--CH_2--] of the above-referenced U.S. Pat. No. 5,489,677, and the amide backbones of the above-referenced U.S. Pat. No. 5,602,240. In some embodiments, the RNAs featured herein have morpholino backbone structures of the above-referenced U.S. Pat. No. 5,034,506.

Modified RNAs can also contain one or more substituted sugar moieties. The iRNAs, *e.g.*, dsRNAs, featured herein can include one of the following at the 2' position: OH; F; O-, S-, or N-alkyl; O-, S-, or N-alkenyl; O-, S- or N-alkynyl; or O-alkyl-O-alkyl, wherein the alkyl, alkenyl and alkynyl may be substituted or unsubstituted C_1 to C_{10} alkyl or C_2 to C_{10} alkenyl and alkynyl. Exemplary suitable modifications include O[$(CH_2)_nO$] $_mCH_3$, O$(CH_2)._nOCH_3$, O$(CH_2)_nNH_2$, O(CH_2) $_nCH_3$, O$(CH_2)_nONH_2$, and O$(CH_2)_nON[(CH_2)_nCH_3)]_2$, where n and m are from 1 to about 10. In other embodiments, dsRNAs include one of the following at the 2' position: C_1 to C_{10} lower alkyl, substituted lower alkyl, alkaryl, aralkyl, O-alkaryl or O-aralkyl, SH, SCH_3, OCN, Cl, Br, CN, 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 the pharmacokinetic properties of an iRNA, or a group for improving the pharmacodynamic properties of an iRNA, and other substituents having similar properties. In some embodiments, the modification includes a 2'-methoxyethoxy (2'-O--$CH_2CH_2OCH_3$, also known as 2'-O-(2-methoxyethyl) or 2'-MOE) (Martin *et al.*, *Helv. Chim. Acta*, 1995, 78:486-504) *i.e.*, an alkoxy-alkoxy group. Another exemplary modification is 2'-dimethylaminooxyethoxy, *i.e.*, a O$(CH_2)_2ON(CH_3)_2$ group, also known as 2'-DMAOE, as described in examples herein below, and 2'-dimethylaminoethoxyethoxy (also known in the art as 2'-O-dimethylaminoethoxyethyl or 2'-DMAEOE), *i.e.*, 2'-O--CH_2--O--CH_2--N$(CH_2)_2$, also described in examples herein below.

Other modifications include 2'-methoxy (2'-OCH_3), 2'-aminopropoxy (2'-$OCH_2CH_2CH_2NH_2$) and 2'-fluoro (2'-F). Similar modifications can also be made at other positions on the RNA of an iRNA, particularly the 3' position of the sugar on the 3' terminal nucleotide or in 2'-5' linked dsRNAs and the 5' position of 5' terminal nucleotide. iRNAs may also have sugar mimetics such as cyclobutyl moieties in place of the pentofuranosyl sugar. Representative U.S. patents that teach the preparation of such modified sugar structures include, but are not limited to, U.S. Pat. Nos. 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; 5,658,873; 5,670,633; and 5,700,920, certain of which are commonly owned with the instant application, and each of which is herein incorporated by reference.

An iRNA can also include nucleobase (often referred to in the art simply as "base") modifications or substitutions. As used herein, "unmodified" or "natural" nucleobases include the purine bases adenine (A) and guanine (G), and the pyrimidine bases thymine (T), cytosine (C) and uracil (U). Modified nucleobases include other synthetic and natural nucleobases such as 5-methylcytosine (5-me-C), 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 uracil and cytosine, 6-azo uracil, cytosine and thymine, 5-uracil (pseudouracil), 4-thiouracil, 8-halo, 8-amino, 8-thiol, 8-thioalkyl, 8-hydroxyl anal 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, 8-azaguanine and 8-azaadenine, 7-deazaguanine and 7-daazaadenine and 3-deazaguanine and 3-deazaadenine. Further nucleobases include those disclosed in U.S. Pat. No. 3,687,808, those disclosed in Modified Nucleosides in Biochemistry, Biotechnology and Medicine, Herdewijn, P. ed. Wiley-VCH, 2008; those disclosed in The Concise Encyclopedia Of Polymer Science And Engineering, pages 858-859, Kroschwitz, J. L, ed. John Wiley & Sons, 1990, these disclosed by Englisch *et al.*, Angewandte Chemie, International Edition, 1991, 30, 613, and those disclosed by Sanghvi, Y S., Chapter 15, dsRNA Research and Applications, pages 289-302, Crooke, S. T. and Lebleu, B., Ed., CRC Press, 1993. Certain of these nucleobases are particularly useful for increasing the binding affinity of the oligomeric compounds featured in the invention. These include 5-substituted pyrimidines, 6-azapyrimidines and N-2, N-6 and 0-6 substituted purines, including 2-aminopropyladenine,

5-propynyluracil and 5-propynylcytosine. 5-methylcytosine substitutions have been shown to increase nucleic acid duplex stability by 0.6-1.2°C (Sanghvi, Y. S., Crooke, S. T. and Lebleu, B., Eds., dsRNA Research and Applications, CRC Press, Boca Raton, 1993, pp. 276-278) and are exemplary base substitutions, even more particularly when combined with 2'-O-methoxyethyl sugar modifications.

Representative U.S. patents that teach the preparation of certain of the above noted modified nucleobases as well as other modified nucleobases include, but are not limited to, the above noted U.S. Pat. No. 3,687,808, as well as U.S. Pat. Nos. 4,845,205; 5,130,30; 5,134,066; 5,175,273; 5,367,066; 5,432,272; 5,457,187; 5,459,255; 5,484,908; 5,502,177; 5,525,711; 5,552,540; 5,587,469; 5,594,121, 5,596,091; 5,614,617; 5,681,941; 6,015,886; 6,147,200; 6,166,197; 6,222,025; 6,235,887; 6,380,368; 6,528,640; 6,639,062; 6,617,438; 7,045,610; 7,427,672; and 7,495,088, each of which is herein incorporated by reference, and U.S. Pat. No. 5,750,692, also herein incorporated by reference.

The RNA of an iRNA can also be modified to include one or more locked nucleic acids (LNA). A locked nucleic acid is a nucleotide having a modified ribose moiety in which the ribose moiety comprises an extra bridge connecting the 2' and 4' carbons. This structure effectively "locks" the ribose in the 3'-endo structural conformation. The addition of locked nucleic acids to siRNAs has been shown to increase siRNA stability in serum, and to reduce off-target effects (Elmen, J. *et al.*, (2005) *Nucleic Acids Research* 33(1):439-447; Mook, OR. *et al.*, (2007) *Mol Canc Ther* 6(3):833-843; Grunweller, A. *et al.*, (2003) *Nucleic Acids Research* 31(12):3185-3193).

Representative U.S. Patents that teach the preparation of locked nucleic acid nucleotides include, but are not limited to, the following: U.S. Pat. Nos. 6,268,490; 6,670,461; 6,794,499; 6,998,484; 7,053,207; 7,084,125; and 7,399,845, each of which is herein incorporated by reference in its entirety.

Another modification of the RNA of an iRNA featured in the invention involves chemically linking to the RNA one or more ligands, moieties or conjugates that enhance the activity, cellular distribution, pharmacokinetic properties, or cellular uptake of the iRNA. Such moieties include but are not limited to lipid moieties such as a cholesterol moiety (Letsinger *et al.*, Proc. Natl. Acid. Sci. USA, 1989, 86: 6553-6556), cholic acid (Manoharan

et al., Biorg. Med. Chem. Let., 1994, 4:1053-1060), a thioether, *e.g.*, beryl-S-tritylthiol (Manoharan *et al.*, Ann. N.Y. Acad. Sci., 1992, 660:306-309; Manoharan *et al.*, Biorg. Med. Chem. Let., 1993, 3:2765-2770), a thiocholesterol (Oberhauser *et al.*, Nucl. Acids Res., 1992, 20:533-538), an aliphatic chain, *e.g.*, dodecandiol 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-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-carbonyloxycholesterol moiety (Crooke *et al.*, J. Pharmacol. Exp. Ther., 1996, 277:923-937).

In one embodiment, a ligand alters the distribution, targeting or lifetime of an iRNA agent into which it is incorporated. In preferred embodiments a ligand provides an enhanced affinity for a selected target, *e.g.*, molecule, cell or cell type (*e.g.*, a liver cell, such as a hepatocyte), compartment, *e.g.*, a cellular or organ compartment, tissue, organ or region of the body, as, *e.g.*, compared to a species absent such a ligand. Preferred ligands will not take part in duplex pairing in a duplexed nucleic acid.

Ligands can include a naturally occurring substance, such as a protein (*e.g.*, human serum albumin (HSA), low-density lipoprotein (LDL), or globulin); carbohydrate (*e.g.*, a dextran, pullulan, chitin, chitosan, inulin, cyclodextrin or hyaluronic acid); or a lipid. The ligand may also be a recombinant or synthetic molecule, such as a synthetic polymer, *e.g.*, a synthetic polyamino acid. Examples of polyamino acids include polyamino acid is a polylysine (PLL), poly L-aspartic acid, poly L-glutamic acid, styrene-maleic acid anhydride copolymer, poly(L-lactide-co-glycolide) copolymer, divinyl ether-maleic anhydride copolymer, N-(2-hydroxypropyl)methacrylamide copolymer (HMPA), polyethylene glycol (PEG), polyvinyl alcohol (PVA), polyurethane, poly(2-ethylacryllic acid), N-isopropylacrylamide polymers, or polyphosphazine. Example of polyamines include: polyethylenimine, polylysine (PLL), spermine, spermidine, polyamine, pseudopeptide-polyamine, peptidomimetic polyamine, dendrimer polyamine, arginine, amidine, protamine, cationic lipid, cationic porphyrin, quaternary salt of a polyamine, or an α helical peptide.

Ligands can also include targeting groups, *e.g.*, a cell or tissue targeting agent, *e.g.*, a lectin, glycoprotein, lipid or protein, *e.g.*, an antibody, that binds to a specified cell type such as a kidney cell. A targeting group can be a thyrotropin, melanotropin, lectin, glycoprotein, surfactant protein A, Mucin carbohydrate, multivalent lactose, multivalent galactose, N-acetyl-galactosamine, N-acetyl-gulucosamine multivalent mannose, multivalent fucose, glycosylated polyaminoacids, multivalent galactose, transferrin, bisphosphonate, polyglutamate, polyaspartate, a lipid, cholesterol, a steroid, bile acid, folate, vitamin B12, vitamin A, biotin, or an RGD peptide or RGD peptide mimetic.

Other examples of ligands include dyes, intercalating agents (*e.g.* acridines), cross-linkers (*e.g.* psoralene, mitomycin C), porphyrins (TPPC4, texaphyrin, Sapphyrin), polycyclic aromatic hydrocarbons (*e.g.*, phenazine, dihydrophenazine), artificial endonucleases (*e.g.* EDTA), lipophilic molecules, *e.g.*, cholesterol, cholic acid, adamantane acetic acid, 1-pyrene butyric acid, dihydrotestosterone, 1,3-Bis-O(hexadecyl)glycerol, geranyloxyhexyl group, hexadecylglycerol, borneol, menthol, 1,3-propanediol, heptadecyl group, palmitic acid, myristic acid,O3-(oleoyl)lithocholic acid, O3-(oleoyl)cholenic acid, dimethoxytrityl, or phenoxazine)and peptide conjugates (*e.g.*, antennapedia peptide, Tat peptide), alkylating agents, phosphate, amino, mercapto, PEG (*e.g.*, PEG-40K), MPEG, $[MPEG]_2$, polyamino, alkyl, substituted alkyl, radiolabeled markers, enzymes, haptens (*e.g.* biotin), transport/absorption facilitators (*e.g.*, aspirin, vitamin E, folic acid), synthetic ribonucleases (*e.g.*, imidazole, bisimidazole, histamine, imidazole clusters, acridine-imidazole conjugates, Eu3+ complexes of tetraazamacrocycles), dinitrophenyl, HRP, or AP.

Ligands can be proteins, *e.g.*, glycoproteins, or peptides, *e.g.*, molecules having a specific affinity for a co-ligand, or antibodies *e.g.*, an antibody, that binds to a specified cell type such as a cancer cell, endothelial cell, or bone cell. Ligands may also include hormones and hormone receptors. They can also include non-peptidic species, such as lipids, lectins, carbohydrates, vitamins, cofactors, multivalent lactose, multivalent galactose, N-acetyl-galactosamine, N-acetyl-gulucosamine multivalent mannose, or multivalent fucose. The ligand can be, for example, a lipopolysaccharide, an activator of p38 MAP kinase, or an activator of NF-κB.

The ligand can be a substance, *e.g.*, a drug, which can increase the uptake of the iRNA agent into the cell, for example, by disrupting the cell's cytoskeleton, *e.g.*, by

disrupting the cell's microtubules, microfilaments, and/or intermediate filaments. The drug can be, for example, taxon, vincristine, vinblastine, cytochalasin, nocodazole, japlakinolide, latrunculin A, phalloidin, swinholide A, indanocine, or myoservin.

In some embodiments, a ligand attached to an iRNA as described herein acts as a PK modulator. As used herein, a "PK modulator" refers to a pharmacokinetic modulator. PK modulators include lipophiles, bile acids, steroids, phospholipid analogues, peptides, protein binding agents, PEG, vitamins *etc*. Exemplary PK modulators include, but are not limited to, cholesterol, fatty acids, cholic acid, lithocholic acid, dialkylglycerides, diacylglyceride, phospholipids, sphingolipids, naproxen, ibuprofen, vitamin E, biotin *etc*. Oligonucleotides that comprise a number of phosphorothioate linkages are also known to bind to serum protein, thus short oligonucleotides, *e.g.*, oligonucleotides of about 5 bases, 10 bases, 15 bases or 20 bases, comprising multiple of phosphorothioate linkages in the backbaone are also amenable to the present invention as ligands (*e.g.* as PK modulating ligands). In addition, aptamers that bind serum components (*e.g.* serum proteins) are also suitable for use as PK modulating ligands in the embodiments described herein.

For macromolecular drugs and hydrophilic drug molecules, which cannot easily cross bilayer membranes, entrapment in endosomal/lysosomal compartments of the cell is thought to be the biggest hurdle for effective delivery to their site of action. In recent years, a number of approaches and strategies have been devised to address this problem. For liposomal formulations, the use of fusogenic lipids in the formulation have been the most common approach (Singh, R. S., Goncalves, C. *et al.* (2004). On the Gene Delivery Efficacies of pH-Sensitive Cationic Lipids via Endosomal Protonation. A Chemical Biology Investigation. Chem. Biol. 11, 713-723.). Other components, which exhibit pH-sensitive endosomolytic activity through protonation and/or pH-induced conformational changes, include charged polymers and peptides. Examples may be found in Hoffman, A. S., Stayton, P. S. *et al.* (2002). Design of "smart" polymers that can direct intracellular drug delivery. Polymers Adv. Technol. 13, 992-999; Kakudo, Chaki, T., S. *et al.* (2004). Transferrin-Modified Liposomes Equipped with a pH-Sensitive Fusogenic Peptide: An Artificial Viral-like Delivery System. Biochemistry 436, 5618-5628; Yessine, M. A. and Leroux, J. C. (2004). Membrane-destabilizing polyanions: interaction with lipid bilayers and endosomal escape of biomacromolecules. Adv. Drug Deliv. Rev. 56, 999-1021; Oliveira, S., van Rooy, I. *et al.* (2007). Fusogenic peptides enhance endosomal escape improving iRNA-induced silencing of

oncogenes. Int. J. Pharm. 331, 211-4. They have generally been used in the context of drug delivery systems, such as liposomes or lipoplexes. For folate receptor-mediated delivery using liposomal formulations, for instance, a pH-sensitive fusogenic peptide has been incorporated into the liposomes and shown to enhance the activity through improving the unloading of drug during the uptake process (Turk, M. J., Reddy, J. A. *et al.* (2002). Characterization of a novel pH-sensitive peptide that enhances drug release from folate-targeted liposomes at endosomal pHs is described in Biochim. Biophys. Acta 1559, 56-68).

In certain embodiments, the endosomolytic components of the present invention can be polyanionic peptides or peptidomimetics which show pH-dependent membrane activity and/or fusogenicity. A peptidomimetic can be a small protein-like chain designed to mimic a peptide. A peptidomimetic can arise from modification of an existing peptide in order to alter the molecule's properties, or the synthesis of a peptide-like molecule using unnatural amino acids or their analogs. In certain embodiments, they have improved stability and/or biological activity when compared to a peptide. In certain embodiments, the endosomolytic component assumes its active conformation at endosomal pH (*e.g.*, pH 5-6). The "active" conformation is that conformation in which the endosomolytic component promotes lysis of the endosome and/or transport of the modular composition featured in the invention, or its any of its components (*e.g.*, a nucleic acid), from the endosome to the cytoplasm of the cell.

Libraries of compounds can be screened for their differential membrane activity at endosomal pH versus neutral pH using a hemolysis assay. Promising candidates isolated by this method may be used as components of the modular compositions featured in the invention. A method for identifying an endosomolytic component for use in the compositions and methods of the present invention may comprise: providing a library of compounds; contacting blood cells with the members of the library, wherein the pH of the medium in which the contact occurs is controlled; determining whether the compounds induce differential lysis of blood cells at a low pH (*e.g.*, about pH 5-6) versus neutral pH (*e.g.*, about pH 7-8).

Exemplary endosomolytic components include the GALA peptide (Subbarao *et al.*, Biochemistry, 1987, 26: 2964-2972), the EALA peptide (Vogel *et al.*, J. Am. Chem. Soc., 1996, 118: 1581-1586), and their derivatives (Turk *et al.*, Biochem. Biophys. Acta, 2002, 1559: 56-68). In certain embodiments, the endosomolytic component can contain a chemical group (*e.g.*, an amino acid) which will undergo a change in charge or protonation in response

to a change in pH. The endosomolytic component may be linear or branched. Exemplary primary sequences of endosomolytic components include H_2N-(AALEALAEALEALAEALEALAEAAAAGGC)-CO2H (SEQ ID NO:2); H_2N-(AALAEALAEALAEALAEALAEALAAAAGGC)-CO2H (SEQ ID NO:3); and H_2N-(ALEALAEALEALAEA)-CONH2 (SEQ ID NO:4).

In certain embodiments, more than one endosomolytic component can be incorporated into the iRNA agent featured in the invention. In some embodiments, this will entail incorporating more than one of the same endosomolytic component into the iRNA agent. In other embodiments, this will entail incorporating two or more different endosomolytic components into iRNA agent.

These endosomolytic components can mediate endosomal escape by, for example, changing conformation at endosomal pH. In certain embodiments, the endosomolytic components can exist in a random coil conformation at neutral pH and rearrange to an amphipathic helix at endosomal pH. As a consequence of this conformational transition, these peptides may insert into the lipid membrane of the endosome, causing leakage of the endosomal contents into the cytoplasm. Because the conformational transition is pH-dependent, the endosomolytic components can display little or no fusogenic activity while circulating in the blood (pH ~7.4). "Fusogenic activity," as used herein, is defined as that activity which results in disruption of a lipid membrane by the endosomolytic component. One example of fusogenic activity is the disruption of the endosomal membrane by the endosomolytic component, leading to endosomal lysis or leakage and transport of one or more components of the modular composition featured in the invention (*e.g.*, the nucleic acid) from the endosome into the cytoplasm.

In addition to hemolysis assays, as described herein, suitable endosomolytic components can be tested and identified by a skilled artisan using other methods. For example, the ability of a compound to respond to, *e.g.*, change charge depending on, the pH environment can be tested by routine methods, *e.g.*, in a cellular assay. In certain embodiments, a test compound is combined with or contacted with a cell, and the cell is allowed to internalize the test compound, *e.g.*, by endocytosis. An endosome preparation can then be made from the contacted cells and the endosome preparation compared to an endosome preparation from control cells. A change, *e.g.*, a decrease, in the endosome fraction from the contacted cell vs. the control cell indicates that the test compound can

function as a fusogenic agent. Alternatively, the contacted cell and control cell can be evaluated, *e.g.*, by microscopy, *e.g.*, by light or electron microscopy, to determine a difference in the endosome population in the cells. The test compound and/or the endosomes can labeled, *e.g.*, to quantify endosomal leakage.

In another type of assay, an iRNA agent described herein is constructed using one or more test or putative fusogenic agents. The iRNA agent can be labeled for easy visualization. The ability of the endosomolytic component to promote endosomal escape, once the iRNA agent is taken up by the cell, can be evaluated, *e.g.*, by preparation of an endosome preparation, or by microscopy techniques, which enable visualization of the labeled iRNA agent in the cytoplasm of the cell. In certain other embodiments, the inhibition of gene expression, or any other physiological parameter, may be used as a surrogate marker for endosomal escape.

In other embodiments, circular dichroism spectroscopy can be used to identify compounds that exhibit a pH-dependent structural transition.

A two-step assay can also be performed, wherein a first assay evaluates the ability of a test compound alone to respond to changes in pH, and a second assay evaluates the ability of a modular composition that includes the test compound to respond to changes in pH.

Lipid Conjugates

In one ligand, the ligand or conjugate is a lipid or lipid-based molecule. Such a lipid or lipid-based molecule preferably binds a serum protein, *e.g.*, human serum albumin (HSA). An HSA binding ligand allows for distribution of the conjugate to a target tissue, *e.g.*, a non-kidney target tissue of the body. For example, the target tissue can be the liver, including parenchymal cells of the liver. Other molecules that can bind HSA can also be used as ligands. For example, neproxin or aspirin can be used. A lipid or lipid-based ligand can (a) increase resistance to degradation of the conjugate, (b) increase targeting or transport into a target cell or cell membrane, and/or (c) can be used to adjust binding to a serum protein, *e.g.*, HSA.

A lipid based ligand can be used to modulate, *e.g.*, control the binding of the conjugate to a target tissue. For example, a lipid or lipid-based ligand that binds to HSA more strongly will be less likely to be targeted to the kidney and therefore less likely to be

cleared from the body. A lipid or lipid-based ligand that binds to HSA less strongly can be used to target the conjugate to the kidney.

In a preferred embodiment, the lipid based ligand binds HSA. Preferably, it binds HSA with a sufficient affinity such that the conjugate will be preferably distributed to a non-kidney tissue. However, it is preferred that the affinity not be so strong that the HSA-ligand binding cannot be reversed.

In another preferred embodiment, the lipid based ligand binds HSA weakly or not at all, such that the conjugate will be preferably distributed to the kidney. Other moieties that target to kidney cells can also be used in place of or in addition to the lipid based ligand.

In another aspect, the ligand is a moiety, *e.g.*, a vitamin, which is taken up by a target cell, *e.g.*, a proliferating cell. These are particularly useful for treating disorders characterized by unwanted cell proliferation, *e.g.*, of the malignant or non-malignant type, *e.g.*, cancer cells. Exemplary vitamins include vitamin A, E, and K. Other exemplary vitamins include are B vitamin, *e.g.*, folic acid, B12, riboflavin, biotin, pyridoxal or other vitamins or nutrients taken up by cancer cells. Also included are HSA and low density lipoprotein (LDL).

In another aspect, the ligand is a cell-permeation agent, preferably a helical cell-permeation agent. Preferably, the agent is amphipathic. An exemplary agent is a peptide such as tat or antennopedia. If the agent is a peptide, it can be modified, including a peptidylmimetic, invertomers, non-peptide or pseudo-peptide linkages, and use of D-amino acids. The helical agent is preferably an α-helical agent, which preferably has a lipophilic and a lipophobic phase.

Cell Permeation Peptides

Peptides suitable for use with the present invention can be a natural peptide, *e.g.*, tat or antennapedia peptide, a synthetic peptide, or a peptidomimetic. Furthermore, the peptide can be a modified peptide, for example peptide can comprise non-peptide or pseudo-peptide linkages, and D-amino acids. A peptidomimetic (also referred to herein as an oligopeptidomimetic) is a molecule capable of folding into a defined three-dimensional structure similar to a natural peptide. The attachment of peptide and peptidomimetics to

iRNA agents can affect pharmacokinetic distribution of the iRNA, such as by enhancing cellular recognition and absorption. The peptide or peptidomimetic moiety can be about 5-50 amino acids long, *e.g.*, about 5, 10, 15, 20, 25, 30, 35, 40, 45, or 50 amino acids long.

A peptide or peptidomimetic can be, for example, a cell permeation peptide, cationic peptide, amphipathic peptide, or hydrophobic peptide (*e.g.*, consisting primarily of Tyr, Trp or Phe). The peptide moiety can be a dendrimer peptide, constrained peptide or crosslinked peptide. In another alternative, the peptide moiety can include a hydrophobic membrane translocation sequence (MTS). An exemplary hydrophobic MTS-containing peptide is RFGF having the amino acid sequence AAVALLPAVLLALLAP (SEQ ID NO:5). An RFGF analogue (*e.g.*, amino acid sequence AALLPVLLAAP (SEQ ID NO:6)) containing a hydrophobic MTS can also be a targeting moiety. The peptide moiety can be a "delivery" peptide, which can carry large polar molecules including peptides, oligonucleotides, and protein across cell membranes. For example, sequences from the HIV Tat protein (GRKKRRQRRRPPQ (SEQ ID NO:7)) and the Drosophila Antennapedia protein (RQIKIWFQNRRMKWKK (SEQ ID NO:8)) have been found to be capable of functioning as delivery peptides. A peptide or peptidomimetic can be encoded by a random sequence of DNA, such as a peptide identified from a phage-display library, or one-bead-one-compound (OBOC) combinatorial library (Lam *et al.*, Nature, 354:82-84, 1991). Preferably, the peptide or peptidomimetic tethered to the lipid is a cell-targeting peptide such as an arginine-glycine-aspartic acid (RGD)-peptide, or RGD mimic. A peptide moiety can range in length from about 5 amino acids to about 40 amino acids. The peptide moieties can have a structural modification, such as to increase stability or direct conformational properties. Any of the structural modifications described below can be utilized.

An RGD peptide moiety can be used to target a tumor cell, such as an endothelial tumor cell or a breast cancer tumor cell (Zitzmann *et al.*, Cancer Res., 62:5139-43, 2002). An RGD peptide can facilitate targeting of an dsRNA agent to tumors of a variety of other tissues, including the lung, kidney, spleen, or liver (Aoki *et al.*, Cancer Gene Therapy 8:783-787, 2001). Preferably, the RGD peptide will facilitate targeting of an iRNA agent to the kidney. The RGD peptide can be linear or cyclic, and can be modified, *e.g.*, glycosylated or methylated to facilitate targeting to specific tissues. For example, a glycosylated RGD peptide can deliver a iRNA agent to a tumor cell expressing $\alpha_V\beta_3$ (Haubner *et al.*, Jour. Nucl. Med., 42:326-336, 2001).

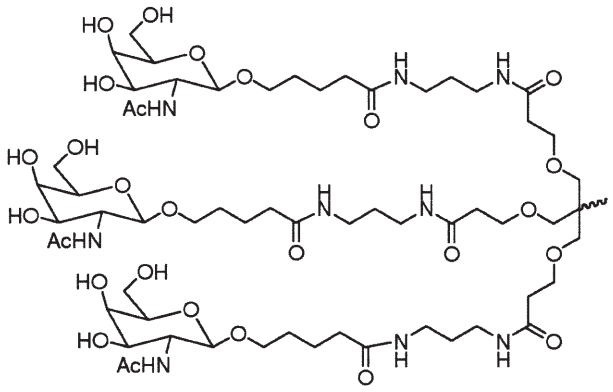
Peptides that target markers enriched in proliferating cells can be used. *E.g.*, RGD containing peptides and peptidomimetics can target cancer cells, in particular cells that exhibit an αvβ3 integrin. Thus, one could use RGD peptides, cyclic peptides containing RGD, RGD peptides that include D-amino acids, as well as synthetic RGD mimics. In addition to RGD, one can use other moieties that target the αvβ3 integrin ligand. Generally, such ligands can be used to control proliferating cells and angiogeneis.

A "cell permeation peptide" is capable of permeating a cell, *e.g.*, a microbial cell, such as a bacterial or fungal cell, or a mammalian cell, such as a human cell. A microbial cell-permeating peptide can be, for example, an α-helical linear peptide (*e.g.*, LL-37 or Ceropin P1), a disulfide bond-containing peptide (*e.g.*, α -defensin, β-defensin or bactenecin), or a peptide containing only one or two dominating amino acids (*e.g.*, PR-39 or indolicidin). A cell permeation peptide can also include a nuclear localization signal (NLS). For example, a cell permeation peptide can be a bipartite amphipathic peptide, such as MPG, which is derived from the fusion peptide domain of HIV-1 gp41 and the NLS of SV40 large T antigen (Simeoni *et al.*, Nucl. Acids Res. 31:2717-2724, 2003).

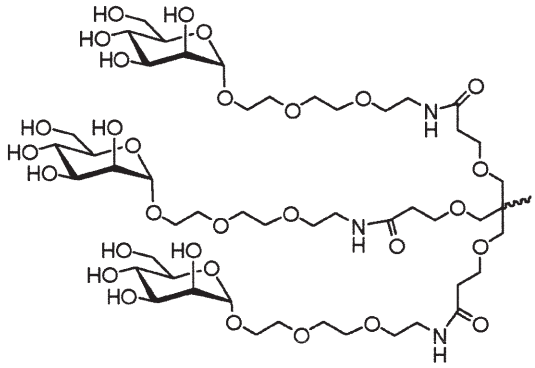
Carbohydrate Conjugates

In some embodiments, the iRNA oligonucleotides described herein further comprise carbohydrate conjugates. The carbohydrate conjugates are advantageous for the *in vivo* delivery of nucleic acids, as well as compositions suitable for *in vivo* therapeutic use, as described herein. As used herein, "carbohydrate" refers to a compound which is either a carbohydrate per se made up of one or more monosaccharide units having at least 6 carbon atoms (which may be linear, branched or cyclic) with an oxygen, nitrogen or sulfur atom bonded to each carbon atom; or a compound having as a part thereof a carbohydrate moiety made up of one or more monosaccharide units each having at least six carbon atoms (which may be linear, branched or cyclic), with an oxygen, nitrogen or sulfur atom bonded to each carbon atom. Representative carbohydrates include the sugars (mono-, di-, tri- and oligosaccharides containing from about 4-9 monosaccharide units), and polysaccharides such as starches, glycogen, cellulose and polysaccharide gums. Specific monosaccharides include C_5 and above (preferably C_5 -C_8) sugars; di- and trisaccharides include sugars having two or three monosaccharide units (preferably C_5 -C_8).

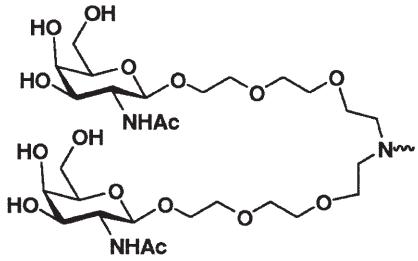
In one embodiment, the carbohydrate conjugate is selected from the group consisting of:



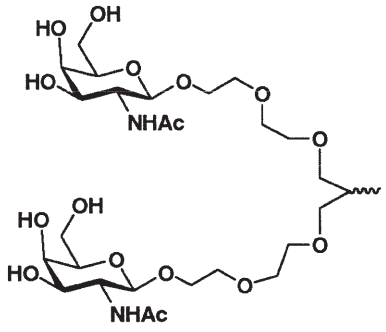
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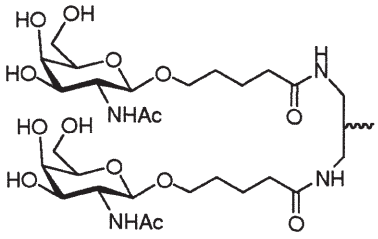
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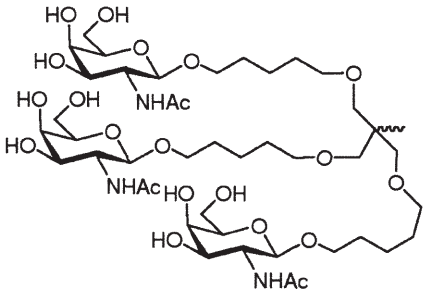
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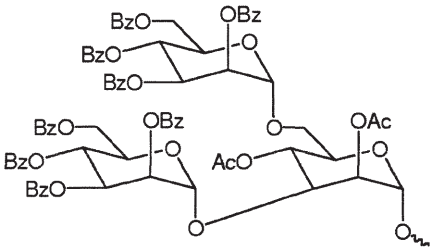
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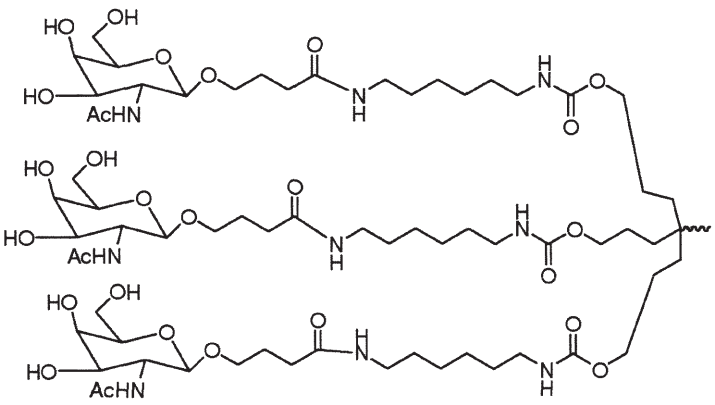
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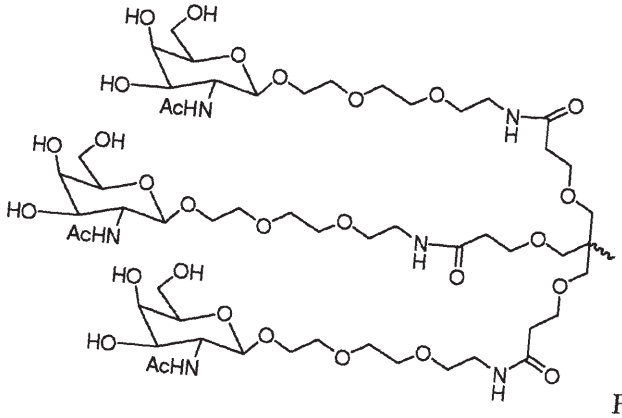
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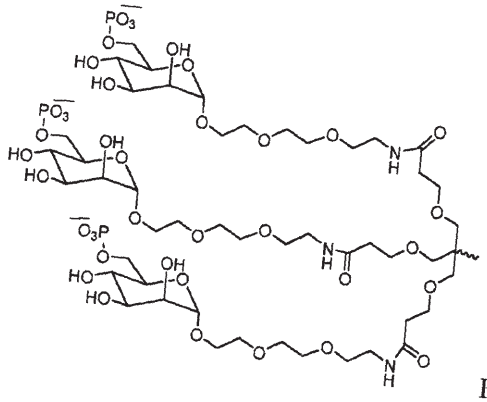
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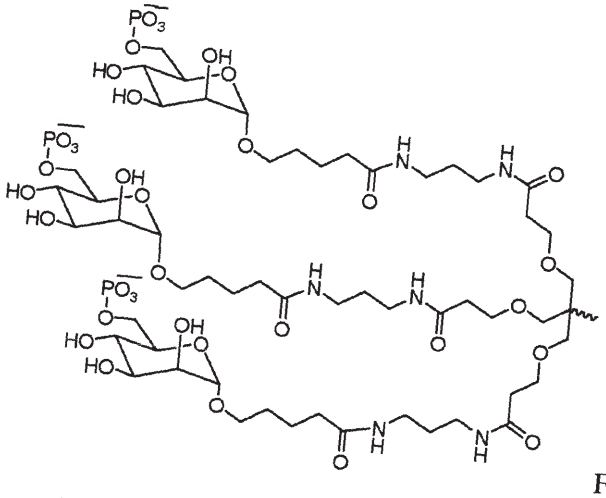
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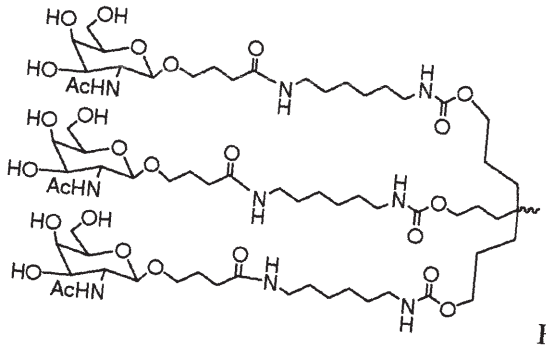
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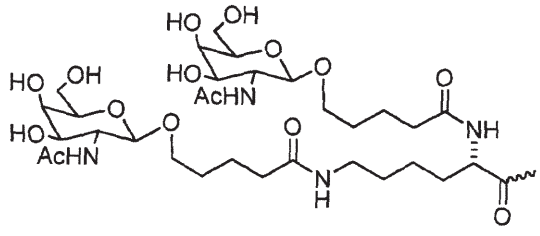
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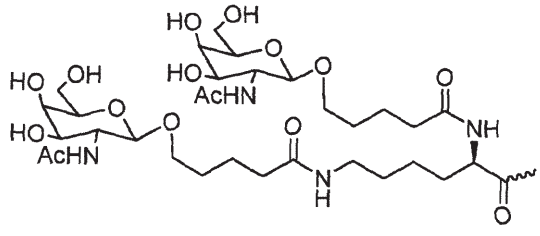
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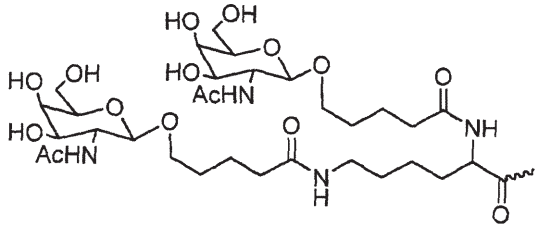
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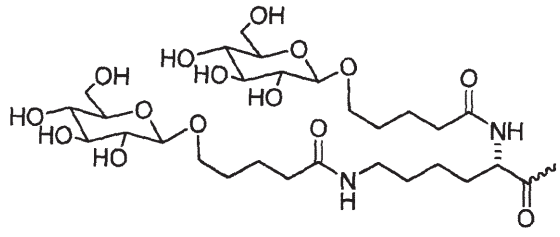
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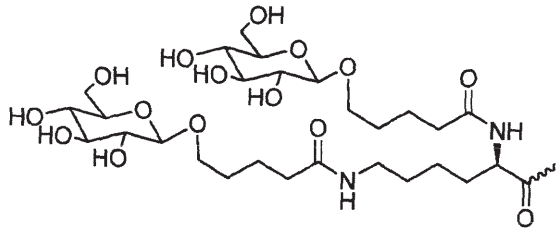
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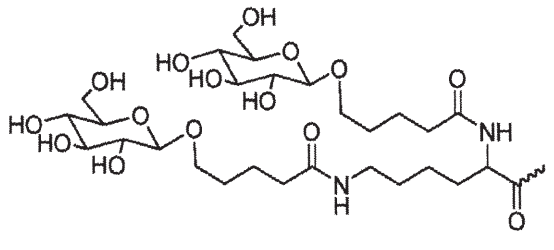
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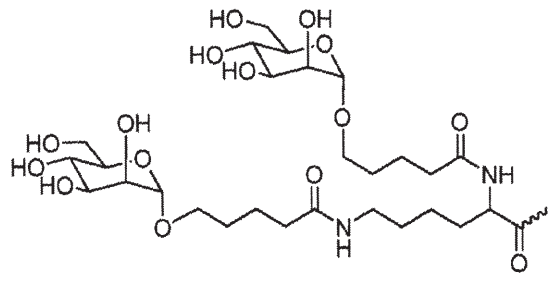
Formula XVII,



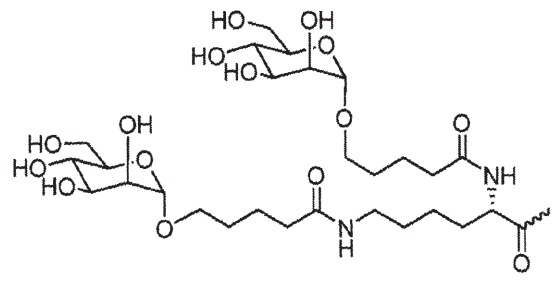
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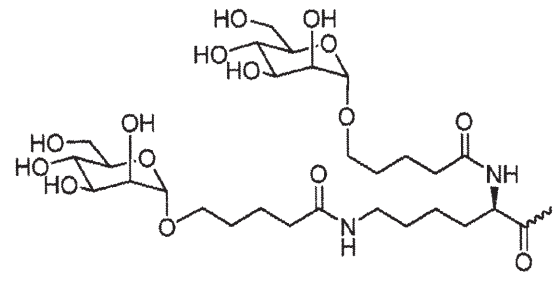
Formula XIX,



Formula XX,

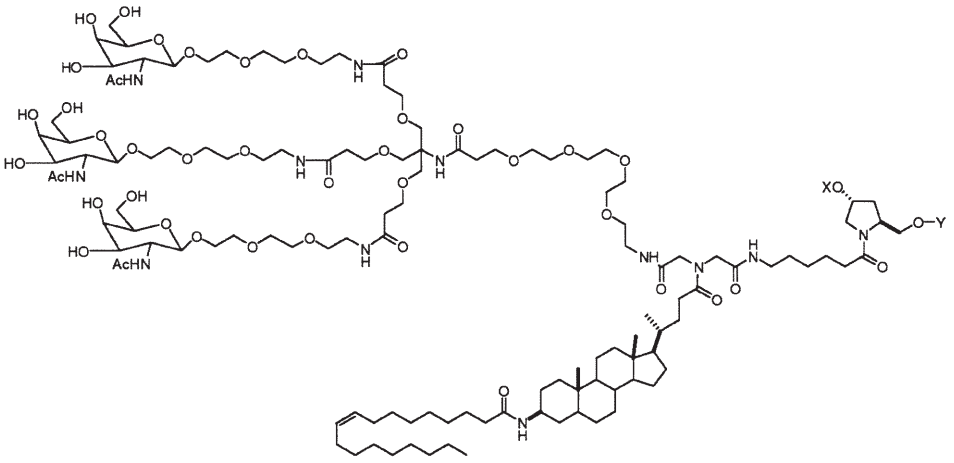


Formula XXI,



Formula XXII, *i.e.*, Formula II – Formula XXII.

Another representative carbohydrate conjugate for use in the embodiments described herein includes, but is not limited to,



(Formula XXIII),

when one of X or Y is an oligonucleotide, the other is a hydrogen.

In some embodiments, the carbohydrate conjugate further comprises other ligand such as, but not limited to, PK modulator, endosomolytic ligand, and cell permeation peptide.

Linkers

In some embodiments, the conjugates described herein can be attached to the iRNA oligonucleotide with various linkers that can be cleavable or non cleavable.

The term "linker" or "linking group" means an organic moiety that connects two parts of a compound. Linkers typically comprise a direct bond or an atom such as oxygen or sulfur, a unit such as NR^8, C(O), C(O)NH, SO, SO_2, SO_2NH or a chain of atoms, such as, but not limited to, substituted or unsubstituted alkyl, substituted or unsubstituted alkenyl, substituted or unsubstituted alkynyl, arylalkyl, arylalkenyl, arylalkynyl, heteroarylalkyl, heteroarylalkenyl, heteroarylalkynyl, heterocyclylalkyl, heterocyclylalkenyl, heterocyclylalkynyl, aryl, heteroaryl, heterocyclyl, cycloalkyl, cycloalkenyl, alkylarylalkyl, alkylarylalkenyl, alkylarylalkynyl, alkenylarylalkyl, alkenylarylalkenyl, alkenylarylalkynyl, alkynylarylalkyl, alkynylarylalkenyl, alkynylarylalkynyl, alkylheteroarylalkyl, alkylheteroarylalkenyl, alkylheteroarylalkynyl, alkenylheteroarylalkyl, alkenylheteroarylalkenyl, alkenylheteroarylalkynyl, alkynylheteroarylalkyl, alkynylheteroarylalkenyl, alkynylheteroarylalkynyl, alkylheterocyclylalkyl, alkylheterocyclylalkenyl, alkylhererocyclylalkynyl, alkenylheterocyclylalkyl, alkenylheterocyclylalkenyl, alkenylheterocyclylalkynyl, alkynylheterocyclylalkyl,

alkynylheterocyclylalkenyl, alkynylheterocyclylalkynyl, alkylaryl, alkenylaryl, alkynylaryl, alkylheteroaryl, alkenylheteroaryl, alkynylhereroaryl, which one or more methylenes can be interrupted or terminated by O, S, S(O), SO_2, $N(R^8)$, C(O), substituted or unsubstituted aryl, substituted or unsubstituted heteroaryl, substituted or unsubstituted heterocyclic; where R^8 is hydrogen, acyl, aliphatic or substituted aliphatic. In one embodiment, the linker is between 1-24 atoms, preferably 4-24 atoms, preferably 6-18 atoms, more preferably 8-18 atoms, and most preferably 8-16 atoms.

A cleavable linking group is one which is sufficiently stable outside the cell, but which upon entry into a target cell is cleaved to release the two parts the linker is holding together. In a preferred embodiment, the cleavable linking group is cleaved at least 10 times or more, preferably at least 100 times faster in the target cell or under a first reference condition (which can, *e.g.*, be selected to mimic or represent intracellular conditions) than in the blood of a subject, or under a second reference condition (which can, *e.g.*, be selected to mimic or represent conditions found in the blood or serum).

Cleavable linking groups are susceptible to cleavage agents, *e.g.*, pH, redox potential or the presence of degradative molecules. Generally, cleavage agents are more prevalent or found at higher levels or activities inside cells than in serum or blood. Examples of such degradative agents include: redox agents which are selected for particular substrates or which have no substrate specificity, including, *e.g.*, oxidative or reductive enzymes or reductive agents such as mercaptans, present in cells, that can degrade a redox cleavable linking group by reduction; esterases; endosomes or agents that can create an acidic environment, *e.g.*, those that result in a pH of five or lower; enzymes that can hydrolyze or degrade an acid cleavable linking group by acting as a general acid, peptidases (which can be substrate specific), and phosphatases.

A cleavable linkage group, such as a disulfide bond can be susceptible to pH. The pH of human serum is 7.4, while the average intracellular pH is slightly lower, ranging from about 7.1-7.3. Endosomes have a more acidic pH, in the range of 5.5-6.0, and lysosomes have an even more acidic pH at around 5.0. Some linkers will have a cleavable linking group that is cleaved at a preferred pH, thereby releasing the cationic lipid from the ligand inside the cell, or into the desired compartment of the cell.

A linker can include a cleavable linking group that is cleavable by a particular enzyme. The type of cleavable linking group incorporated into a linker can depend on the

cell to be targeted. For example, liver targeting ligands can be linked to the cationic lipids through a linker that includes an ester group. Liver cells are rich in esterases, and therefore the linker will be cleaved more efficiently in liver cells than in cell types that are not esterase-rich. Other cell-types rich in esterases include cells of the lung, renal cortex, and testis.

Linkers that contain peptide bonds can be used when targeting cell types rich in peptidases, such as liver cells and synoviocytes.

In general, the suitability of a candidate cleavable linking group can be evaluated by testing the ability of a degradative agent (or condition) to cleave the candidate linking group. It will also be desirable to also test the candidate cleavable linking group for the ability to resist cleavage in the blood or when in contact with other non-target tissue. Thus, one can determine the relative susceptibility to cleavage between a first and a second condition, where the first is selected to be indicative of cleavage in a target cell and the second is selected to be indicative of cleavage in other tissues or biological fluids, *e.g.*, blood or serum. The evaluations can be carried out in cell free systems, in cells, in cell culture, in organ or tissue culture, or in whole animals. It may be useful to make initial evaluations in cell-free or culture conditions and to confirm by further evaluations in whole animals. In preferred embodiments, useful candidate compounds are cleaved at least 2, 4, 10 or 100 times faster in the cell (or under *in vitro* conditions selected to mimic intracellular conditions) as compared to blood or serum (or under *in vitro* conditions selected to mimic extracellular conditions).

Redox cleavable linking groups

One class of cleavable linking groups are redox cleavable linking groups that are cleaved upon reduction or oxidation. An example of reductively cleavable linking group is a disulphide linking group (-S-S-). To determine if a candidate cleavable linking group is a suitable "reductively cleavable linking group," or for example is suitable for use with a particular iRNA moiety and particular targeting agent one can look to methods described herein. For example, a candidate can be evaluated by incubation with dithiothreitol (DTT), or other reducing agent using reagents know in the art, which mimic the rate of cleavage which would be observed in a cell, *e.g.*, a target cell. The candidates can also be evaluated under conditions which are selected to mimic blood or serum conditions. In a preferred embodiment, candidate compounds are cleaved by at most 10% in the blood. In preferred embodiments, useful candidate compounds are degraded at least 2, 4, 10 or 100 times faster in the cell (or under *in vitro* conditions selected to mimic intracellular conditions) as

compared to blood (or under *in vitro* conditions selected to mimic extracellular conditions). The rate of cleavage of candidate compounds can be determined using standard enzyme kinetics assays under conditions chosen to mimic intracellular media and compared to conditions chosen to mimic extracellular media.

Phosphate-based cleavable linking groups

Phosphate-based cleavable linking groups are cleaved by agents that degrade or hydrolyze the phosphate group. An example of an agent that cleaves phosphate groups in cells are enzymes such as phosphatases in cells. Examples of phosphate-based linking groups are -O-P(O)(ORk)-O-, -O-P(S)(ORk)-O-, -O-P(S)(SRk)-O-, -S-P(O)(ORk)-O-, -O-P(O)(ORk)-S-, -S-P(O)(ORk)-S-, -O-P(S)(ORk)-S-, -S-P(S)(ORk)-O-, -O-P(O)(Rk)-O-, -O-P(S)(Rk)-O-, -S-P(O)(Rk)-O-, -S-P(S)(Rk)-O-, -S-P(O)(Rk)-S-, -O-P(S)(Rk)-S-. Preferred embodiments are -O-P(O)(OH)-O-, -O-P(S)(OH)-O-, -O-P(S)(SH)-O-, -S-P(O)(OH)-O-, -O-P(O)(OH)-S-, -S-P(O)(OH)-S-, -O-P(S)(OH)-S-, -S-P(S)(OH)-O-, -O-P(O)(H)-O-, -O-P(S)(H)-O-, -S-P(O)(H)-O-, -S-P(S)(H)-O-, -S-P(O)(H)-S-, -O-P(S)(H)-S-. A preferred embodiment is -O-P(O)(OH)-O-. These candidates can be evaluated using methods analogous to those described above.

Acid cleavable linking groups

Acid cleavable linking groups are linking groups that are cleaved under acidic conditions. In preferred embodiments acid cleavable linking groups are cleaved in an acidic environment with a pH of about 6.5 or lower (*e.g.*, about 6.0, 5.5, 5.0, or lower), or by agents such as enzymes that can act as a general acid. In a cell, specific low pH organelles, such as endosomes and lysosomes can provide a cleaving environment for acid cleavable linking groups. Examples of acid cleavable linking groups include but are not limited to hydrazones, esters, and esters of amino acids. Acid cleavable groups can have the general formula -C=NN-, C(O)O, or -OC(O). A preferred embodiment is when the carbon attached to the oxygen of the ester (the alkoxy group) is an aryl group, substituted alkyl group, or tertiary alkyl group such as dimethyl pentyl or t-butyl. These candidates can be evaluated using methods analogous to those described above.

Ester-based linking groups

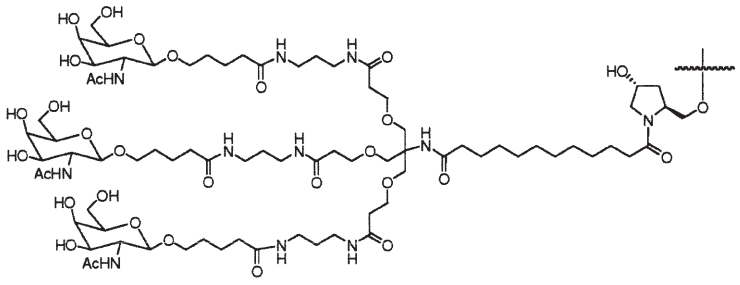
Ester-based cleavable linking groups are cleaved by enzymes such as esterases and amidases in cells. Examples of ester-based cleavable linking groups include but are not

limited to esters of alkylene, alkenylene and alkynylene groups. Ester cleavable linking groups have the general formula -C(O)O-, or -OC(O)-. These candidates can be evaluated using methods analogous to those described above.

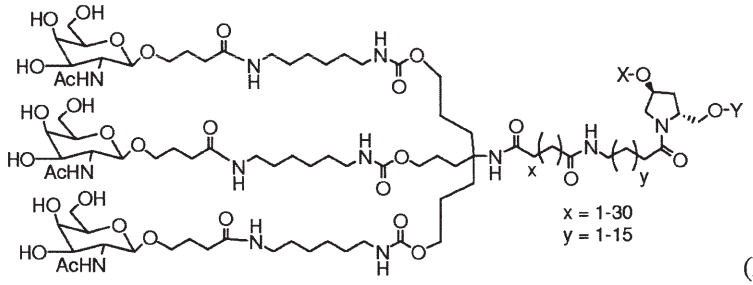
Peptide-based cleaving groups

Peptide-based cleavable linking groups are cleaved by enzymes such as peptidases and proteases in cells. Peptide-based cleavable linking groups are peptide bonds formed between amino acids to yield oligopeptides (*e.g.*, dipeptides, tripeptides *etc.*) and polypeptides. Peptide-based cleavable groups do not include the amide group (-C(O)NH-). The amide group can be formed between any alkylene, alkenylene or alkynelene. A peptide bond is a special type of amide bond formed between amino acids to yield peptides and proteins. The peptide based cleavage group is generally limited to the peptide bond (*i.e.*, the amide bond) formed between amino acids yielding peptides and proteins and does not include the entire amide functional group. Peptide-based cleavable linking groups have the general formula – $NHCHR^ACO)NHCHR^BC(O)-$, where R^A and R^B are the R groups of the two adjacent amino acids. These candidates can be evaluated using methods analogous to those described above.

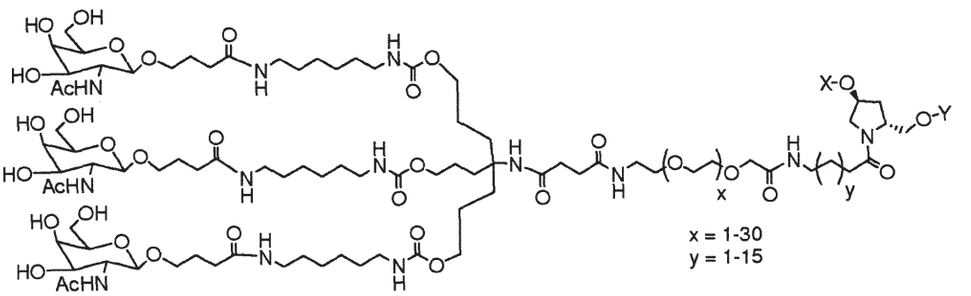
Representative carbohydrate conjugates with linkers include, but are not limited to,



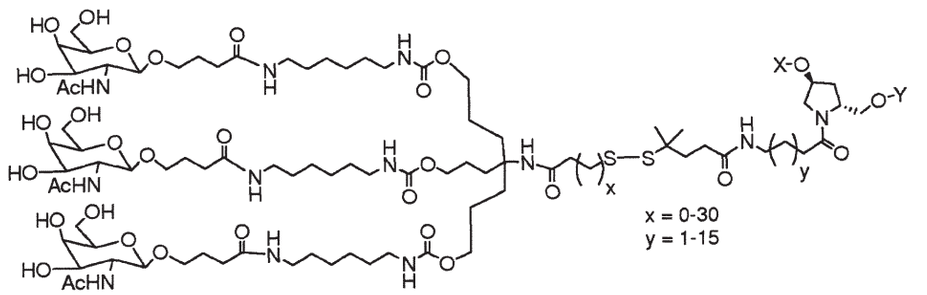
(Formula XXIV),



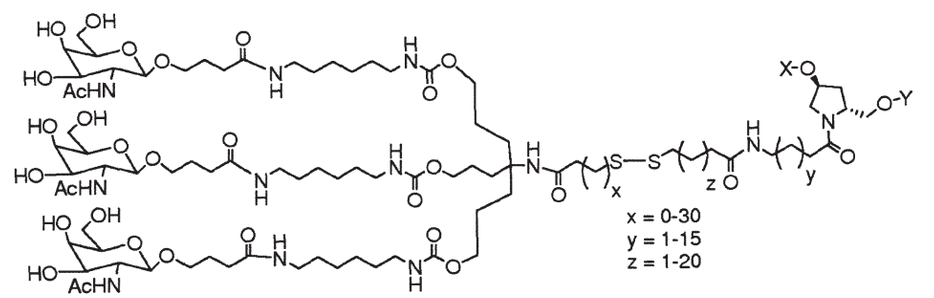
(Formula XXV),



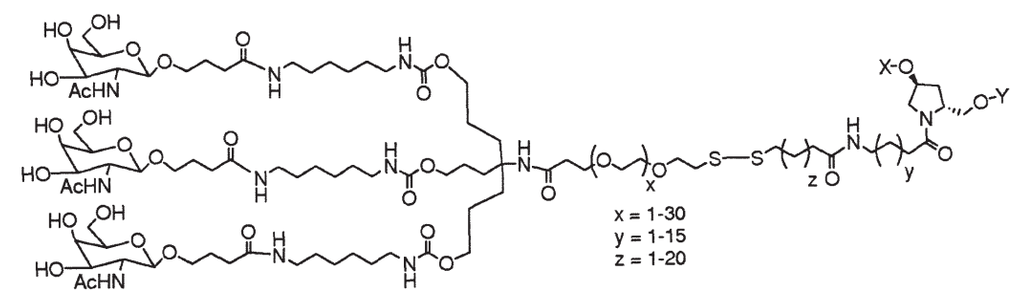
(Formula XXVI),



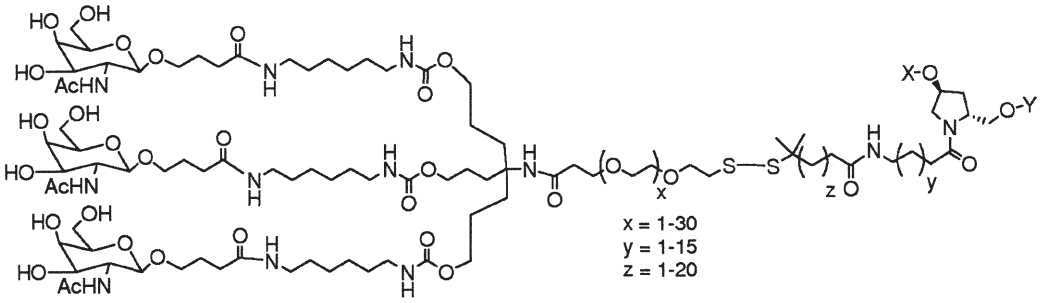
(Formula XXVII),



(Formula XXVIII),



(Formula XXIX), and



(Formula XXX),

when one of X or Y is an oligonucleotide, the other is a hydrogen.

Representative U.S. patents that teach the preparation of RNA conjugates include, but are not limited to, U.S. Pat. Nos. 4,828,979; 4,948,882; 5,218,105; 5,525,465; 5,541,313; 5,545,730; 5,552,538; 5,578,717, 5,580,731; 5,591,584; 5,109,124; 5,118,802; 5,138,045; 5,414,077; 5,486,603; 5,512,439; 5,578,718; 5,608,046; 4,587,044; 4,605,735; 4,667,025; 4,762,779; 4,789,737; 4,824,941; 4,835,263; 4,876,335; 4,904,582; 4,958,013; 5,082,830; 5,112,963; 5,214,136; 5,082,830; 5,112,963; 5,214,136; 5,245,022; 5,254,469; 5,258,506; 5,262,536; 5,272,250; 5,292,873; 5,317,098; 5,371,241, 5,391,723; 5,416,203, 5,451,463; 5,510,475; 5,512,667; 5,514,785; 5,565,552; 5,567,810; 5,574,142; 5,585,481; 5,587,371; 5,595,726; 5,597,696; 5,599,923; 5,599,928 and 5,688,941; 6,294,664; 6,320,017; 6,576,752; 6,783,931; 6,900,297; 7,037,646; each of which is herein incorporated by reference.

It is not necessary for all positions in a given compound to be uniformly modified, and in fact more than one of the aforementioned modifications can be incorporated in a single compound or even at a single nucleoside within an iRNA. The present invention also includes iRNA compounds that are chimeric compounds. "Chimeric" iRNA compounds or "chimeras," in the context of this invention, are iRNA compounds, preferably dsRNAs, which contain two or more chemically distinct regions, each made up of at least one monomer unit, *i.e.*, a nucleotide in the case of a dsRNA compound. These iRNAs typically contain at least one region wherein the RNA is modified so as to confer upon the iRNA increased resistance to nuclease degradation, increased cellular uptake, and/or increased binding affinity for the target nucleic acid. An additional region of the iRNA may serve as a

substrate for enzymes capable of cleaving RNA:DNA or RNA:RNA hybrids. By way of example, RNase H is a cellular endonuclease which cleaves the RNA strand of an RNA:DNA duplex. Activation of RNase H, therefore, results in cleavage of the RNA target, thereby greatly enhancing the efficiency of iRNA inhibition of gene expression. Consequently, comparable results can often be obtained with shorter iRNAs when chimeric dsRNAs are used, compared to phosphorothioate deoxy dsRNAs hybridizing to the same target region. Cleavage of the RNA target can be routinely detected by gel electrophoresis and, if necessary, associated nucleic acid hybridization techniques known in the art.

In certain instances, the RNA of an iRNA can be modified by a non-ligand group. A number of non-ligand molecules have been conjugated to iRNAs in order to enhance the activity, cellular distribution or cellular uptake of the iRNA, and procedures for performing such conjugations are available in the scientific literature. Such non-ligand moieties have included lipid moieties, such as cholesterol (Kubo, T. *et al.,* Biochem. Biophys. Res. Comm., 2007, 365(1):54-61; Letsinger *et al.*, Proc. Natl. Acad. Sci. USA, 1989, 86:6553), cholic acid (Manoharan *et al.*, Bioorg. Med. Chem. Lett., 1994, 4:1053), a thioether, *e.g.*, hexyl-S-tritylthiol (Manoharan *et al.*, Ann. N.Y. Acad. Sci., 1992, 660:306; Manoharan *et al.*, Bioorg. Med. Chem. Let., 1993, 3:2765), a thiocholesterol (Oberhauser *et al.*, Nucl. Acids Res., 1992, 20:533), an aliphatic chain, *e.g.*, dodecandiol or undecyl residues (Saison-Behmoaras *et al.*, EMBO J., 1991, 10:111; Kabanov *et al.*, FEBS Lett., 1990, 259:327; Svinarchuk *et al.*, Biochimie, 1993, 75:49), a phospholipid, *e.g.*, di-hexadecyl-rac-glycerol or triethylammonium 1,2-di-O-hexadecyl-rac-glycero-3-H-phosphonate (Manoharan *et al.*, Tetrahedron Lett., 1995, 36:3651; Shea *et al.*, Nucl. Acids Res., 1990, 18:3777), a polyamine or a polyethylene glycol chain (Manoharan *et al.*, Nucleosides & Nucleotides, 1995, 14:969), or adamantane acetic acid (Manoharan *et al.*, Tetrahedron Lett., 1995, 36:3651), a palmityl moiety (Mishra *et al.*, Biochim. Biophys. Acta, 1995, 1264:229), or an octadecylamine or hexylamino-carbonyl-oxycholesterol moiety (Crooke *et al.*, J. Pharmacol. Exp. Ther., 1996, 277:923). Representative United States patents that teach the preparation of such RNA conjugates have been listed above. Typical conjugation protocols involve the synthesis of an RNA bearing an amino linker at one or more positions of the sequence. The amino group is then reacted with the molecule being conjugated using appropriate coupling or activating reagents. The conjugation reaction may be performed either with the RNA still bound to the solid support or following cleavage of the RNA, in solution phase. Purification of the RNA conjugate by HPLC typically affords the pure conjugate.

Delivery of iRNA

The delivery of an iRNA to a subject in need thereof can be achieved in a number of different ways. *In vivo* delivery can be performed directly by administering a composition comprising an iRNA, *e.g.* a dsRNA, to a subject. Alternatively, delivery can be performed indirectly by administering one or more vectors that encode and direct the expression of the iRNA.

Direct Delivery of an iRNA composition

In general, any method of delivering a nucleic acid molecule can be adapted for use with an iRNA (see *e.g.*, Akhtar S. and Julian RL. (1992) Trends Cell. Biol. 2(5):139-144 and WO94/02595, which are incorporated herein by reference in their entireties). However, there are three factors that are important to consider in order to successfully deliver an iRNA molecule *in vivo*: (a) biological stability of the delivered molecule, (2) preventing non-specific effects, and (3) accumulation of the delivered molecule in the target tissue. The non-specific effects of an iRNA can be minimized by local administration, for example by direct injection or implantation into a tissue (as a non-limiting example, a tumor) or topically administering the preparation. Local administration to a treatment site maximizes local concentration of the agent, limits the exposure of the agent to systemic tissues that may otherwise be harmed by the agent or that may degrade the agent, and permits a lower total dose of the iRNA molecule to be administered. Several studies have shown successful knockdown of gene products when an iRNA is administered locally. For example, intraocular delivery of a VEGF dsRNA by intravitreal injection in cynomolgus monkeys (Tolentino, MJ., *et al* (2004) Retina 24:132-138) and subretinal injections in mice (Reich, SJ., *et al* (2003) Mol. Vis. 9:210-216) were both shown to prevent neovascularization in an experimental model of age-related macular degeneration. In addition, direct intratumoral injection of a dsRNA in mice reduces tumor volume (Pille, J., *et al* (2005) Mol. Ther.11:267-274) and can prolong survival of tumor-bearing mice (Kim, WJ., *et al* (2006) Mol. Ther. 14:343-350; Li, S., *et al* (2007) Mol. Ther. 15:515-523). RNA interference has also shown success with local delivery to the CNS by direct injection (Dorn, G., *et al.* (2004) Nucleic Acids 32:e49; Tan, PH., *et al* (2005) Gene Ther. 12:59-66; Makimura, H., *et al* (2002) BMC Neurosci. 3:18; Shishkina, GT., *et al* (2004) Neuroscience 129:521-528; Thakker, ER., *et al* (2004) Proc. Natl. Acad. Sci. U.S.A. 101:17270-17275; Akaneya,Y., *et al* (2005) J. Neurophysiol. 93:594-

602) and to the lungs by intranasal administration (Howard, KA., *et al* (2006) Mol. Ther. 14:476-484; Zhang, X., *et al* (2004) J. Biol. Chem. 279:10677-10684; Bitko, V., *et al* (2005) Nat. Med. 11:50-55). For administering an iRNA systemically for the treatment of a disease, the RNA can be modified or alternatively delivered using a drug delivery system; both methods act to prevent the rapid degradation of the dsRNA by endo- and exo-nucleases *in vivo*. Modification of the RNA or the pharmaceutical carrier can also permit targeting of the iRNA composition to the target tissue and avoid undesirable off-target effects. iRNA molecules can be modified by chemical conjugation to lipophilic groups such as cholesterol to enhance cellular uptake and prevent degradation. For example, an iRNA directed against ApoB conjugated to a lipophilic cholesterol moiety was injected systemically into mice and resulted in knockdown of apoB mRNA in both the liver and jejunum (Soutschek, J., *et al* (2004) Nature 432:173-178). Conjugation of an iRNA to an aptamer has been shown to inhibit tumor growth and mediate tumor regression in a mouse model of prostate cancer (McNamara, JO., *et al* (2006) Nat. Biotechnol. 24:1005-1015). In an alternative embodiment, the iRNA can be delivered using drug delivery systems such as a nanoparticle, a dendrimer, a polymer, a liposome, or a cationic delivery system. Positively charged cationic delivery systems facilitate binding of an iRNA molecule (negatively charged) and also enhance interactions at the negatively charged cell membrane to permit efficient uptake of an iRNA by the cell. Cationic lipids, dendrimers, or polymers can either be bound to an iRNA, or induced to form a vesicle or micelle (see *e.g.*, Kim SH., *et al* (2008) Journal of Controlled Release 129(2):107-116) that encases an iRNA. The formation of vesicles or micelles further prevents degradation of the iRNA when administered systemically. Methods for making and administering cationic- iRNA complexes are well within the abilities of one skilled in the art (see *e.g.*, Sorensen, DR., *et al* (2003) J. Mol. Biol 327:761-766; Verma, UN., *et al* (2003) Clin. Cancer Res. 9:1291-1300; Arnold, AS *et al* (2007) J. Hypertens. 25:197-205, which are incorporated herein by reference in their entirety). Some non-limiting examples of drug delivery systems useful for systemic delivery of iRNAs include DOTAP (Sorensen, DR., *et al* (2003), supra; Verma, UN., *et al* (2003), supra), Oligofectamine, "solid nucleic acid lipid particles" (Zimmermann, TS., *et al* (2006) Nature 441:111-114), cardiolipin (Chien, PY., *et al* (2005) Cancer Gene Ther. 12:321-328; Pal, A., *et al* (2005) Int J. Oncol. 26:1087-1091), polyethyleneimine (Bonnet ME., *et al* (2008) Pharm. Res. Aug 16 Epub ahead of print; Aigner, A. (2006) J. Biomed. Biotechnol. 71659), Arg-Gly-Asp (RGD) peptides (Liu, S. (2006) Mol. Pharm. 3:472-487), and polyamidoamines (Tomalia, DA., *et al* (2007) Biochem. Soc. Trans. 35:61-67; Yoo, H., *et al* (1999) Pharm. Res. 16:1799-1804). In some

embodiments, an iRNA forms a complex with cyclodextrin for systemic administration. Methods for administration and pharmaceutical compositions of iRNAs and cyclodextrins can be found in U.S. Patent No. 7, 427, 605, which is herein incorporated by reference in its entirety.

Vector encoded iRNAs

In another aspect, iRNA targeting the TMPRSS6 gene can be expressed from transcription units inserted into DNA or RNA vectors (see, *e.g.*, Couture, A, *et al.*, *TIG.* (1996), 12:5-10; Skillern, A., *et al.*, International PCT Publication No. WO 00/22113, Conrad, PCT Publication No. WO 00/22114, and Conrad, U.S. Pat. No. 6,054,299). Expression can be transient (on the order of hours to weeks) or sustained (weeks to months or longer), depending upon the specific construct used and the target tissue or cell type. These transgenes can be introduced as a linear construct, a circular plasmid, or a viral vector, which can be an integrating or non-integrating vector. The transgene can also be constructed to permit it to be inherited as an extrachromosomal plasmid (Gassmann, *et al.*, *Proc. Natl. Acad. Sci. USA* (1995) 92:1292).

The individual strand or strands of an iRNA can be transcribed from a promoter on an expression vector. Where two separate strands are to be expressed to generate, for example, a dsRNA, two separate expression vectors can be co-introduced (*e.g.*, by transfection or infection) into a target cell. Alternatively each individual strand of a dsRNA can be transcribed by promoters both of which are located on the same expression plasmid. In one embodiment, the strands of a dsRNA are expressed as inverted repeat polynucleotides joined by a linker polynucleotide sequence such that the dsRNA has a stem and loop structure.

iRNA expression vectors are generally DNA plasmids or viral vectors. Expression vectors compatible with eukaryotic cells, preferably those compatible with vertebrate cells, can be used to produce recombinant constructs for the expression of an iRNA as described herein. Eukaryotic cell expression vectors are well known in the art and are available from a number of commercial sources. Typically, such vectors are provided containing convenient restriction sites for insertion of the desired nucleic acid segment. Delivery of iRNA expressing vectors can be systemic, such as by intravenous or intramuscular administration, by administration to target cells ex-planted from the patient followed by reintroduction into the patient, or by any other means that allows for introduction into a desired target cell.

iRNA expression plasmids can be transfected into target cells as a complex with cationic lipid carriers (*e.g.*, Oligofectamine) or non-cationic lipid-based carriers (*e.g.*, Transit-TKO™). Multiple lipid transfections for iRNA-mediated knockdowns targeting different regions of a target RNA over a period of a week or more are also contemplated by the invention. Successful introduction of vectors into host cells can be monitored using various known methods. For example, transient transfection can be signaled with a reporter, such as a fluorescent marker, such as Green Fluorescent Protein (GFP). Stable transfection of cells *ex vivo* can be ensured using markers that provide the transfected cell with resistance to specific environmental factors (*e.g.*, antibiotics and drugs), such as hygromycin B resistance.

Viral vector systems which can be utilized with the methods and compositions described herein include, but are not limited to, (a) adenovirus vectors; (b) retrovirus vectors, including but not limited to lentiviral vectors, moloney murine leukemia virus, *etc.*; (c) adeno-associated virus vectors; (d) herpes simplex virus vectors; (e) SV40 vectors; (f) polyoma virus vectors; (g) papilloma virus vectors; (h) picornavirus vectors; (i) pox virus vectors such as an orthopox, *e.g.*, vaccinia virus vectors or avipox, *e.g.* canary pox or fowl pox; and (j) a helper-dependent or gutless adenovirus. Replication-defective viruses can also be advantageous. Different vectors will or will not become incorporated into the cells' genome. The constructs can include viral sequences for transfection, if desired. Alternatively, the construct may be incorporated into vectors capable of episomal replication, *e.g.* EPV and EBV vectors. Constructs for the recombinant expression of an iRNA will generally require regulatory elements, *e.g.*, promoters, enhancers, *etc.*, to ensure the expression of the iRNA in target cells. Other aspects to consider for vectors and constructs are further described below.

Vectors useful for the delivery of an iRNA will include regulatory elements (promoter, enhancer, *etc.*) sufficient for expression of the iRNA in the desired target cell or tissue. The regulatory elements can be chosen to provide either constitutive or regulated/inducible expression.

Expression of the iRNA can be precisely regulated, for example, by using an inducible regulatory sequence that is sensitive to certain physiological regulators, *e.g.*, circulating glucose levels, or hormones (Docherty *et al.*, 1994, FASEB J. 8:20-24). Such inducible expression systems, suitable for the control of dsRNA expression in cells or in mammals include, for example, regulation by ecdysone, by estrogen, progesterone, tetracycline, chemical inducers of dimerization, and isopropyl-β-D1 -thiogalactopyranoside

(IPTG). A person skilled in the art would be able to choose the appropriate regulatory/promoter sequence based on the intended use of the iRNA transgene.

In a specific embodiment, viral vectors that contain nucleic acid sequences encoding an iRNA can be used. For example, a retroviral vector can be used (see Miller *et al.*, Meth. Enzymol. 217:581-599 (1993)). These retroviral vectors contain the components necessary for the correct packaging of the viral genome and integration into the host cell DNA. The nucleic acid sequences encoding an iRNA are cloned into one or more vectors, which facilitates delivery of the nucleic acid into a patient. More detail about retroviral vectors can be found, for example, in Boesen *et al.*, Biotherapy 6:291-302 (1994), which describes the use of a retroviral vector to deliver the mdr1 gene to hematopoietic stem cells in order to make the stem cells more resistant to chemotherapy. Other references illustrating the use of retroviral vectors in gene therapy are: Clowes *et al.*, J. Clin. Invest. 93:644-651 (1994); Kiem *et al.*, Blood 83:1467-1473 (1994); Salmons and Gunzberg, Human Gene Therapy 4:129-141 (1993); and Grossman and Wilson, Curr. Opin. in Genetics and Devel. 3:110-114 (1993). Lentiviral vectors contemplated for use include, for example, the HIV based vectors described in U.S. Patent Nos. 6,143,520; 5,665,557; and 5,981,276, which are herein incorporated by reference.

Adenoviruses are also contemplated for use in delivery of iRNAs. Adenoviruses are especially attractive vehicles, *e.g.*, for delivering genes to respiratory epithelia. Adenoviruses naturally infect respiratory epithelia where they cause a mild disease. Other targets for adenovirus-based delivery systems are liver, the central nervous system, endothelial cells, and muscle. Adenoviruses have the advantage of being capable of infecting non-dividing cells. Kozarsky and Wilson, Current Opinion in Genetics and Development 3:499-503 (1993) present a review of adenovirus-based gene therapy. Bout *et al.*, Human Gene Therapy 5:3-10 (1994) demonstrated the use of adenovirus vectors to transfer genes to the respiratory epithelia of rhesus monkeys. Other instances of the use of adenoviruses in gene therapy can be found in Rosenfeld *et al.*, Science 252:431-434 (1991); Rosenfeld *et al.*, Cell 68:143-155 (1992); Mastrangeli *et al.*, J. Clin. Invest. 91:225-234 (1993); PCT Publication WO94/12649; and Wang, *et al.*, Gene Therapy 2:775-783 (1995). A suitable AV vector for expressing an iRNA featured in the invention, a method for constructing the recombinant AV vector, and a method for delivering the vector into target cells, are described in Xia H *et al.* (2002), *Nat. Biotech.* 20: 1006-1010.

Use of Adeno-associated virus (AAV) vectors is also contemplated (Walsh *et al.*, Proc. Soc. Exp. Biol. Med. 204:289-300 (1993); U.S. Pat. No. 5,436,146). In one embodiment, the iRNA can be expressed as two separate, complementary single-stranded RNA molecules from a recombinant AAV vector having, for example, either the U6 or H1 RNA promoters, or the cytomegalovirus (CMV) promoter. Suitable AAV vectors for expressing the dsRNA featured in the invention, methods for constructing the recombinant AV vector, and methods for delivering the vectors into target cells are described in Samulski R *et al.* (1987), J. Virol. 61: 3096-3101; Fisher K J *et al.* (1996), J. Virol, 70: 520-532; Samulski R *et al.* (1989), J. Virol. 63: 3822-3826; U.S. Pat. No. 5,252,479; U.S. Pat. No. 5,139,941; International Patent Application No. WO 94/13788; and International Patent Application No. WO 93/24641, the entire disclosures of which are herein incorporated by reference.

Another preferred viral vector is a pox virus such as a vaccinia virus, for example an attenuated vaccinia such as Modified Virus Ankara (MVA) or NYVAC, an avipox such as fowl pox or canary pox.

The tropism of viral vectors can be modified by pseudotyping the vectors with envelope proteins or other surface antigens from other viruses, or by substituting different viral capsid proteins, as appropriate. For example, lentiviral vectors can be pseudotyped with surface proteins from vesicular stomatitis virus (VSV), rabies, Ebola, Mokola, and the like. AAV vectors can be made to target different cells by engineering the vectors to express different capsid protein serotypes; see, *e.g.*, Rabinowitz J E *et al.* (2002), J Virol 76:791-801, the entire disclosure of which is herein incorporated by reference.

The pharmaceutical preparation of a vector can include the vector in an acceptable diluent, or can include a slow release matrix in which the gene delivery vehicle is imbedded. Alternatively, where the complete gene delivery vector can be produced intact from recombinant cells, *e.g.*, retroviral vectors, the pharmaceutical preparation can include one or more cells which produce the gene delivery system.

III. Pharmaceutical compositions containing iRNA

In one embodiment, provided herein are pharmaceutical compositions containing an iRNA and a pharmaceutically acceptable carrier. The pharmaceutical composition containing

the iRNA is useful for treating a disease or disorder associated with the expression or activity of a TMPRSS6 gene, such as pathological processes mediated by TMPRSS6 expression. Such pharmaceutical compositions are formulated based on the mode of delivery. One example is compositions that are formulated for systemic administration via parenteral delivery, *e.g.*, by intravenous (IV) delivery.

The pharmaceutical compositions featured herein are administered in dosages sufficient to inhibit expression of TMPRSS6 genes. In general, a suitable dose of iRNA will be in the range of 0.01 to 200.0 milligrams per kilogram body weight of the recipient per day, generally in the range of 1 to 50 mg per kilogram body weight per day. For example, the dsRNA can be administered at 0.05 mg/kg, 0.5 mg/kg, 1 mg/kg, 1.5 mg/kg, 2 mg/kg, 3 mg/kg, 10 mg/kg, 20 mg/kg, 30 mg/kg, 40 mg/kg, or 50 mg/kg per single dose. The pharmaceutical composition may be administered once daily, or once weekly, or once monthly, or once every other month. The composition can alternatively be administered twice per week or twice per month, or once every two, three or four weeks. In some embodiments, the iRNA is administered as two, three, or more sub-doses at appropriate intervals throughout the day or even using continuous infusion or delivery through a controlled release formulation. In that case, the iRNA contained in each sub-dose must be correspondingly smaller in order to achieve the total daily dosage. The dosage unit can also be compounded for delivery over several days, *e.g.*, using a conventional sustained release formulation which provides sustained release of the iRNA over a several day period. Sustained release formulations are well known in the art and are particularly useful for delivery of agents at a particular site, such as could be used with the agents of the present invention. In this embodiment, the dosage unit contains a corresponding multiple of the daily dose.

The effect of a single dose on TMPRSS6 levels can be long lasting, such that subsequent doses are administered at not more than 3, 4, or 5 day intervals, or at not more than 1, 2, 3, or 4 week intervals.

The skilled artisan will appreciate that certain factors may influence the dosage and timing required to effectively treat a subject, including but not limited to the severity of the disease or disorder, previous treatments, the general health and/or age of the subject, and other diseases present. Moreover, treatment of a subject with a therapeutically effective amount of a composition can include a single treatment or a series of treatments. Estimates

of effective dosages and *in vivo* half-lives for the individual iRNAs encompassed by the invention can be made using conventional methodologies or on the basis of *in vivo* testing using an appropriate animal model, as described elsewhere herein.

Advances in mouse genetics have generated a number of mouse models for the study of various human diseases, such as pathological processes mediated by TMPRSS6 expression. Such models can be used for *in vivo* testing of iRNA, as well as for determining a therapeutically effective dose. A suitable mouse model is, for example, a mouse containing a transgene expressing human TMPRSS6.

The present invention also includes pharmaceutical compositions and formulations that include the iRNA compounds featured in the invention. The pharmaceutical compositions of the present invention may be administered in a number of ways depending upon whether local or systemic treatment is desired and upon the area to be treated. Administration may be topical (*e.g.*, by a transdermal patch), pulmonary, *e.g.*, by inhalation or insufflation of powders or aerosols, including by nebulizer; intratracheal, intranasal, epidermal and transdermal, oral or parenteral. Parenteral administration includes intravenous, intraarterial, subcutaneous, intraperitoneal or intramuscular injection or infusion; subdermal, *e.g.*, via an implanted device; or intracranial, *e.g.*, by intraparenchymal, intrathecal or intraventricular, administration.

The iRNA can be delivered in a manner to target a particular tissue, such as the liver (*e.g.*, the hepatocytes of the liver).

Pharmaceutical compositions and formulations for topical administration may include transdermal patches, ointments, lotions, creams, gels, drops, suppositories, sprays, liquids and powders. Conventional pharmaceutical carriers, aqueous, powder or oily bases, thickeners and the like may be necessary or desirable. Coated condoms, gloves and the like may also be useful. Suitable topical formulations include those in which the iRNAs featured in the invention are in admixture with a topical delivery agent such as lipids, liposomes, fatty acids, fatty acid esters, steroids, chelating agents and surfactants. Suitable lipids and liposomes include neutral (*e.g.*, dioleoylphosphatidyl DOPE ethanolamine, dimyristoylphosphatidyl choline DMPC, distearolyphosphatidyl choline) negative (*e.g.*, dimyristoylphosphatidyl glycerol DMPG) and cationic (*e.g.*, dioleoyltetramethylaminopropyl DOTAP and dioleoylphosphatidyl ethanolamine DOTMA). iRNAs featured in the invention may be

encapsulated within liposomes or may form complexes thereto, in particular to cationic liposomes. Alternatively, iRNAs may be complexed to lipids, in particular to cationic lipids. Suitable fatty acids and esters include but are not limited to arachidonic acid, oleic acid, eicosanoic acid, lauric acid, caprylic acid, capric acid, myristic acid, palmitic acid, stearic acid, linoleic acid, linolenic acid, dicaprate, tricaprate, monoolein, dilaurin, glyceryl 1-monocaprate, 1-dodecylazacycloheptan-2-one, an acylcarnitine, an acylcholine, or a C_{1-20} alkyl ester (*e.g.*, isopropylmyristate IPM), monoglyceride, diglyceride or pharmaceutically acceptable salt thereof. Topical formulations are described in detail in U.S. Patent No. 6,747,014, which is incorporated herein by reference.

Liposomal formulations

There are many organized surfactant structures besides microemulsions that have been studied and used for the formulation of drugs. These include monolayers, micelles, bilayers and vesicles. Vesicles, such as liposomes, have attracted great interest because of their specificity and the duration of action they offer from the standpoint of drug delivery. As used in the present invention, the term "liposome" means a vesicle composed of amphiphilic lipids arranged in a spherical bilayer or bilayers.

Liposomes are unilamellar or multilamellar vesicles which have a membrane formed from a lipophilic material and an aqueous interior. The aqueous portion contains the composition to be delivered. Cationic liposomes possess the advantage of being able to fuse to the cell wall. Non-cationic liposomes, although not able to fuse as efficiently with the cell wall, are taken up by macrophages *in vivo*.

In order to traverse intact mammalian skin, lipid vesicles must pass through a series of fine pores, each with a diameter less than 50 nm, under the influence of a suitable transdermal gradient. Therefore, it is desirable to use a liposome which is highly deformable and able to pass through such fine pores.

Further advantages of liposomes include; liposomes obtained from natural phospholipids are biocompatible and biodegradable; liposomes can incorporate a wide range of water and lipid soluble drugs; liposomes can protect encapsulated drugs in their internal compartments from metabolism and degradation (Rosoff, in Pharmaceutical Dosage Forms, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume

1, p. 245). Important considerations in the preparation of liposome formulations are the lipid surface charge, vesicle size and the aqueous volume of the liposomes.

Liposomes are useful for the transfer and delivery of active ingredients to the site of action. Because the liposomal membrane is structurally similar to biological membranes, when liposomes are applied to a tissue, the liposomes start to merge with the cellular membranes and as the merging of the liposome and cell progresses, the liposomal contents are emptied into the cell where the active agent may act.

Liposomal formulations have been the focus of extensive investigation as the mode of delivery for many drugs. There is growing evidence that for topical administration, liposomes present several advantages over other formulations. Such advantages include reduced side-effects related to high systemic absorption of the administered drug, increased accumulation of the administered drug at the desired target, and the ability to administer a wide variety of drugs, both hydrophilic and hydrophobic, into the skin.

Several reports have detailed the ability of liposomes to deliver agents including high-molecular weight DNA into the skin. Compounds including analgesics, antibodies, hormones and high-molecular weight DNAs have been administered to the skin. The majority of applications resulted in the targeting of the upper epidermis

Liposomes fall into two broad classes. Cationic liposomes are positively charged liposomes which interact with the negatively charged DNA molecules to form a stable complex. The positively charged DNA/liposome complex binds to the negatively charged cell surface and is internalized in an endosome. Due to the acidic pH within the endosome, the liposomes are ruptured, releasing their contents into the cell cytoplasm (Wang *et al.*, Biochem. Biophys. Res. Commun., 1987, 147, 980-985).

Liposomes which are pH-sensitive or negatively-charged, entrap nucleic acids rather than complex with it. Since both the DNA and the lipid are similarly charged, repulsion rather than complex formation occurs. Nevertheless, some DNA is entrapped within the aqueous interior of these liposomes. pH-sensitive liposomes have been used to deliver nucleic acids encoding the thymidine kinase gene to cell monolayers in culture. Expression of the exogenous gene was detected in the target cells (Zhou *et al.*, Journal of Controlled Release, 1992, 19, 269-274).

One major type of liposomal composition includes phospholipids other than naturally-derived phosphatidylcholine. Neutral liposome compositions, for example, can be formed from dimyristoyl phosphatidylcholine (DMPC) or dipalmitoyl phosphatidylcholine (DPPC). Anionic liposome compositions generally are formed from dimyristoyl phosphatidylglycerol, while anionic fusogenic liposomes are formed primarily from dioleoyl phosphatidylethanolamine (DOPE). Another type of liposomal composition is formed from phosphatidylcholine (PC) such as, for example, soybean PC, and egg PC. Another type is formed from mixtures of phospholipid and/or phosphatidylcholine and/or cholesterol.

Several studies have assessed the topical delivery of liposomal drug formulations to the skin. Application of liposomes containing interferon to guinea pig skin resulted in a reduction of skin herpes sores while delivery of interferon via other means (*e.g.*, as a solution or as an emulsion) were ineffective (Weiner *et al.*, Journal of Drug Targeting, 1992, 2, 405-410). Further, an additional study tested the efficacy of interferon administered as part of a liposomal formulation to the administration of interferon using an aqueous system, and concluded that the liposomal formulation was superior to aqueous administration (du Plessis *et al.*, Antiviral Research, 1992, 18, 259-265).

Non-ionic liposomal systems have also been examined to determine their utility in the delivery of drugs to the skin, in particular systems comprising non-ionic surfactant and cholesterol. Non-ionic liposomal formulations comprising Novasome™ I (glyceryl dilaurate/cholesterol/polyoxyethylene-10-stearyl ether) and Novasome™ II (glyceryl distearate/cholesterol/polyoxyethylene-10-stearyl ether) were used to deliver cyclosporin-A into the dermis of mouse skin. Results indicated that such non-ionic liposomal systems were effective in facilitating the deposition of cyclosporine A into different layers of the skin (Hu *et al.* S.T.P.Pharma. Sci., 1994, 4, 6, 466).

Liposomes also include "sterically stabilized" liposomes, a term which, as used herein, refers to liposomes comprising one or more specialized lipids that, when incorporated into liposomes, result in enhanced circulation lifetimes relative to liposomes lacking such specialized lipids. Examples of sterically stabilized liposomes are those in which part of the vesicle-forming lipid portion of the liposome (A) comprises one or more glycolipids, such as monosialoganglioside G_{M1}, or (B) is derivatized with one or more hydrophilic polymers, such as a polyethylene glycol (PEG) moiety. While not wishing to be bound by any particular theory, it is thought in the art that, at least for sterically stabilized liposomes containing

gangliosides, sphingomyelin, or PEG-derivatized lipids, the enhanced circulation half-life of these sterically stabilized liposomes derives from a reduced uptake into cells of the reticuloendothelial system (RES) (Allen *et al.*, FEBS Letters, 1987, 223, 42; Wu *et al.*, Cancer Research, 1993, 53, 3765).

Various liposomes comprising one or more glycolipids are known in the art. Papahadjopoulos *et al.* (Ann. N.Y. Acad. Sci., 1987, 507, 64) reported the ability of monosialoganglioside G_{M1}, galactocerebroside sulfate and phosphatidylinositol to improve blood half-lives of liposomes. These findings were expounded upon by Gabizon *et al.* (Proc. Natl. Acad. Sci. U.S.A., 1988, 85, 6949). U.S. Pat. No. 4,837,028 and WO 88/04924, both to Allen *et al.*, disclose liposomes comprising (1) sphingomyelin and (2) the ganglioside G_{M1} or a galactocerebroside sulfate ester. U.S. Pat. No. 5,543,152 (Webb *et al.*) discloses liposomes comprising sphingomyelin. Liposomes comprising 1,2-sn-dimyristoylphosphatidylcholine are disclosed in WO 97/13499 (Lim *et al*).

Many liposomes comprising lipids derivatized with one or more hydrophilic polymers, and methods of preparation thereof, are known in the art. Sunamoto *et al.* (Bull. Chem. Soc. Jpn., 1980, 53, 2778) described liposomes comprising a nonionic detergent, $2C_{1215G}$, that contains a PEG moiety. Illum *et al.* (FEBS Lett., 1984, 167, 79) noted that hydrophilic coating of polystyrene particles with polymeric glycols results in significantly enhanced blood half-lives. Synthetic phospholipids modified by the attachment of carboxylic groups of polyalkylene glycols (*e.g.*, PEG) are described by Sears (U.S. Pat. Nos. 4,426,330 and 4,534,899). Klibanov *et al.* (FEBS Lett., 1990, 268, 235) described experiments demonstrating that liposomes comprising phosphatidylethanolamine (PE) derivatized with PEG or PEG stearate have significant increases in blood circulation half-lives. Blume *et al.* (Biochimica et Biophysica Acta, 1990, 1029, 91) extended such observations to other PEG-derivatized phospholipids, *e.g.*, DSPE-PEG, formed from the combination of distearoylphosphatidylethanolamine (DSPE) and PEG. Liposomes having covalently bound PEG moieties on their external surface are described in European Patent No. EP 0 445 131 B1 and WO 90/04384 to Fisher. Liposome compositions containing 1-20 mole percent of PE derivatized with PEG, and methods of use thereof, are described by Woodle *et al.* (U.S. Pat. Nos. 5,013,556 and 5,356,633) and Martin *et al.* (U.S. Pat. No. 5,213,804 and European Patent No. EP 0 496 813 B1). Liposomes comprising a number of other lipid-polymer conjugates are disclosed in WO 91/05545 and U.S. Pat. No. 5,225,212 (both to Martin *et al.*)

and in WO 94/20073 (Zalipsky *et al.*) Liposomes comprising PEG-modified ceramide lipids are described in WO 96/10391 (Choi *et al*). U.S. Pat. No. 5,540,935 (Miyazaki *et al.*) and U.S. Pat. No. 5,556,948 (Tagawa *et al.*) describes PEG-containing liposomes that can be further derivatized with functional moieties on their surfaces.

A number of liposomes comprising nucleic acids are known in the art. WO 96/40062 to Thierry *et al.* discloses methods for encapsulating high molecular weight nucleic acids in liposomes. U.S. Pat. No. 5,264,221 to Tagawa *et al.* discloses protein-bonded liposomes and asserts that the contents of such liposomes may include a dsRNA. U.S. Pat. No. 5,665,710 to Rahman *et al.* describes certain methods of encapsulating oligodeoxynucleotides in liposomes. WO 97/04787 to Love *et al.* discloses liposomes comprising dsRNAs targeted to the raf gene.

Transfersomes are yet another type of liposomes, and are highly deformable lipid aggregates which are attractive candidates for drug delivery vehicles. Transfersomes may be described as lipid droplets which are so highly deformable that they are easily able to penetrate through pores which are smaller than the droplet. Transfersomes are adaptable to the environment in which they are used, *e.g.*, they are self-optimizing (adaptive to the shape of pores in the skin), self-repairing, frequently reach their targets without fragmenting, and often self-loading. To make transfersomes it is possible to add surface edge-activators, usually surfactants, to a standard liposomal composition. Transfersomes have been used to deliver serum albumin to the skin. The transfersome-mediated delivery of serum albumin has been shown to be as effective as subcutaneous injection of a solution containing serum albumin.

Surfactants find wide application in formulations such as emulsions (including microemulsions) and liposomes. The most common way of classifying and ranking the properties of the many different types of surfactants, both natural and synthetic, is by the use of the hydrophile/lipophile balance (HLB). The nature of the hydrophilic group (also known as the "head") provides the most useful means for categorizing the different surfactants used in formulations (Rieger, in Pharmaceutical Dosage Forms, Marcel Dekker, Inc., New York, N.Y., 1988, p. 285).

If the surfactant molecule is not ionized, it is classified as a nonionic surfactant. Nonionic surfactants find wide application in pharmaceutical and cosmetic products and are

usable over a wide range of pH values. In general their HLB values range from 2 to about 18 depending on their structure. Nonionic surfactants include nonionic esters such as ethylene glycol esters, propylene glycol esters, glyceryl esters, polyglyceryl esters, sorbitan esters, sucrose esters, and ethoxylated esters. Nonionic alkanolamides and ethers such as fatty alcohol ethoxylates, propoxylated alcohols, and ethoxylated/propoxylated block polymers are also included in this class. The polyoxyethylene surfactants are the most popular members of the nonionic surfactant class.

If the surfactant molecule carries a negative charge when it is dissolved or dispersed in water, the surfactant is classified as anionic. Anionic surfactants include carboxylates such as soaps, acyl lactylates, acyl amides of amino acids, esters of sulfuric acid such as alkyl sulfates and ethoxylated alkyl sulfates, sulfonates such as alkyl benzene sulfonates, acyl isethionates, acyl taurates and sulfosuccinates, and phosphates. The most important members of the anionic surfactant class are the alkyl sulfates and the soaps.

If the surfactant molecule carries a positive charge when it is dissolved or dispersed in water, the surfactant is classified as cationic. Cationic surfactants include quaternary ammonium salts and ethoxylated amines. The quaternary ammonium salts are the most used members of this class.

If the surfactant molecule has the ability to carry either a positive or negative charge, the surfactant is classified as amphoteric. Amphoteric surfactants include acrylic acid derivatives, substituted alkylamides, N-alkylbetaines and phosphatides.

The use of surfactants in drug products, formulations and in emulsions has been reviewed (Rieger, in Pharmaceutical Dosage Forms, Marcel Dekker, Inc., New York, N.Y., 1988, p. 285).

Nucleic acid lipid particles

In one embodiment, a TMPRSS6 dsRNA featured in the invention is fully encapsulated in the lipid formulation, *e.g.*, to form a SPLP, pSPLP, SNALP, or other nucleic acid-lipid particle. As used herein, the term "SNALP" refers to a stable nucleic acid-lipid particle, including SPLP. As used herein, the term "SPLP" refers to a nucleic acid-lipid particle comprising plasmid DNA encapsulated within a lipid vesicle. SNALPs and SPLPs typically contain a cationic lipid, a non-cationic lipid, and a lipid that prevents aggregation of

the particle (*e.g.*, a PEG-lipid conjugate). SNALPs and SPLPs are extremely useful for systemic applications, as they exhibit extended circulation lifetimes following intravenous (i.v.) injection and accumulate at distal sites (*e.g.*, sites physically separated from the administration site). SPLPs include "pSPLP," which include an encapsulated condensing agent-nucleic acid complex as set forth in PCT Publication No. WO 00/03683. The particles of the present invention typically have a mean diameter of about 50 nm to about 150 nm, more typically about 60 nm to about 130 nm, more typically about 70 nm to about 110 nm, most typically about 70 nm to about 90 nm, and are substantially nontoxic. In addition, the nucleic acids when present in the nucleic acid- lipid particles of the present invention are resistant in aqueous solution to degradation with a nuclease. Nucleic acid-lipid particles and their method of preparation are disclosed in, *e.g.*, U.S. Patent Nos. 5,976,567; 5,981,501; 6,534,484; 6,586,410; 6,815,432; and PCT Publication No. WO 96/40964.

In one embodiment, the lipid to drug ratio (mass/mass ratio) (*e.g.*, lipid to dsRNA ratio) will be in the range of from about 1:1 to about 50:1, from about 1:1 to about 25:1, from about 3:1 to about 15:1, from about 4:1 to about 10:1, from about 5:1 to about 9:1, or about 6:1 to about 9:1.

The cationic lipid can be, for example, N,N-dioleyl-N,N-dimethylammonium chloride (DODAC), N,N-distearyl-N,N-dimethylammonium bromide (DDAB), N-(I -(2,3-dioleoyloxy)propyl)-N,N,N-trimethylammonium chloride (DOTAP), N-(I -(2,3-dioleyloxy)propyl)-N,N,N-trimethylammonium chloride (DOTMA), N,N-dimethyl-2,3-dioleyloxy)propylamine (DODMA), 1,2-DiLinoleyloxy-N,N-dimethylaminopropane (DLinDMA), 1,2-Dilinolenyloxy-N,N-dimethylaminopropane (DLenDMA), 1,2-Dilinoleylcarbamoyloxy-3-dimethylaminopropane (DLin-C-DAP), 1,2-Dilinoleyoxy-3-(dimethylamino)acetoxypropane (DLin-DAC), 1,2-Dilinoleyoxy-3-morpholinopropane (DLin-MA), 1,2-Dilinoleoyl-3-dimethylaminopropane (DLinDAP), 1,2-Dilinoleylthio-3-dimethylaminopropane (DLin-S-DMA), 1-Linoleoyl-2-linoleyloxy-3-dimethylaminopropane (DLin-2-DMAP), 1,2-Dilinoleyloxy-3-trimethylaminopropane chloride salt (DLin-TMA.Cl), 1,2-Dilinoleoyl-3-trimethylaminopropane chloride salt (DLin-TAP.Cl), 1,2-Dilinoleyloxy-3-(N-methylpiperazino)propane (DLin-MPZ), or 3-(N,N-Dilinoleylamino)-1,2-propanediol (DLinAP), 3-(N,N-Dioleylamino)-1,2-propanedio (DOAP), 1,2-Dilinoleyloxo-3-(2-N,N-dimethylamino)ethoxypropane (DLin-EG-DMA), 1,2-Dilinolenyloxy-N,N-dimethylaminopropane (DLinDMA), 2,2-Dilinoleyl-4-dimethylaminomethyl-[1,3]-dioxolane

(DLin-K-DMA) or analogs thereof, (3aR,5s,6aS)-N,N-dimethyl-2,2-di((9Z,12Z)-octadeca-9,12-dienyl)tetrahydro-3aH-cyclopenta[d][1,3]dioxol-5-amine (ALN100), (6Z,9Z,28Z,31Z)-heptatriaconta-6,9,28,31-tetraen-19-yl 4-(dimethylamino)butanoate (MC3), 1,1'-(2-(4-(2-((2-(bis(2-hydroxydodecyl)amino)ethyl)(2-hydroxydodecyl)amino)ethyl)piperazin-1-yl)ethylazanediyl)didodecan-2-ol (Tech G1), or a mixture thereof. The cationic lipid may comprise from about 20 mol % to about 50 mol % or about 40 mol % of the total lipid present in the particle.

In another embodiment, the compound 2,2-Dilinoleyl-4-dimethylaminoethyl-[1,3]-dioxolane can be used to prepare lipid-siRNA nanoparticles. Synthesis of 2,2-Dilinoleyl-4-dimethylaminoethyl-[1,3]-dioxolane is described in United States provisional patent application number 61/107,998 filed on October 23, 2008, which is herein incorporated by reference.

In one embodiment, the lipid-siRNA particle includes 40% 2, 2-Dilinoleyl-4-dimethylaminoethyl-[1,3]-dioxolane: 10% DSPC: 40% Cholesterol: 10% PEG-C-DOMG (mole percent) with a particle size of 63.0 ± 20 nm and a 0.027 siRNA/Lipid Ratio.

The non-cationic lipid may be an anionic lipid or a neutral lipid including, but not limited to, distearoylphosphatidylcholine (DSPC), dioleoylphosphatidylcholine (DOPC), dipalmitoylphosphatidylcholine (DPPC), dioleoylphosphatidylglycerol (DOPG), dipalmitoylphosphatidylglycerol (DPPG), dioleoyl-phosphatidylethanolamine (DOPE), palmitoyloleoylphosphatidylcholine (POPC), palmitoyloleoylphosphatidylethanolamine (POPE), dioleoyl- phosphatidylethanolamine 4-(N-maleimidomethyl)-cyclohexane-l-carboxylate (DOPE-mal), dipalmitoyl phosphatidyl ethanolamine (DPPE), dimyristoylphosphoethanolamine (DMPE), distearoyl-phosphatidyl-ethanolamine (DSPE), 16-O-monomethyl PE, 16-O-dimethyl PE, 18-1 -trans PE, 1 -stearoyl-2-oleoyl-phosphatidyethanolamine (SOPE), cholesterol, or a mixture thereof. The non-cationic lipid may be from about 5 mol % to about 90 mol %, about 10 mol %, or about 58 mol % if cholesterol is included, of the total lipid present in the particle.

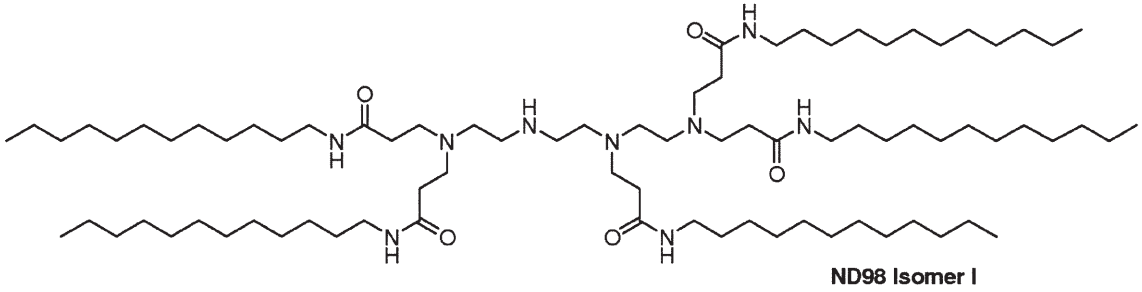
The conjugated lipid that inhibits aggregation of particles may be, for example, a polyethyleneglycol (PEG)-lipid including, without limitation, a PEG-diacylglycerol (DAG), a PEG-dialkyloxypropyl (DAA), a PEG-phospholipid, a PEG-ceramide (Cer), or a mixture thereof. The PEG-DAA conjugate may be, for example, a PEG-dilauryloxypropyl (C_{12}), a

PEG-dimyristyloxypropyl (C_{i4}), a PEG-dipalmityloxypropyl (C_{i6}), or a PEG-distearyloxypropyl ($C]_8$). The conjugated lipid that prevents aggregation of particles may be from 0 mol % to about 20 mol % or about 2 mol % of the total lipid present in the particle.

In some embodiments, the nucleic acid-lipid particle further includes cholesterol at, *e.g.*, about 10 mol % to about 60 mol % or about 48 mol % of the total lipid present in the particle.

LNP01

In one embodiment, the lipidoid ND98·4HCl (MW 1487) (see U.S. Patent Application No. 12/056,230, filed 3/26/2008, which is herein incorporated by reference in its entirety), Cholesterol (Sigma-Aldrich), and PEG-Ceramide C16 (Avanti Polar Lipids) can be used to prepare lipid-dsRNA nanoparticles (*i.e.*, LNP01 particles). Stock solutions of each in ethanol can be prepared as follows: ND98, 133 mg/ml; Cholesterol, 25 mg/ml, PEG-Ceramide C16, 100 mg/ml. The ND98, Cholesterol, and PEG-Ceramide C16 stock solutions can then be combined in a, *e.g.*, 42:48:10 molar ratio. The combined lipid solution can be mixed with aqueous dsRNA (*e.g.*, in sodium acetate pH 5) such that the final ethanol concentration is about 35-45% and the final sodium acetate concentration is about 100-300 mM. Lipid-dsRNA nanoparticles typically form spontaneously upon mixing. Depending on the desired particle size distribution, the resultant nanoparticle mixture can be extruded through a polycarbonate membrane (*e.g.*, 100 nm cut-off) using, for example, a thermobarrel extruder, such as Lipex Extruder (Northern Lipids, Inc). In some cases, the extrusion step can be omitted. Ethanol removal and simultaneous buffer exchange can be accomplished by, for example, dialysis or tangential flow filtration. Buffer can be exchanged with, for example, phosphate buffered saline (PBS) at about pH 7, *e.g.*, about pH 6.9, about pH 7.0, about pH 7.1, about pH 7.2, about pH 7.3, or about pH 7.4.



Formula I

LNP01 formulations are described, *e.g.*, in International Application Publication No. WO 2008/042973, which is hereby incorporated by reference.

Additional exemplary lipid-dsRNA formulations are as follows:

	Cationic Lipid	**cationic lipid/non-cationic lipid/cholesterol/PEG-lipid conjugate** **Lipid:siRNA ratio**
SNALP-1	l,2-Dilinolenyloxy-N,N-dimethylaminopropane (DLinDMA)	DLinDMA/DPPC/Cholesterol/PEG-cDMA (57.1/7.1/34.4/1.4) lipid:siRNA ~ 7:1
S-XTC	2,2-Dilinoleyl-4-dimethylaminoethyl-[1,3]-dioxolane (XTC)	XTC/DPPC/Cholesterol/PEG-cDMA 57.1/7.1/34.4/1.4 lipid:siRNA ~ 7:1
LNP05	2,2-Dilinoleyl-4-dimethylaminoethyl-[1,3]-dioxolane (XTC)	XTC/DSPC/Cholesterol/PEG-DMG 57.5/7.5/31.5/3.5 lipid:siRNA ~ 6:1
LNP06	2,2-Dilinoleyl-4-dimethylaminoethyl-[1,3]-dioxolane (XTC)	XTC/DSPC/Cholesterol/PEG-DMG 57.5/7.5/31.5/3.5 lipid:siRNA ~ 11:1
LNP07	2,2-Dilinoleyl-4-dimethylaminoethyl-[1,3]-dioxolane (XTC)	XTC/DSPC/Cholesterol/PEG-DMG 60/7.5/31/1.5, lipid:siRNA ~ 6:1
LNP08	2,2-Dilinoleyl-4-dimethylaminoethyl-[1,3]-dioxolane (XTC)	XTC/DSPC/Cholesterol/PEG-DMG 60/7.5/31/1.5, lipid:siRNA ~ 11:1
LNP09	2,2-Dilinoleyl-4-dimethylaminoethyl-[1,3]-dioxolane (XTC)	XTC/DSPC/Cholesterol/PEG-DMG 50/10/38.5/1.5 Lipid:siRNA 10:1

	Cationic Lipid	**cationic lipid/non-cationic lipid/cholesterol/PEG-lipid conjugate** **Lipid:siRNA ratio**
LNP10	(3aR,5s,6aS)-N,N-dimethyl-2,2-di((9Z,12Z)-octadeca-9,12-dienyl)tetrahydro-3aH-cyclopenta[d][1,3]dioxol-5-amine (ALN100)	ALN100/DSPC/Cholesterol/PEG-DMG 50/10/38.5/1.5 Lipid:siRNA 10:1
LNP11	(6Z,9Z,28Z,31Z)-heptatriaconta-6,9,28,31-tetraen-19-yl 4-(dimethylamino)butanoate (MC3)	MC-3/DSPC/Cholesterol/PEG-DMG 50/10/38.5/1.5 Lipid:siRNA 10:1
LNP12	1,1'-(2-(4-(2-((2-(bis(2-hydroxydodecyl)amino)ethyl)(2-hydroxydodecyl)amino)ethyl)piperazin-1-yl)ethylazanediyl)didodecan-2-ol (C12-200)	C12-200/DSPC/Cholesterol/PEG-DMG 50/10/38.5/1.5 Lipid:siRNA 10:1
LNP13	XTC	XTC/DSPC/Chol/PEG-DMG 50/10/38.5/1.5 Lipid:siRNA: 33:1
LNP14	MC3	MC3/DSPC/Chol/PEG-DMG 40/15/40/5 Lipid:siRNA: 11:1
LNP15	MC3	MC3/DSPC/Chol/PEG-DSG/GalNAc-PEG-DSG 50/10/35/4.5/0.5 Lipid:siRNA: 11:1
LNP16	MC3	MC3/DSPC/Chol/PEG-DMG 50/10/38.5/1.5 Lipid:siRNA: 7:1
LNP17	MC3	MC3/DSPC/Chol/PEG-DSG 50/10/38.5/1.5 Lipid:siRNA: 10:1
LNP18	MC3	MC3/DSPC/Chol/PEG-DMG 50/10/38.5/1.5 Lipid:siRNA: 12:1
LNP19	MC3	MC3/DSPC/Chol/PEG-DMG 50/10/35/5 Lipid:siRNA: 8:1
LNP20	MC3	MC3/DSPC/Chol/PEG-DPG 50/10/38.5/1.5 Lipid:siRNA: 10:1
LNP21	C12-200	C12-200/DSPC/Chol/PEG-DSG 50/10/38.5/1.5 Lipid:siRNA: 7:1

	Cationic Lipid	cationic lipid/non-cationic lipid/cholesterol/PEG-lipid conjugate Lipid:siRNA ratio
LNP22	XTC	XTC/DSPC/Chol/PEG-DSG 50/10/38.5/1.5 Lipid:siRNA: 10:1

DSPC: distearoylphosphatidylcholine

DPPC: dipalmitoylphosphatidylcholine

PEG-DMG: PEG-didimyristoyl glycerol (C14-PEG, or PEG-C14) (PEG with avg mol wt of 2000)

PEG-DSG: PEG-distyryl glycerol (C18-PEG, or PEG-C18) (PEG with avg mol wt of 2000)

PEG-cDMA: PEG-carbamoyl-1,2-dimyristyloxypropylamine (PEG with avg mol wt of 2000)

SNALP (1,2-Dilinolenyloxy-N,N-dimethylaminopropane (DLinDMA)) comprising formulations are described in International Publication No. WO2009/127060, filed April 15, 2009, which is hereby incorporated by reference.

XTC comprising formulations are described, e.g., in U.S. Provisional Serial No. 61/148,366, filed January 29, 2009; U.S. Provisional Serial No. 61/156,851, filed March 2, 2009; U.S. Provisional Serial No. filed June 10, 2009; U.S. Provisional Serial No. 61/228,373, filed July 24, 2009; U.S. Provisional Serial No. 61/239,686, filed September 3, 2009, and International Application No. PCT/US2010/022614, filed January 29, 2010, which are hereby incorporated by reference.

MC3 comprising formulations are described, *e.g.*, in U.S. Provisional Serial No. 61/244,834, filed September 22, 2009, U.S. Provisional Serial No. 61/185,800, filed June 10, 2009, and International Application No. PCT/US10/28224, filed June 10, 2010, which are hereby incorporated by reference.

ALNY-100 comprising formulations are described, *e.g.*, International patent application number PCT/US09/63933, filed on November 10, 2009, which is hereby incorporated by reference.

C12-200 comprising formulations are described in U.S. Provisional Serial No. 61/175,770, filed May 5, 2009and International Application No. PCT/US10/33777, filed May 5, 2010, which are hereby incorporated by reference.

As used herein, the term "LNPXX", wherein the "XX" are numerals, is also referred to as "AFXX" herein. For example, LNP09 is also referred to AF09 and LNP12 is also known as or referred to as AF12.

Synthesis of cationic lipids

Any of the compounds, *e.g.*, cationic lipids and the like, used in the nucleic acid-lipid particles featured in the invention can be prepared by known organic synthesis techniques, including the methods described in more detail in the Examples. All substituents are as defined below unless indicated otherwise.

"Alkyl" means a straight chain or branched, noncyclic or cyclic, saturated aliphatic hydrocarbon containing from 1 to 24 carbon atoms. Representative saturated straight chain alkyls include methyl, ethyl, n-propyl, n-butyl, n-pentyl, n-hexyl, and the like; while saturated branched alkyls include isopropyl, sec-butyl, isobutyl, tert-butyl, isopentyl, and the like. Representative saturated cyclic alkyls include cyclopropyl, cyclobutyl, cyclopentyl, cyclohexyl, and the like; while unsaturated cyclic alkyls include cyclopentenyl and cyclohexenyl, and the like.

"Alkenyl" means an alkyl, as defined above, containing at least one double bond between adjacent carbon atoms. Alkenyls include both cis and trans isomers. Representative straight chain and branched alkenyls include ethylenyl, propylenyl, 1-butenyl, 2-butenyl, isobutylenyl, 1-pentenyl, 2-pentenyl, 3-methyl-1-butenyl, 2-methyl-2-butenyl, 2,3-dimethyl-2-butenyl, and the like.

"Alkynyl" means any alkyl or alkenyl, as defined above, which additionally contains at least one triple bond between adjacent carbons. Representative straight chain and branched alkynyls include acetylenyl, propynyl, 1-butynyl, 2-butynyl, 1-pentynyl, 2-pentynyl, 3-methyl-1 butynyl, and the like.

"Acyl" means any alkyl, alkenyl, or alkynyl wherein the carbon at the point of attachment is substituted with an oxo group, as defined below. For example, -C(=O)alkyl, -C(=O)alkenyl, and -C(=O)alkynyl are acyl groups.

"Heterocycle" means a 5- to 7-membered monocyclic, or 7- to 10-membered bicyclic, heterocyclic ring which is either saturated, unsaturated, or aromatic, and which contains from 1 or 2 heteroatoms independently selected from nitrogen, oxygen and sulfur, and wherein the nitrogen and sulfur heteroatoms may be optionally oxidized, and the nitrogen heteroatom may be optionally quaternized, including bicyclic rings in which any of the above heterocycles are fused to a benzene ring. The heterocycle can be attached via any heteroatom or carbon atom. Heterocycles include heteroaryls as defined below. Heterocycles include morpholinyl, pyrrolidinonyl, pyrrolidinyl, piperidinyl, piperizynyl, hydantoinyl, valerolactamyl, oxiranyl, oxetanyl, tetrahydrofuranyl, tetrahydropyranyl, tetrahydropyridinyl, tetrahydroprimidinyl, tetrahydrothiophenyl, tetrahydrothiopyranyl, tetrahydropyrimidinyl, tetrahydrothiophenyl, tetrahydrothiopyranyl, and the like.

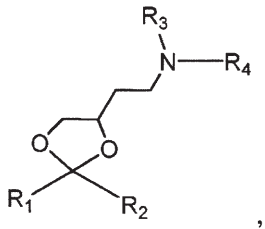
The terms "optionally substituted alkyl," "optionally substituted alkenyl," "optionally substituted alkynyl," "optionally substituted acyl," and "optionally substituted heterocycle" means that, when substituted, at least one hydrogen atom is replaced with a substituent. In the case of an oxo substituent (=O) two hydrogen atoms are replaced. In this regard, substituents include oxo, halogen, heterocycle, -CN, $-OR^x$, $-NR^xR^y$, $-NR^xC(=O)R^y$, $-NR^xSO_2R^y$, $-C(=O)R^x$, $-C(=O)OR^x$, $-C(=O)NR^xR^y$, $-SO_nR^x$ and $-SO_nNR^xR^y$, wherein n is 0, 1 or 2, R^x and R^y are the same or different and independently hydrogen, alkyl or heterocycle, and each of said alkyl and heterocycle substituents may be further substituted with one or more of oxo, halogen, -OH, -CN, alkyl, $-OR^x$, heterocycle, $-NR^xR^y$, $-NR^xC(=O)R^y$, $-NR^xSO_2R^y$, $-C(=O)R^x$, $-C(=O)OR^x$, $-C(=O)NR^xR^y$, $-SO_nR^x$ and $-SO_nNR^xR^y$.

"Halogen" means fluoro, chloro, bromo and iodo.

In some embodiments, the methods featured in the invention can require the use of protecting groups. Protecting group methodology is well known to those skilled in the art (see, for example, Protective Groups in Organic Synthesis, Green, T.W. *et al.*, Wiley-Interscience, New York City, 1999). Briefly, protecting groups within the context of this invention are any group that reduces or eliminates unwanted reactivity of a functional group. A protecting group can be added to a functional group to mask its reactivity during certain reactions and then removed to reveal the original functional group. In some embodiments, an "alcohol protecting group" is used. An "alcohol protecting group" is any group which decreases or eliminates unwanted reactivity of an alcohol functional group. Protecting groups can be added and removed using techniques well known in the art.

Synthesis of Formula A

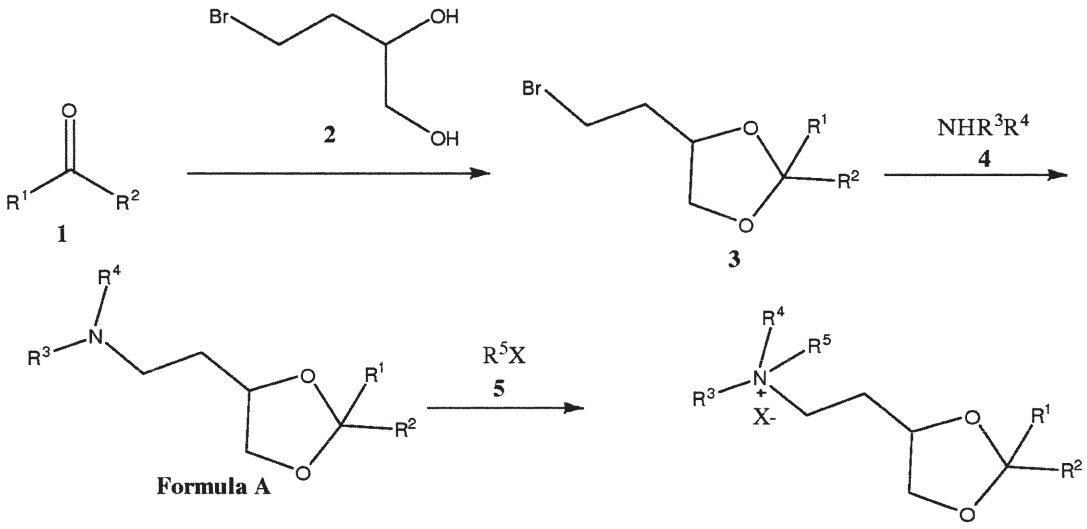
In some embodiments, nucleic acid-lipid particles featured in the invention are formulated using a cationic lipid of formula A:



,

where R1 and R2 are independently alkyl, alkenyl or alkynyl, each can be optionally substituted, and R3 and R4 are independently lower alkyl or R3 and R4 can be taken together to form an optionally substituted heterocyclic ring. In some embodiments, the cationic lipid is XTC (2,2-Dilinoleyl-4-dimethylaminoethyl-[1,3]-dioxolane). In general, the lipid of formula A above may be made by the following Reaction Schemes 1 or 2, wherein all substituents are as defined above unless indicated otherwise.

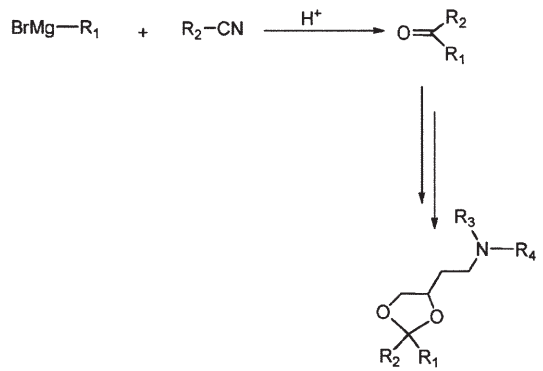
Scheme 1



Lipid A, where R_1 and R_2 are independently alkyl, alkenyl or alkynyl, each can be optionally substituted, and R_3 and R_4 are independently lower alkyl or R_3 and R_4 can be taken together to form an optionally substituted heterocyclic ring, can be prepared according to Scheme 1.

Ketone 1 and bromide 2 can be purchased or prepared according to methods known to those of ordinary skill in the art. Reaction of 1 and 2 yields ketal 3. Treatment of ketal 3 with amine 4 yields lipids of formula A. The lipids of formula A can be converted to the corresponding ammonium salt with an organic salt of formula 5, where X is anion counter ion selected from halogen, hydroxide, phosphate, sulfate, or the like.

Scheme 2



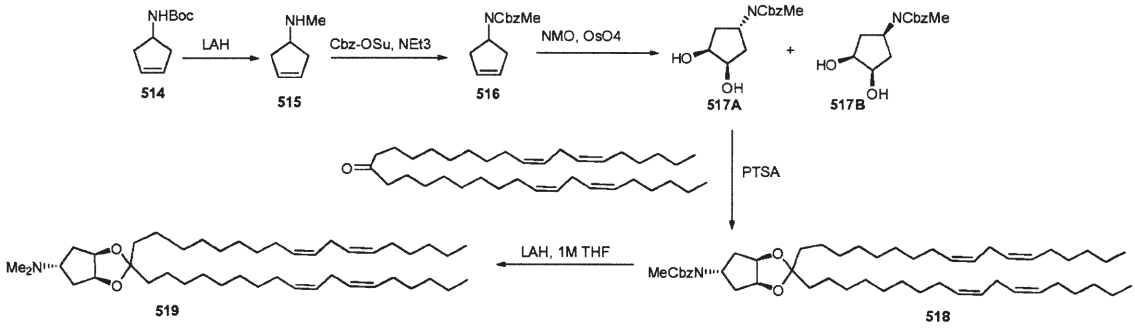
Alternatively, the ketone 1 starting material can be prepared according to Scheme 2. Grignard reagent 6 and cyanide 7 can be purchased or prepared according to methods known to those of ordinary skill in the art. Reaction of 6 and 7 yields ketone 1. Conversion of ketone 1 to the corresponding lipids of formula A is as described in Scheme 1.

Synthesis of MC3

Preparation of DLin-M-C3-DMA (*i.e.*, (6Z,9Z,28Z,31Z)-heptatriaconta-6,9,28,31-tetraen-19-yl 4-(dimethylamino)butanoate) was as follows. A solution of (6Z,9Z,28Z,31Z)-heptatriaconta-6,9,28,31-tetraen-19-ol (0.53 g), 4-N,N-dimethylaminobutyric acid hydrochloride (0.51 g), 4-N,N-dimethylaminopyridine (0.61g) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (0.53 g) in dichloromethane (5 mL) was stirred at room temperature overnight. The solution was washed with dilute hydrochloric acid followed by dilute aqueous sodium bicarbonate. The organic fractions were dried over anhydrous magnesium sulphate, filtered and the solvent removed on a rotovap. The residue was passed down a silica gel column (20 g) using a 1-5% methanol/dichloromethane elution gradient. Fractions containing the purified product were combined and the solvent removed, yielding a colorless oil (0.54 g).

Synthesis of ALNY-100

Synthesis of ketal 519 [ALNY-100] was performed using the following scheme 3:



Synthesis of 515

To a stirred suspension of LiAlH4 (3.74 g, 0.09852 mol) in 200 ml anhydrous THF in a two neck RBF (1L), was added a solution of 514 (10g, 0.04926mol) in 70 mL of THF slowly at 0 0C under nitrogen atmosphere. After complete addition, reaction mixture was warmed to room temperature and then heated to reflux for 4 h. Progress of the reaction was monitored by TLC. After completion of reaction (by TLC) the mixture was cooled to 0 0C and quenched with careful addition of saturated Na2SO4 solution. Reaction mixture was stirred for 4 h at room temperature and filtered off. Residue was washed well with THF. The filtrate and washings were mixed and diluted with 400 mL dioxane and 26 mL conc. HCl and stirred for 20 minutes at room temperature. The volatilities were stripped off under vacuum to furnish the hydrochloride salt of 515 as a white solid. Yield: 7.12 g 1H-NMR (DMSO, 400MHz): δ= 9.34 (broad, 2H), 5.68 (s, 2H), 3.74 (m, 1H), 2.66-2.60 (m, 2H), 2.50-2.45 (m, 5H).

Synthesis of 516

To a stirred solution of compound 515 in 100 mL dry DCM in a 250 mL two neck RBF, was added NEt3 (37.2 mL, 0.2669 mol) and cooled to 0 0C under nitrogen atmosphere. After a slow addition of N-(benzyloxy-carbonyloxy)-succinimide (20 g, 0.08007 mol) in 50 mL dry DCM, reaction mixture was allowed to warm to room temperature. After completion of the reaction (2-3 h by TLC) mixture was washed successively with 1N HCl solution (1 x 100 mL) and saturated NaHCO3 solution (1 x 50 mL). The organic layer was then dried over

anhyd. Na2SO4 and the solvent was evaporated to give crude material which was purified by silica gel column chromatography to get 516 as sticky mass. Yield: 11g (89%). 1H-NMR (CDCl3, 400MHz): δ = 7.36-7.27(m, 5H), 5.69 (s, 2H), 5.12 (s, 2H), 4.96 (br., 1H) 2.74 (s, 3H), 2.60(m, 2H), 2.30-2.25(m, 2H). LC-MS [M+H] -232.3 (96.94%).

Synthesis of 517A and 517B

The cyclopentene 516 (5 g, 0.02164 mol) was dissolved in a solution of 220 mL acetone and water (10:1) in a single neck 500 mL RBF and to it was added N-methyl morpholine-N-oxide (7.6 g, 0.06492 mol) followed by 4.2 mL of 7.6% solution of OsO4 (0.275 g, 0.00108 mol) in tert-butanol at room temperature. After completion of the reaction (~ 3 h), the mixture was quenched with addition of solid Na2SO3 and resulting mixture was stirred for 1.5 h at room temperature. Reaction mixture was diluted with DCM (300 mL) and washed with water (2 x 100 mL) followed by saturated NaHCO3 (1 x 50 mL) solution, water (1 x 30 mL) and finally with brine (1x 50 mL). Organic phase was dried over an.Na2SO4 and solvent was removed in vacuum. Silica gel column chromatographic purification of the crude material was afforded a mixture of diastereomers, which were separated by prep HPLC. Yield: - 6 g crude

517A - Peak-1 (white solid), 5.13 g (96%). 1H-NMR (DMSO, 400MHz): δ= 7.39-7.31(m, 5H), 5.04(s, 2H), 4.78-4.73 (m, 1H), 4.48-4.47(d, 2H), 3.94-3.93(m, 2H), 2.71(s, 3H), 1.72-1.67(m, 4H). LC-MS - [M+H]-266.3, [M+NH4 +]-283.5 present, HPLC-97.86%. Stereochemistry confirmed by X-ray.

Synthesis of 518

Using a procedure analogous to that described for the synthesis of compound 505, compound 518 (1.2 g, 41%) was obtained as a colorless oil. 1H-NMR (CDCl3, 400MHz): δ= 7.35-7.33(m, 4H), 7.30-7.27(m, 1H), 5.37-5.27(m, 8H), 5.12(s, 2H), 4.75(m,1H), 4.58-4.57(m,2H), 2.78-2.74(m,7H), 2.06-2.00(m,8H), 1.96-1.91(m, 2H), 1.62(m, 4H), 1.48(m, 2H), 1.37-1.25(br m, 36H), 0.87(m, 6H). HPLC-98.65%.

General Procedure for the Synthesis of Compound 519

A solution of compound 518 (1 eq) in hexane (15 mL) was added in a drop-wise fashion to an ice-cold solution of LAH in THF (1 M, 2 eq). After complete addition, the

mixture was heated at 40°C over 0.5 h then cooled again on an ice bath. The mixture was carefully hydrolyzed with saturated aqueous Na2SO4 then filtered through celite and reduced to an oil. Column chromatography provided the pure 519 (1.3 g, 68%) which was obtained as a colorless oil. 13C NMR = 130.2, 130.1 (x2), 127.9 (x3), 112.3, 79.3, 64.4, 44.7, 38.3, 35.4, 31.5, 29.9 (x2), 29.7, 29.6 (x2), 29.5 (x3), 29.3 (x2), 27.2 (x3), 25.6, 24.5, 23.3, 226, 14.1; Electrospray MS (+ve): Molecular weight for C44H80NO2 (M + H)+ Calc. 654.6, Found 654.6.

Formulations prepared by either the standard or extrusion-free method can be characterized in similar manners. For example, formulations are typically characterized by visual inspection. They should be whitish translucent solutions free from aggregates or sediment. Particle size and particle size distribution of lipid-nanoparticles can be measured by light scattering using, for example, a Malvern Zetasizer Nano ZS (Malvern, USA). Particles should be about 20-300 nm, such as 40-100 nm in size. The particle size distribution should be unimodal. The total dsRNA concentration in the formulation, as well as the entrapped fraction, is estimated using a dye exclusion assay. A sample of the formulated dsRNA can be incubated with an RNA-binding dye, such as Ribogreen (Molecular Probes) in the presence or absence of a formulation disrupting surfactant, *e.g.*, 0.5% Triton-X100. The total dsRNA in the formulation can be determined by the signal from the sample containing the surfactant, relative to a standard curve. The entrapped fraction is determined by subtracting the "free" dsRNA content (as measured by the signal in the absence of surfactant) from the total dsRNA content. Percent entrapped dsRNA is typically >85%. For SNALP formulation, the particle size is at least 30 nm, at least 40 nm, at least 50 nm, at least 60 nm, at least 70 nm, at least 80 nm, at least 90 nm, at least 100 nm, at least 110 nm, and at least 120 nm. The suitable range is typically about at least 50 nm to about at least 110 nm, about at least 60 nm to about at least 100 nm, or about at least 80 nm to about at least 90 nm.

Compositions and formulations for oral administration include 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 may be desirable. In some embodiments, oral formulations are those in which dsRNAs featured in the invention are administered in conjunction with one or more penetration enhancers surfactants and chelators. Suitable

surfactants include fatty acids and/or esters or salts thereof, bile acids and/or salts thereof. Suitable bile acids/salts include chenodeoxycholic acid (CDCA) and ursodeoxychenodeoxycholic acid (UDCA), cholic acid, dehydrocholic acid, deoxycholic acid, glucholic acid, glycholic acid, glycodeoxycholic acid, taurocholic acid, taurodeoxycholic acid, sodium tauro-24,25-dihydro-fusidate and sodium glycodihydrofusidate. Suitable fatty acids include arachidonic acid, undecanoic acid, oleic acid, lauric acid, caprylic acid, capric acid, myristic acid, palmitic acid, stearic acid, linoleic acid, linolenic acid, dicaprate, tricaprate, monoolein, dilaurin, glyceryl 1-monocaprate, 1-dodecylazacycloheptan-2-one, an acylcarnitine, an acylcholine, or a monoglyceride, a diglyceride or a pharmaceutically acceptable salt thereof (*e.g.*, sodium). In some embodiments, combinations of penetration enhancers are used, for example, fatty acids/salts in combination with bile acids/salts. One exemplary combination is the sodium salt of lauric acid, capric acid and UDCA. Further penetration enhancers include polyoxyethylene-9-lauryl ether, polyoxyethylene-20-cetyl ether. DsRNAs featured in the invention may be delivered orally, in granular form including sprayed dried particles, or complexed to form micro or nanoparticles. DsRNA complexing agents include poly-amino acids; polyimines; polyacrylates; polyalkylacrylates, polyoxethanes, polyalkylcyanoacrylates; cationized gelatins, albumins, starches, acrylates, polyethyleneglycols (PEG) and starches; polyalkylcyanoacrylates; DEAE-derivatized polyimines, pollulans, celluloses and starches. Suitable complexing agents include chitosan, N-trimethylchitosan, poly-L-lysine, polyhistidine, polyornithine, polyspermines, protamine, polyvinylpyridine, polythiodiethylaminomethylethylene P(TDAE), polyaminostyrene (*e.g.*, p-amino), poly(methylcyanoacrylate), poly(ethylcyanoacrylate), poly(butylcyanoacrylate), poly(isobutylcyanoacrylate), poly(isohexylcynaoacrylate), DEAE-methacrylate, DEAE-hexylacrylate, DEAE-acrylamide, DEAE-albumin and DEAE-dextran, polymethylacrylate, polyhexylacrylate, poly(D,L-lactic acid), poly(DL-lactic-co-glycolic acid (PLGA), alginate, and polyethyleneglycol (PEG). Oral formulations for dsRNAs and their preparation are described in detail in U.S. Patent 6,887,906, US Publn. No. 20030027780, and U.S. Patent No. 6,747,014, each of which is incorporated herein by reference.

Compositions and formulations for parenteral, intraparenchymal (into the brain), intrathecal, intraventricular or intrahepatic administration may include sterile aqueous solutions which may 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.

Pharmaceutical compositions of the present invention include, but are not limited to, solutions, emulsions, and liposome-containing formulations. These compositions may be generated from a variety of components that include, but are not limited to, preformed liquids, self-emulsifying solids and self-emulsifying semisolids. Particularly preferred are formulations that target the liver when treating hepatic disorders such as hepatic carcinoma.

The pharmaceutical formulations of the present invention, which may conveniently be presented in unit dosage form, may be prepared according to conventional techniques well known in the pharmaceutical industry. Such techniques include the step of bringing into association the active ingredients with the pharmaceutical carrier(s) or excipient(s). In general, the formulations are prepared by uniformly and intimately bringing into association the active ingredients with liquid carriers or finely divided solid carriers or both, and then, if necessary, shaping the product.

The compositions of the present invention may be formulated into any of many possible dosage forms such as, but not limited to, tablets, capsules, gel capsules, liquid syrups, soft gels, suppositories, and enemas. The compositions of the present invention may also be formulated as suspensions in aqueous, non-aqueous or mixed media. Aqueous suspensions may further contain substances which increase the viscosity of the suspension including, for example, sodium carboxymethylcellulose, sorbitol and/or dextran. The suspension may also contain stabilizers.

Additional Formulations

Emulsions

The compositions of the present invention can be prepared and formulated as emulsions. Emulsions are typically heterogeneous systems of one liquid dispersed in another in the form of droplets usually exceeding 0.1μm in diameter (see *e.g.*, Ansel's Pharmaceutical Dosage Forms and Drug Delivery Systems, Allen, LV., Popovich NG., and Ansel HC., 2004, Lippincott Williams & Wilkins (8th ed.), New York, NY; Idson, in Pharmaceutical Dosage Forms, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 199; Rosoff, in Pharmaceutical Dosage Forms, Lieberman, Rieger and Banker

(Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., Volume 1, p. 245; Block in Pharmaceutical Dosage Forms, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 2, p. 335; Higuchi *et al.*, in Remington's Pharmaceutical Sciences, Mack Publishing Co., Easton, Pa., 1985, p. 301). Emulsions are often biphasic systems comprising two immiscible liquid phases intimately mixed and dispersed with each other. In general, emulsions may be of either the water-in-oil (w/o) or the oil-in-water (o/w) variety. When an aqueous phase is finely divided into and dispersed as minute droplets into a bulk oily phase, the resulting composition is called a water-in-oil (w/o) emulsion. Alternatively, when an oily phase is finely divided into and dispersed as minute droplets into a bulk aqueous phase, the resulting composition is called an oil-in-water (o/w) emulsion. Emulsions may contain additional components in addition to the dispersed phases, and the active drug which may be present as a solution in either the aqueous phase, oily phase or itself as a separate phase. Pharmaceutical excipients such as emulsifiers, stabilizers, dyes, and anti-oxidants may also be present in emulsions as needed. Pharmaceutical emulsions may also be multiple emulsions that are comprised of more than two phases such as, for example, in the case of oil-in-water-in-oil (o/w/o) and water-in-oil-in-water (w/o/w) emulsions. Such complex formulations often provide certain advantages that simple binary emulsions do not. Multiple emulsions in which individual oil droplets of an o/w emulsion enclose small water droplets constitute a w/o/w emulsion. Likewise a system of oil droplets enclosed in globules of water stabilized in an oily continuous phase provides an o/w/o emulsion.

Emulsions are characterized by little or no thermodynamic stability. Often, the dispersed or discontinuous phase of the emulsion is well dispersed into the external or continuous phase and maintained in this form through the means of emulsifiers or the viscosity of the formulation. Either of the phases of the emulsion may be a semisolid or a solid, as is the case of emulsion-style ointment bases and creams. Other means of stabilizing emulsions entail the use of emulsifiers that may be incorporated into either phase of the emulsion. Emulsifiers may broadly be classified into four categories: synthetic surfactants, naturally occurring emulsifiers, absorption bases, and finely dispersed solids (see *e.g.*, Ansel's Pharmaceutical Dosage Forms and Drug Delivery Systems, Allen, LV., Popovich NG., and Ansel HC., 2004, Lippincott Williams & Wilkins (8th ed.), New York, NY; Idson, in Pharmaceutical Dosage Forms, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 199).

Synthetic surfactants, also known as surface active agents, have found wide applicability in the formulation of emulsions and have been reviewed in the literature (see *e.g.*, Ansel's Pharmaceutical Dosage Forms and Drug Delivery Systems, Allen, LV., Popovich NG., and Ansel HC., 2004, Lippincott Williams & Wilkins (8th ed.), New York, NY; Rieger, in Pharmaceutical Dosage Forms, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 285; Idson, in Pharmaceutical Dosage Forms, Lieberman, Rieger and Banker (Eds.), Marcel Dekker, Inc., New York, N.Y., 1988, volume 1, p. 199). Surfactants are typically amphiphilic and comprise a hydrophilic and a hydrophobic portion. The ratio of the hydrophilic to the hydrophobic nature of the surfactant has been termed the hydrophile/lipophile balance (HLB) and is a valuable tool in categorizing and selecting surfactants in the preparation of formulations. Surfactants may be classified into different classes based on the nature of the hydrophilic group: nonionic, anionic, cationic and amphoteric (see *e.g.*, Ansel's Pharmaceutical Dosage Forms and Drug Delivery Systems, Allen, LV., Popovich NG., and Ansel HC., 2004, Lippincott Williams & Wilkins (8th ed.), New York, NY Rieger, in Pharmaceutical Dosage Forms, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 285).

Naturally occurring emulsifiers used in emulsion formulations include lanolin, beeswax, phosphatides, lecithin and acacia. Absorption bases possess hydrophilic properties such that they can soak up water to form w/o emulsions yet retain their semisolid consistencies, such as anhydrous lanolin and hydrophilic petrolatum. Finely divided solids have also been used as good emulsifiers especially in combination with surfactants and in viscous preparations. These include polar inorganic solids, such as heavy metal hydroxides, nonswelling clays such as bentonite, attapulgite, hectorite, kaolin, montmorillonite, colloidal aluminum silicate and colloidal magnesium aluminum silicate, pigments and nonpolar solids such as carbon or glyceryl tristearate.

A large variety of non-emulsifying materials are also included in emulsion formulations and contribute to the properties of emulsions. These include fats, oils, waxes, fatty acids, fatty alcohols, fatty esters, humectants, hydrophilic colloids, preservatives and antioxidants (Block, in Pharmaceutical Dosage Forms, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 335; Idson, in Pharmaceutical Dosage Forms, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 199).

Hydrophilic colloids or hydrocolloids include naturally occurring gums and synthetic polymers such as polysaccharides (for example, acacia, agar, alginic acid, carrageenan, guar gum, karaya gum, and tragacanth), cellulose derivatives (for example, carboxymethylcellulose and carboxypropylcellulose), and synthetic polymers (for example, carbomers, cellulose ethers, and carboxyvinyl polymers). These disperse or swell in water to form colloidal solutions that stabilize emulsions by forming strong interfacial films around the dispersed-phase droplets and by increasing the viscosity of the external phase.

Since emulsions often contain a number of ingredients such as carbohydrates, proteins, sterols and phosphatides that may readily support the growth of microbes, these formulations often incorporate preservatives. Commonly used preservatives included in emulsion formulations include methyl paraben, propyl paraben, quaternary ammonium salts, benzalkonium chloride, esters of p-hydroxybenzoic acid, and boric acid. Antioxidants are also commonly added to emulsion formulations to prevent deterioration of the formulation. Antioxidants used may be free radical scavengers such as tocopherols, alkyl gallates, butylated hydroxyanisole, butylated hydroxytoluene, or reducing agents such as ascorbic acid and sodium metabisulfite, and antioxidant synergists such as citric acid, tartaric acid, and lecithin.

The application of emulsion formulations via dermatological, oral and parenteral routes and methods for their manufacture have been reviewed in the literature (see *e.g.*, Ansel's Pharmaceutical Dosage Forms and Drug Delivery Systems, Allen, LV., Popovich NG., and Ansel HC., 2004, Lippincott Williams & Wilkins (8th ed.), New York, NY; Idson, in Pharmaceutical Dosage Forms, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 199). Emulsion formulations for oral delivery have been very widely used because of ease of formulation, as well as efficacy from an absorption and bioavailability standpoint (see *e.g.*, Ansel's Pharmaceutical Dosage Forms and Drug Delivery Systems, Allen, LV., Popovich NG., and Ansel HC., 2004, Lippincott Williams & Wilkins (8th ed.), New York, NY; Rosoff, in Pharmaceutical Dosage Forms, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 245; Idson, in Pharmaceutical Dosage Forms, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 199). Mineral-oil base laxatives, oil-soluble vitamins and high fat nutritive preparations are among the materials that have commonly been administered orally as o/w emulsions.

In one embodiment of the present invention, the compositions of iRNAs and nucleic acids are formulated as microemulsions. A microemulsion may be defined as a system of water, oil and amphiphile which is a single optically isotropic and thermodynamically stable liquid solution (see *e.g.*, Ansel's Pharmaceutical Dosage Forms and Drug Delivery Systems, Allen, LV., Popovich NG., and Ansel HC., 2004, Lippincott Williams & Wilkins (8th ed.), New York, NY; Rosoff, in Pharmaceutical Dosage Forms, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 245). Typically microemulsions are systems that are prepared by first dispersing an oil in an aqueous surfactant solution and then adding a sufficient amount of a fourth component, generally an intermediate chain-length alcohol to form a transparent system. Therefore, microemulsions have also been described as thermodynamically stable, isotropically clear dispersions of two immiscible liquids that are stabilized by interfacial films of surface-active molecules (Leung and Shah, in: Controlled Release of Drugs: Polymers and Aggregate Systems, Rosoff, M., Ed., 1989, VCH Publishers, New York, pages 185-215). Microemulsions commonly are prepared via a combination of three to five components that include oil, water, surfactant, cosurfactant and electrolyte. Whether the microemulsion is of the water-in-oil (w/o) or an oil-in-water (o/w) type is dependent on the properties of the oil and surfactant used and on the structure and geometric packing of the polar heads and hydrocarbon tails of the surfactant molecules (Schott, in Remington's Pharmaceutical Sciences, Mack Publishing Co., Easton, Pa., 1985, p. 271).

The phenomenological approach utilizing phase diagrams has been extensively studied and has yielded a comprehensive knowledge, to one skilled in the art, of how to formulate microemulsions (see *e.g.*, Ansel's Pharmaceutical Dosage Forms and Drug Delivery Systems, Allen, LV., Popovich NG., and Ansel HC., 2004, Lippincott Williams & Wilkins (8th ed.), New York, NY; Rosoff, in Pharmaceutical Dosage Forms, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 245; Block, in Pharmaceutical Dosage Forms, Lieberman, Rieger and Banker (Eds.), 1988, Marcel Dekker, Inc., New York, N.Y., volume 1, p. 335). Compared to conventional emulsions, microemulsions offer the advantage of solubilizing water-insoluble drugs in a formulation of thermodynamically stable droplets that are formed spontaneously.

Surfactants used in the preparation of microemulsions include, but are not limited to, ionic surfactants, non-ionic surfactants, Brij 96, polyoxyethylene oleyl ethers, polyglycerol

fatty acid esters, tetraglycerol monolaurate (ML310), tetraglycerol monooleate (MO310), hexaglycerol monooleate (PO310), hexaglycerol pentaoleate (PO500), decaglycerol monocaprate (MCA750), decaglycerol monooleate (MO750), decaglycerol sequioleate (SO750), decaglycerol decaoleate (DAO750), alone or in combination with cosurfactants. The cosurfactant, usually a short-chain alcohol such as ethanol, 1-propanol, and 1-butanol, serves to increase the interfacial fluidity by penetrating into the surfactant film and consequently creating a disordered film because of the void space generated among surfactant molecules. Microemulsions may, however, be prepared without the use of cosurfactants and alcohol-free self-emulsifying microemulsion systems are known in the art. The aqueous phase may typically be, but is not limited to, water, an aqueous solution of the drug, glycerol, PEG300, PEG400, polyglycerols, propylene glycols, and derivatives of ethylene glycol. The oil phase may include, but is not limited to, materials such as Captex 300, Captex 355, Capmul MCM, fatty acid esters, medium chain (C8-C12) mono, di, and tri-glycerides, polyoxyethylated glyceryl fatty acid esters, fatty alcohols, polyglycolized glycerides, saturated polyglycolized C8-C10 glycerides, vegetable oils and silicone oil.

Microemulsions are particularly of interest from the standpoint of drug solubilization and the enhanced absorption of drugs. Lipid based microemulsions (both o/w and w/o) have been proposed to enhance the oral bioavailability of drugs, including peptides (see *e.g.*, U.S. Patent Nos. 6,191,105; 7,063,860; 7,070,802; 7,157,099; Constantinides *et al.*, Pharmaceutical Research, 1994, 11, 1385-1390; Ritschel, Meth. Find. Exp. Clin. Pharmacol., 1993, 13, 205). Microemulsions afford advantages of improved drug solubilization, protection of drug from enzymatic hydrolysis, possible enhancement of drug absorption due to surfactant-induced alterations in membrane fluidity and permeability, ease of preparation, ease of oral administration over solid dosage forms, improved clinical potency, and decreased toxicity (see *e.g.*, U.S. Patent Nos. 6,191,105; 7,063,860; 7,070,802; 7,157,099; Constantinides *et al.*, Pharmaceutical Research, 1994, 11, 1385; Ho *et al.*, J. Pharm. Sci., 1996, 85, 138-143). Often microemulsions may form spontaneously when their components are brought together at ambient temperature. This may be particularly advantageous when formulating thermolabile drugs, peptides or iRNAs. Microemulsions have also been effective in the transdermal delivery of active components in both cosmetic and pharmaceutical applications. It is expected that the microemulsion compositions and formulations of the present invention will facilitate the increased systemic absorption of iRNAs and nucleic acids

from the gastrointestinal tract, as well as improve the local cellular uptake of iRNAs and nucleic acids.

Microemulsions of the present invention may also contain additional components and additives such as sorbitan monostearate (Grill 3), Labrasol, and penetration enhancers to improve the properties of the formulation and to enhance the absorption of the iRNAs and nucleic acids of the present invention. Penetration enhancers used in the microemulsions of the present invention may be classified as belonging to one of five broad categories--surfactants, fatty acids, bile salts, chelating agents, and non-chelating non-surfactants (Lee *et al.*, Critical Reviews in Therapeutic Drug Carrier Systems, 1991, p. 92). Each of these classes has been discussed above.

Penetration Enhancers

In one embodiment, the present invention employs various penetration enhancers to effect the efficient delivery of nucleic acids, particularly iRNAs, to the skin of animals. Most drugs are present in solution in both ionized and nonionized forms. However, usually only lipid soluble or lipophilic drugs readily cross cell membranes. It has been discovered that even non-lipophilic drugs may cross cell membranes if the membrane to be crossed is treated with a penetration enhancer. In addition to aiding the diffusion of non-lipophilic drugs across cell membranes, penetration enhancers also enhance the permeability of lipophilic drugs.

Penetration enhancers may be classified as belonging to one of five broad categories, *i.e.*, surfactants, fatty acids, bile salts, chelating agents, and non-chelating non-surfactants (see *e.g.*, Malmsten, M. Surfactants and polymers in drug delivery, Informa Health Care, New York, NY, 2002; Lee *et al.*, Critical Reviews in Therapeutic Drug Carrier Systems, 1991, p.92). Each of the above mentioned classes of penetration enhancers are described below in greater detail.

Surfactants: In connection with the present invention, surfactants (or "surface-active agents") are chemical entities which, when dissolved in an aqueous solution, reduce the surface tension of the solution or the interfacial tension between the aqueous solution and another liquid, with the result that absorption of iRNAs through the mucosa is enhanced. In addition to bile salts and fatty acids, these penetration enhancers include, for example, sodium lauryl sulfate, polyoxyethylene-9-lauryl ether and polyoxyethylene-20-cetyl ether) (see *e.g.*, Malmsten, M. Surfactants and polymers in drug delivery, Informa Health Care,

New York, NY, 2002; Lee *et al.*, Critical Reviews in Therapeutic Drug Carrier Systems, 1991, p.92); and perfluorochemical emulsions, such as FC-43. Takahashi *et al.*, J. Pharm. Pharmacol., 1988, 40, 252).

Fatty acids: Various fatty acids and their derivatives which act as penetration enhancers include, for example, oleic acid, lauric acid, capric acid (n-decanoic acid), myristic acid, palmitic acid, stearic acid, linoleic acid, linolenic acid, dicaprate, tricaprate, monoolein (1-monooleoyl-rac-glycerol), dilaurin, caprylic acid, arachidonic acid, glycerol 1-monocaprate, 1-dodecylazacycloheptan-2-one, acylcarnitines, acylcholines, C_{1-20} alkyl esters thereof (*e.g.*, methyl, isopropyl and t-butyl), and mono- and di-glycerides thereof (*i.e.*, oleate, laurate, caprate, myristate, palmitate, stearate, linoleate, *etc.*) (see *e.g.*, Touitou, E., *et al.* Enhancement in Drug Delivery, CRC Press, Danvers, MA, 2006; Lee *et al.*, Critical Reviews in Therapeutic Drug Carrier Systems, 1991, p.92; Muranishi, Critical Reviews in Therapeutic Drug Carrier Systems, 1990, 7, 1-33; El Hariri *et al.*, J. Pharm. Pharmacol., 1992, 44, 651-654).

Bile salts: The physiological role of bile includes the facilitation of dispersion and absorption of lipids and fat-soluble vitamins (see *e.g.*, Malmsten, M. Surfactants and polymers in drug delivery, Informa Health Care, New York, NY, 2002; Brunton, Chapter 38 in: Goodman & Gilman's The Pharmacological Basis of Therapeutics, 9th Ed., Hardman *et al.* Eds., McGraw-Hill, New York, 1996, pp. 934-935). Various natural bile salts, and their synthetic derivatives, act as penetration enhancers. Thus, the term "bile salts" includes any of the naturally occurring components of bile as well as any of their synthetic derivatives. Suitable bile salts include, for example, cholic acid (or its pharmaceutically acceptable sodium salt, sodium cholate), dehydrocholic acid (sodium dehydrocholate), deoxycholic acid (sodium deoxycholate), glucholic acid (sodium glucholate), glycholic acid (sodium glycocholate), glycodeoxycholic acid (sodium glycodeoxycholate), taurocholic acid (sodium taurocholate), taurodeoxycholic acid (sodium taurodeoxycholate), chenodeoxycholic acid (sodium chenodeoxycholate), ursodeoxycholic acid (UDCA), sodium tauro-24,25-dihydro-fusidate (STDHF), sodium glycodihydrofusidate and polyoxyethylene-9-lauryl ether (POE) (see *e.g.*, Malmsten, M. Surfactants and polymers in drug delivery, Informa Health Care, New York, NY, 2002; Lee *et al.*, Critical Reviews in Therapeutic Drug Carrier Systems, 1991, page 92; Swinyard, Chapter 39 In: Remington's Pharmaceutical Sciences, 18th Ed., Gennaro, ed., Mack Publishing Co., Easton, Pa., 1990, pages 782-783; Muranishi, Critical

Reviews in Therapeutic Drug Carrier Systems, 1990, 7, 1-33; Yamamoto *et al.*, J. Pharm. Exp. Ther., 1992, 263, 25; Yamashita *et al.*, J. Pharm. Sci., 1990, 79, 579-583).

Chelating Agents: Chelating agents, as used in connection with the present invention, can be defined as compounds that remove metallic ions from solution by forming complexes therewith, with the result that absorption of iRNAs through the mucosa is enhanced. With regards to their use as penetration enhancers in the present invention, chelating agents have the added advantage of also serving as DNase inhibitors, as most characterized DNA nucleases require a divalent metal ion for catalysis and are thus inhibited by chelating agents (Jarrett, J. Chromatogr., 1993, 618, 315-339). Suitable chelating agents include but are not limited to disodium ethylenediaminetetraacetate (EDTA), citric acid, salicylates (*e.g.*, sodium salicylate, 5-methoxysalicylate and homovanilate), N-acyl derivatives of collagen, laureth-9 and N-amino acyl derivatives of β-diketones (enamines)(see *e.g.*, Katdare, A. *et al.*, Excipient development for pharmaceutical, biotechnology, and drug delivery, CRC Press, Danvers, MA, 2006; Lee *et al.*, Critical Reviews in Therapeutic Drug Carrier Systems, 1991, page 92; Muranishi, Critical Reviews in Therapeutic Drug Carrier Systems, 1990, 7, 1-33; Buur *et al.*, J. Control Rel., 1990, 14, 43-51).

Non-chelating non-surfactants: As used herein, non-chelating non-surfactant penetration enhancing compounds can be defined as compounds that demonstrate insignificant activity as chelating agents or as surfactants but that nonetheless enhance absorption of iRNAs through the alimentary mucosa (see *e.g.*, Muranishi, Critical Reviews in Therapeutic Drug Carrier Systems, 1990, 7, 1-33). This class of penetration enhancers includes, for example, unsaturated cyclic ureas, 1-alkyl- and 1-alkenylazacyclo-alkanone derivatives (Lee *et al.*, Critical Reviews in Therapeutic Drug Carrier Systems, 1991, page 92); and non-steroidal anti-inflammatory agents such as diclofenac sodium, indomethacin and phenylbutazone (Yamashita *et al.*, J. Pharm. Pharmacol., 1987, 39, 621-626).

Agents that enhance uptake of iRNAs at the cellular level may also be added to the pharmaceutical and other compositions of the present invention. For example, cationic lipids, such as lipofectin (Junichi *et al*, U.S. Pat. No. 5,705,188), cationic glycerol derivatives, and polycationic molecules, such as polylysine (Lollo *et al.*, PCT Application WO 97/30731), are also known to enhance the cellular uptake of dsRNAs. Examples of commercially available transfection reagents include, for example Lipofectamine™ (Invitrogen; Carlsbad, CA), Lipofectamine 2000™ (Invitrogen; Carlsbad, CA), 293fectin™ (Invitrogen; Carlsbad, CA),

Cellfectin™ (Invitrogen; Carlsbad, CA), DMRIE-C™ (Invitrogen; Carlsbad, CA), FreeStyle™ MAX (Invitrogen; Carlsbad, CA), Lipofectamine™ 2000 CD (Invitrogen; Carlsbad, CA), Lipofectamine™ (Invitrogen; Carlsbad, CA), RNAiMAX (Invitrogen; Carlsbad, CA), Oligofectamine™ (Invitrogen; Carlsbad, CA), Optifect™ (Invitrogen; Carlsbad, CA), X-tremeGENE Q2 Transfection Reagent (Roche; Grenzacherstrasse, Switzerland), DOTAP Liposomal Transfection Reagent (Grenzacherstrasse, Switzerland), DOSPER Liposomal Transfection Reagent (Grenzacherstrasse, Switzerland), or Fugene (Grenzacherstrasse, Switzerland), Transfectam® Reagent (Promega; Madison, WI), TransFast™ Transfection Reagent (Promega; Madison, WI), Tfx™-20 Reagent (Promega; Madison, WI), Tfx™-50 Reagent (Promega; Madison, WI), DreamFect™ (OZ Biosciences; Marseille, France), EcoTransfect (OZ Biosciences; Marseille, France), TransPass[a] D1 Transfection Reagent (New England Biolabs; Ipswich, MA, USA), LyoVec™/LipoGen™ (Invivogen; San Diego, CA, USA), PerFectin Transfection Reagent (Genlantis; San Diego, CA, USA), NeuroPORTER Transfection Reagent (Genlantis; San Diego, CA, USA), GenePORTER Transfection reagent (Genlantis; San Diego, CA, USA), GenePORTER 2 Transfection reagent (Genlantis; San Diego, CA, USA), Cytofectin Transfection Reagent (Genlantis; San Diego, CA, USA), BaculoPORTER Transfection Reagent (Genlantis; San Diego, CA, USA), TroganPORTER™ transfection Reagent (Genlantis; San Diego, CA, USA), RiboFect (Bioline; Taunton, MA, USA), PlasFect (Bioline; Taunton, MA, USA), UniFECTOR (B-Bridge International; Mountain View, CA, USA), SureFECTOR (B-Bridge International; Mountain View, CA, USA), or HiFect™ (B-Bridge International, Mountain View, CA, USA), among others.

Other agents may be utilized to enhance the penetration of the administered nucleic acids, including glycols such as ethylene glycol and propylene glycol, pyrrols such as 2-pyrrol, azones, and terpenes such as limonene and menthone.

Carriers

Certain compositions of the present invention also incorporate carrier compounds in the formulation. As used herein, "carrier compound" or "carrier" can refer to a nucleic acid, or analog thereof, which is inert (*i.e.*, does not possess biological activity per se) but is recognized as a nucleic acid by *in vivo* processes that reduce the bioavailability of a nucleic acid having biological activity by, for example, degrading the biologically active nucleic acid or promoting its removal from circulation. The coadministration of a nucleic acid and a

carrier compound, typically with an excess of the latter substance, can result in a substantial reduction of the amount of nucleic acid recovered in the liver, kidney or other extracirculatory reservoirs, presumably due to competition between the carrier compound and the nucleic acid for a common receptor. For example, the recovery of a partially phosphorothioate dsRNA in hepatic tissue can be reduced when it is coadministered with polyinosinic acid, dextran sulfate, polycytidic acid or 4-acetamido-4'isothiocyano-stilbene-2,2'-disulfonic acid (Miyao *et al.*, DsRNA Res. Dev., 1995, 5, 115-121; Takakura *et al.*, DsRNA & Nucl. Acid Drug Dev., 1996, 6, 177-183.

Excipients

In contrast to a carrier compound, a "pharmaceutical carrier" or "excipient" is a pharmaceutically acceptable solvent, suspending agent or any other pharmacologically inert vehicle for delivering one or more nucleic acids to an animal. The excipient may be liquid or solid and is selected, with the planned manner of administration in mind, so as to provide for the desired bulk, consistency, *etc.*, when combined with a nucleic acid and the other components of a given pharmaceutical composition. Typical pharmaceutical carriers include, but are not limited to, binding agents (*e.g.*, pregelatinized maize starch, polyvinylpyrrolidone or hydroxypropyl methylcellulose, *etc.*); fillers (*e.g.*, lactose and other sugars, microcrystalline cellulose, pectin, gelatin, calcium sulfate, ethyl cellulose, polyacrylates or calcium hydrogen phosphate, *etc.*); lubricants (*e.g.*, magnesium stearate, talc, silica, colloidal silicon dioxide, stearic acid, metallic stearates, hydrogenated vegetable oils, corn starch, polyethylene glycols, sodium benzoate, sodium acetate, *etc.*); disintegrants (*e.g.*, starch, sodium starch glycolate, *etc.*); and wetting agents (*e.g.*, sodium lauryl sulphate, *etc*).

Pharmaceutically acceptable organic or inorganic excipients suitable for non-parenteral administration which do not deleteriously react with nucleic acids can also be used to formulate the compositions of the present invention. Suitable pharmaceutically acceptable carriers include, but are not limited to, water, salt solutions, alcohols, polyethylene glycols, gelatin, lactose, amylose, magnesium stearate, talc, silicic acid, viscous paraffin, hydroxymethylcellulose, polyvinylpyrrolidone and the like.

Formulations for topical administration of nucleic acids may include sterile and non-sterile aqueous solutions, non-aqueous solutions in common solvents such as alcohols, or solutions of the nucleic acids in liquid or solid oil bases. The solutions may also contain

buffers, diluents and other suitable additives. Pharmaceutically acceptable organic or inorganic excipients suitable for non-parenteral administration which do not deleteriously react with nucleic acids can be used.

Suitable pharmaceutically acceptable excipients include, but are not limited to, water, salt solutions, alcohol, polyethylene glycols, gelatin, lactose, amylose, magnesium stearate, talc, silicic acid, viscous paraffin, hydroxymethylcellulose, polyvinylpyrrolidone and the like.

Other Components

The compositions of the present invention may additionally contain other adjunct components conventionally found in pharmaceutical compositions, at their art-established usage levels. Thus, for example, the compositions may contain additional, compatible, pharmaceutically-active materials such as, for example, antipruritics, astringents, local anesthetics or anti-inflammatory agents, or may contain additional materials useful in physically formulating various dosage forms of the compositions of the present invention, such as dyes, flavoring agents, preservatives, antioxidants, opacifiers, thickening agents and stabilizers. However, such materials, when added, should not unduly interfere with the biological activities of the components of the compositions of the present invention. The formulations can be sterilized and, if desired, mixed with auxiliary agents, *e.g.*, lubricants, preservatives, stabilizers, wetting agents, emulsifiers, salts for influencing osmotic pressure, buffers, colorings, flavorings and/or aromatic substances and the like which do not deleteriously interact with the nucleic acid(s) of the formulation.

Aqueous suspensions can contain substances that increase the viscosity of the suspension including, for example, sodium carboxymethylcellulose, sorbitol and/or dextran. The suspension may also contain stabilizers.

In some embodiments, pharmaceutical compositions featured in the invention include (a) one or more iRNA compounds and (b) one or more anti-cytokine biologic agents which function by a non-RNAi mechanism. Examples of such biologics include, biologics that target IL1β (*e.g.*, anakinra), IL6 (*e.g.*, tocilizumab), or TNF (*e.g.*, etanercept, infliximab, adlimumab, or certolizumab).

Toxicity and therapeutic efficacy of such compounds can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, *e.g.*, for determining the

LD50 (the dose lethal to 50% of the population) and the ED50 (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio LD50/ED50. Compounds that exhibit high therapeutic indices are preferred.

The data obtained from cell culture assays and animal studies can be used in formulating a range of dosage for use in humans. The dosage of compositions featured herein lies generally within a range of circulating concentrations that include the ED50 with little or no toxicity. The dosage can vary within this range depending upon the dosage form employed and the route of administration utilized. For any compound used in the methods featured in the invention, the therapeutically effective dose can be estimated initially from cell culture assays. A dose can be formulated in animal models to achieve a circulating plasma concentration range of the compound or, when appropriate, of the polypeptide product of a target sequence (*e.g.*, achieving a decreased concentration of the polypeptide) that includes the IC50 (*i.e.*, the concentration of the test compound which achieves a half-maximal inhibition of symptoms) as determined in cell culture. Such information can be used to more accurately determine useful doses in humans. Levels in plasma may be measured, for example, by high performance liquid chromatography.

In addition to their administration, as discussed above, the iRNAs described herein can be administered in combination with other known agents effective in treatment of pathological processes mediated by TMPRSS6 expression. In any event, the administering physician can adjust the amount and timing of iRNA administration on the basis of results observed using standard measures of efficacy known in the art or described herein.

Methods for Treating Diseases caused by Expression of a TMPRSS6 Gene

The invention relates in particular to the use of an iRNA targeting TMPRSS6 and compositions containing at least one such iRNA for the treatment of a TMPRSS6-mediated disorder or disease. For example, a composition containing an iRNA targeting a TMPRSS6 gene is used for treatment of a disorder associated with elevated iron levels, such as a thalassemia, (*e.g.*, β-thalassemia intermedia or α-thalassemia), primary hemochromatosis, secondary hemochromatosis, severe juvenile hemochromatosis, sideroblastic anemia, hemolytic anemia, dyserythropoietic anemia, or sickle-cell anemia. In one embodiment, a TMPRSS6 iRNA is used to treat a hemoglobinopathy. The TMPRSS6 iRNAs featured in the

invention can also be used to treat elevated levels of iron due to other conditions, such as, chronic alcoholism.

In thalassemias, the bone marrow synthesizes insufficient amounts of a hemoglobin chain; this in turn reduces the production of red blood cells and causes anemia. Either the α or the β chain may be affected, but β thalassemias are more common; newborn babies are healthy because their bodies still produce HbF, which does not have β chains; during the first few months of life, the bone marrow switches to producing HbA, and symptoms start to appear.

β-thalassemias result from mutation with either non-expressing (β^0) or low expressing (β^+) alleles of the HBB gene. β-thalassemias vary in severity depending on the genotype, and include minor/trait β-thalassemia (β/ β^0 or β/ β+), intermedia β-thalassemia (β^0/β+), and major β-thalassemia (β^0 /β^0 or β^+/ β^+).

Thalassemia intermedia (TI) typically presents with little hemolysis, while major β-thalassemia (TM) is typically accompanied by abundant hemolysis which causes, *e.g.*, anemia and splenomegaly; and highly ineffective erythropoiesis, which causes bone marrow drive (skeletal changes, oteopenia), increased erythropoietin synthesis, hepato-splenomegaly, consumption of haematinics (megablastic anemia), and high uric acid in blood. The iRNAs featured in the invention, *e.g.*, TMPRSS6 iRNAs, are better suited for treating the iron overload that typically accompanies thalassemia's that are more TI like (*e.g.*, for treating individuals having a β^0/β+, β/ β^0 or β/ β+ genotype).

Symptoms of β-thalassemias also include, *e.g.*, complication due to therapy, *e.g.*, iron overload, which causes endocrinopathy, liver fibrosis and cardiac fibrosis. Administration of an iRNA agent that targets TMPRSS6 can be effective to treat one or more of these symptoms.

α-thalassemias result from mutation with either non-expressing (α^0) or low expressing (α^+) alleles of the HBA1 or HBA2 genes. α-thalassemias vary in severity depending on the genotype, and include trait thalassemia (-α/αα), Hb Bart and Hydrops fetalis (α^0 /α^0), α-Thalaseemia minor (--/αα), (-α/-α), and HbH disease (--/-α). Lower α-globin chains are produced, resulting in an excess of β chains in adults and excess γ chains in newborns. The excess β chains form unstable tetramers (called Hemoglobin H or HbH of 4 beta chains), which have abnormal oxygen dissociation curves. Administration of an iRNA agent that

targets TMPRSS6 can be effective to treat iron overload in a subject who has an α-thalassemias.

Symptoms of hemochromatosis include, *e.g.*, abdominal pain, joint pain, fatigue, lack of energy, weakness, darkening of the skin (often referred to as "bronzing"), and loss of body hair. Administration of an iRNA agent that targets TMPRSS6 can be effective to treat one or more of these symptoms.

Other symptoms associated with iron overload include increased risk for liver disease (cirrhosis, cancer), heart attack or heart failure, diabetes mellitus, osteoarthritis, osteoporosis, metabolic syndrome, hypothyroidism, hypogonadism, and in some cases premature death. Iron mismanagement resulting in overload can also accelerate such neurodegenerative diseases as Alzheimer's, early-onset Parkinson's, Huntington's, epilepsy and multiple sclerosis. Administration of an iRNA that targets TMPRSS6, *e.g.*, an iRNA described in Tables 2, 3 or 4 can treat one or more of these symptoms, or prevent the development or progression of a disease or disorder that is aggrevated by increased iron levels.

The invention further relates to the use of an iRNA or a pharmaceutical composition thereof, *e.g.*, for treating a disorder associated with elevated iron levels, in combination with other pharmaceuticals and/or other therapeutic methods, *e.g.*, with known pharmaceuticals and/or known therapeutic methods, such as, for example, those which are currently employed for treating these disorders. For example, in certain embodiments, an iRNA targeting TMPRSS6 is administered in combination with, *e.g.*, iron chelators (*e.g.*, desferroxamine), folic acid, a blood transfusion, a phlebotomy, agents to manage ulcers, agents to increase fetal hemoglobin levels (*e.g.*, hydroxyurea), agents to control infection (*e.g.*, antibiotics and antivirals), agents to treat thrombotic state, or a stem cell or bone marrow transplant. A stem cell transplant can utilize stem cells from an umbilical cord, such as from a relative, *e.g.*, a sibling. Exemplary iron chelators include desferroxamine, Deferasirox (Exjade), deferoprone, vitamin E, wheat germ oil, tocophersolan, and indicaxanthin.

The iRNA and an additional therapeutic agent can be administered in the same composition, *e.g.*, parenterally, or the additional therapeutic agent can be administered as part of a separate composition or by another method described herein. Administration of the TMPRSS6 iRNA and the additional therapeutic agent can be at the same time, or at different times and, in any order.

The invention features a method of administering an iRNA agent targeting TMPRSS6 to a patient having a disease or disorder mediated by TMPRSS6 expression, such as a disorder associated with elevated iron levels. Administration of the dsRNA can lower iron levels, lower ferritin levels, and/or lower transferrin saturation levels. For example, administration of the dsRNA can lower serum iron levels and/or lower serum ferritin levels. Transferrin saturation levels can be lowered by 5%, 10%, 15%, 20%, 25% or more. Transferrin saturation levels can be lowered to below 50%, below 45%, below 40%, below 35%, below 35% or lower. Transferrin saturation is a measure of the amount of iron bound to serum transferrin, and corresponds to the ratio of serum iron and total iron-binding capacity.

By "lower" in this context is meant a statistically significant decrease in such level. The decrease can be, for example, at least 10%, at least 20%, at least 30%, at least 40% or more, and is preferably down to a level accepted as within the range of normal for an individual without such disorder.

Efficacy of treatment or prevention of disease can be assessed, for example by measuring disease progression, disease remission, symptom severity, reduction in pain, quality of life, dose of a medication required to sustain a treatment effect, level of a disease marker or any other measurable parameter appropriate for a given disease being treated or targeted for prevention. It is well within the ability of one skilled in the art to monitor efficacy of treatment or prevention by measuring any one of such parameters, or any combination of parameters. For example, the levels of transferrin saturation or serum ferritin can be monitored for efficacy of a given treatment regime.

Iron level tests are typically performed on a sample of a pateint's blood. An iron level test measure the amount of iron in the blood serum that is being carried by the proteins trasferrin. A TIBC (Total iron-binding capacity) test measures the amount of iron that the blood would carry if the transferrin were fully saturated. Since transferrin is produced by the liver, the TIBC can be used to monitor liver function and nutrition. The transferrin test is a direct measure of transferrin (also called siderophilin) levels in the blood. The saturation level of transferrin can be calculated by dividing the serum iron level by the TIBC. The ferritin test measures the level of a protein in the blood that stores iron for later use by the body.

The iRNA treatments described herein can be used to treat individuals having elevated iron levels, as may be indicated by iron levels in serum *e.g.*, iron levels measuring greater than 350 μg/dL, greater than 500 μg/dL, greater than 1000 μg/dL, or more. In an embodiment, elevated levels of iron in serum, *e.g.*, greater than 15, 20, 25, or 30 mg/g dry weight.

The iRNA treatments described herein can be used to treat individuals having elevated iron levels, as may be indicated by elevated ferritin levels in serum, *e.g.*, ferritin levels measuring greater than 300 μg/L, greater than 500 μg/L, greater than 1000 μg/L, greater than 1500 μg/L, greater than 2000 μg/L, greater than 2500 μg/L, or 3000 μg/L, or more.

The iRNA treatments described herein can be used to treat individuals having elevated iron levels, as may be indicated by elevated transferrin levels in serum, *e.g.*, transferrin levels measuring greater than 400 mg/dL, greater than 500 mg/L, greater than 1000 mg/dL, or more.

The iRNA treatments described herein can be used to treat individuals having moderately elevated iron levels, as may be indicated by moderately elevated transferrin saturation levels, *e.g.*, saturation levels of 40%, 45%, or 50% or more. In addition, the treatment described herein may also be used to prevent elevated iron levels in individuals with only minor elevations in transferrin saturation. One of skill in the art can easily monitor the transferrin saturation levels in subjects receiving treatment with iRNA as described herein and assay for a reduction in transferrin saturation levels of at least 5% or 10%.

The iRNA treatments described herein can be used to treat individuals having elevated iron levels, as may be indicated by a TIBC value greater than 400 μg/dL, greater than 500 μg/dL, or greater than 1000 μg/dL, or more.

In some embodiments, individuals in need of treatment with a TMPRSS6 siRNA have decreased hematocrit levels, decreased hemoglobin levels, increased red blood cell distribution width, increased number of reticulocytes, decreased number of mature red blood cells, increased unsaturated iron binding capacity, decreased ineffective erythropoiesis, decreased extradedullary hematopoiesis, and/or decreased HAMP1 expression levels.

A patient can be further monitored by assay of blood sugar (glucose) level or α fetoprotein level, by echocardiogram (*e.g.*, to examine the heart's function), electrocardiogram (ECG) (*e.g.*, to look at the electrical activity of the heart), imaging tests (such as CT scans, MRI and ultrasound), and liver function tests. Excess iron staining or iron

concentrations can be measured on liver biopsy samples, or to confirm the extent of liver damage, *e.g.*, the stage of liver disease.

A treatment or preventive effect is evident when there is a statistically significant improvement in one or more parameters of disease status, or by a failure to worsen or to develop symptoms where they would otherwise be anticipated. As an example, a favorable change of at least 10% in a measurable parameter of disease, and preferably at least 20%, 30%, 40%, 50% or more can be indicative of effective treatment. Efficacy for a given iRNA drug or formulation of that drug can also be judged using an experimental animal model for the given disease as known in the art. When using an experimental animal model, efficacy of treatment is evidenced when a statistically significant reduction in a marker or symptom is observed.

Alternatively, the efficacy can be measured by a reduction in the severity of disease as determined by one skilled in the art of diagnosis based on a clinically accepted disease severity grading scale.

Patients can be administered a therapeutic amount of iRNA, such as 0.01 mg/kg, 0.05 mg/kg, 0.1 mg/kg, 0.5 mg/kg, 1.0 mg/kg, 1.5 mg/kg, 2.0 mg/kg, or 2.5 mg/kg dsRNA. The iRNA can be administered by intravenous infusion over a period of time, such as over a 5 minute, 10 minute, 15 minute, 20 minute, or 25 minute period. The administration is repeated, for example, on a regular basis, such as biweekly (*i.e.*, every two weeks) for one month, two months, three months, four months or longer. After an initial treatment regimen, the treatments can be administered on a less frequent basis. For example, after administration biweekly for three months, administration can be repeated once per month, for six months or a year or longer. Administration of the iRNA can reduce TMPRSS6 levels, *e.g.*, in a cell, tissue, blood, urine or other compartment of the patient by at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80 % or at least 90% or more.

Before administration of a full dose of the iRNA, patients can be administered a smaller dose, such as a 5% infusion reaction, and monitored for adverse effects, such as an allergic reaction or a worsening of symptoms. In another example, the patient can be monitored for unwanted immunostimulatory effects, such as increased cytokine (*e.g.*, TNF-α or INF-α) levels.

Many disorders associated with elevated iron levels are hereditary. Therefore, a patient in need of a TMPRSS6 iRNA may be identified by taking a family history. A healthcare provider, such as a doctor, nurse, or family member, can take a family history before prescribing or administering a TMPRSS6 dsRNA. A DNA test may also be performed on the patient to identify a mutation in the TMPRSS6 gene, before a TMPRSS6 dsRNA is administered to the patient. For example, diagnosis of hereditary hemochromatosis can be confirmed by identifying the two HFE (Hemochromatosis) gene mutations C282Y and H63D, according to GenBank Accession No. CAB07442.1 (GI:1890180, record dated October 23, 2008).

Owing to the inhibitory effects on TMPRSS6 expression, a composition according to the invention or a pharmaceutical composition prepared therefrom can enhance the quality of life.

Methods for modulating expression of a TMPRSS6 gene

In yet another aspect, the invention provides a method for modulating (*e.g.*, inhibiting or activating) the expression of a TMPRSS6 gene in a mammal.

In one embodiment, the method includes administering a composition featured in the invention to the mammal such that expression of the target TMPRSS6 gene is decreased, such as for an extended duration, *e.g.*, at least two, three, four days or more, *e.g.*, one week, two weeks, three weeks, or four weeks or longer. The effect of the decreased target TMPRSS6 gene preferably results in a decrease in iron absorption and/or mobilization in the body. Decreased iron absorption or mobilization can be manifested by an observed decrease in serum ferritin levels, serum or liver iron levels, and/or serum transferrin saturation levels. In some embodiments, one or more of serum ferritin levels, serum or liver iron levels, or serum transferrin saturation levels are decreased by at least 5%, at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 40%, at least 50%, or at least 60%, or more, as compared to pretreatment levels. In some embodiments, serum ferritin levels are decreased by at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 40%, at least 50%, or at least 60%, or more, as compared to pretreatment levels.

In another embodiment, the method includes administering a composition as described herein to a mammal such that expression of the target TMPRSS6 gene is increased by *e.g.*, at least 10% compared to an untreated animal. In some embodiments, the activation

of TMPRSS6 occurs over an extended duration, *e.g.*, at least two, three, four days or more, *e.g.*, one week, two weeks, three weeks, four weeks, or more. Without wishing to be bound by theory, an iRNA can activate TMPRSS6 expression by stabilizing the TMPRSS6 mRNA transcript, interacting with a promoter in the genome, and/or inhibiting an inhibitor of TMPRSS6 expression.

The iRNAs useful for the methods and compositions featured in the invention specifically target RNAs (primary or processed) of the target TMPRSS6 gene. Compositions and methods for inhibiting the expression of these TMPRSS6 genes using iRNAs can be prepared and performed as described elsewhere herein.

In one embodiment, the method includes administering a composition containing an iRNA, where the iRNA includes a nucleotide sequence that is complementary to at least a part of an RNA transcript of the TMPRSS6 gene of the mammal to be treated. When the organism to be treated is a mammal such as a human, the composition may be administered by any means known in the art including, but not limited to oral, intraperitoneal, or parenteral routes, including intracranial (*e.g.*, intraventricular, intraparenchymal and intrathecal), intravenous, intramuscular, subcutaneous, transdermal, airway (aerosol), nasal, rectal, and topical (including buccal and sublingual) administration. In certain embodiments, the compositions are administered by intravenous infusion or injection.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the iRNAs and methods featured in the invention, suitable methods and materials are described below. All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

EXAMPLES

Example 1. Interference RNA (iRNA) synthesis

Source of reagents

Where the source of a reagent is not specifically given herein, such reagent may be obtained from any supplier of reagents for molecular biology at a quality/purity standard for application in molecular biology.

Oligonucleotide Synthesis

Applicants have used several different methods to generate the iRNA molecules described herein. This Example describes one approach that has been used. The ordinarily skilled artisan can use any method known in the art to prepare iRNAs as described herein.

Oligonucleotides are synthesized on an AKTAoligopilot synthesizer. Commercially available controlled pore glass solid support (dT-CPG, 500Å, Prime Synthesis) and RNA phosphoramidites with standard protecting groups, 5'-*O*-dimethoxytrityl N6-benzoyl-2'-*t*-butyldimethylsilyl-adenosine-3'-*O*-N,N'-diisopropyl-2-cyanoethylphosphoramidite, 5'-*O*-dimethoxytrityl-N4-acetyl-2'-*t*-butyldimethylsilyl-cytidine-3'-*O*-N,N'-diisopropyl-2-cyanoethylphosphoramidite, 5'-*O*-dimethoxytrityl-N2--isobutryl-2'-*t*-butyldimethylsilyl-guanosine-3'-*O*-N,N'-diisopropyl-2-cyanoethylphosphoramidite, and 5'-*O*-dimethoxytrityl-2'-*t*-butyldimethylsilyl-uridine-3'-O-N,N'-diisopropyl-2-cyanoethylphosphoramidite (Pierce Nucleic Acids Technologies) were used for the oligonucleotide synthesis. The 2'-F phosphoramidites, 5'-*O*-dimethoxytrityl-N4-acetyl-2'-fluro-cytidine-3'-*O*-N,N'-diisopropyl-2-cyanoethyl-phosphoramidite and 5'-*O*-dimethoxytrityl-2'-fluro-uridine-3'-O-N,N'-diisopropyl-2-cyanoethyl-phosphoramidite are purchased from (Promega). All phosphoramidites are used at a concentration of 0.2M in acetonitrile (CH_3CN) except for guanosine which is used at 0.2M concentration in 10% THF/ANC (v/v). Coupling/recycling time of 16 minutes is used. The activator is 5-ethyl thiotetrazole (0.75M, American International Chemicals); for the PO-oxidation iodine/water/pyridine is used and for the PS-oxidation PADS (2%) in 2,6-lutidine/ACN (1:1 v/v) is used.

3'-ligand conjugated strands are synthesized using solid support containing the corresponding ligand. For example, the introduction of cholesterol unit in the sequence is

performed from a hydroxyprolinol-cholesterol phosphoramidite. Cholesterol is tethered to *trans*-4-hydroxyprolinol via a 6-aminohexanoate linkage to obtain a hydroxyprolinol-cholesterol moiety. 5'-end Cy-3 and Cy-5.5 (fluorophore) labeled iRNAs are synthesized from the corresponding Quasar-570 (Cy-3) phosphoramidite are purchased from Biosearch Technologies. Conjugation of ligands to 5'-end and or internal position is achieved by using appropriately protected ligand-phosphoramidite building block. An extended 15 min coupling of 0.1 M solution of phosphoramidite in anhydrous CH_3CN in the presence of 5-(ethylthio)-1*H*-tetrazole activator to a solid-support-bound oligonucleotide. Oxidation of the internucleotide phosphite to the phosphate is carried out using standard iodine-water as reported (1) or by treatment with *tert*-butyl hydroperoxide/acetonitrile/water (10: 87: 3) with 10 min oxidation wait time conjugated oligonucleotide. Phosphorothioate is introduced by the oxidation of phosphite to phosphorothioate by using a sulfur transfer reagent such as DDTT (purchased from AM Chemicals), PADS and or Beaucage reagent. The cholesterol phosphoramidite is synthesized in house and used at a concentration of 0.1 M in dichloromethane. Coupling time for the cholesterol phosphoramidite is 16 minutes.

Deprotection I (Nucleobase Deprotection)

After completion of synthesis, the support is transferred to a 100 mL glass bottle (VWR). The oligonucleotide is cleaved from the support with simultaneous deprotection of base and phosphate groups with 80 mL of a mixture of ethanolic ammonia [ammonia: ethanol (3:1)] for 6.5 h at 55°C. The bottle is cooled briefly on ice and then the ethanolic ammonia mixture is filtered into a new 250-mL bottle. The CPG is washed with 2 x 40 mL portions of ethanol/water (1:1 v/v). The volume of the mixture is then reduced to ~ 30 mL by roto-vap. The mixture is then frozen on dry ice and dried under vacuum on a speed vac.

Deprotection II (Removal of 2'-TBDMS group)

The dried residue is resuspended in 26 mL of triethylamine, triethylamine trihydrofluoride (TEA•3HF) or pyridine-HF and DMSO (3:4:6) and heated at 60°C for 90 minutes to remove the *tert*-butyldimethylsilyl (TBDMS) groups at the 2' position. The reaction is then quenched with 50 mL of 20 mM sodium acetate and the pH is adjusted to 6.5. Oligonucleotide is stored in a freezer until purification.

Analysis

The oligonucleotides are analyzed by high-performance liquid chromatography (HPLC) prior to purification and selection of buffer and column depends on nature of the sequence and or conjugated ligand.

HPLC Purification

The ligand-conjugated oligonucleotides are purified by reverse-phase preparative HPLC. The unconjugated oligonucleotides are purified by anion-exchange HPLC on a TSK gel column packed in house. The buffers are 20 mM sodium phosphate (pH 8.5) in 10% CH_3CN (buffer A) and 20 mM sodium phosphate (pH 8.5) in 10% CH_3CN, 1M NaBr (buffer B). Fractions containing full-length oligonucleotides are pooled, desalted, and lyophilized. Approximately 0.15 OD of desalted oligonucleotides are diluted in water to 150 µL and then pipetted into special vials for CGE and LC/MS analysis. Compounds are then analyzed by LC-ESMS and CGE.

iRNA preparation

For the general preparation of iRNA, equimolar amounts of sense and antisense strand are heated in 1xPBS at 95°C for 5 min and slowly cooled to room temperature. Integrity of the duplex is confirmed by HPLC analysis.

Nucleic acid sequences are represented below using standard nomenclature, and specifically the abbreviations of Table 1.

Table 1: Abbreviations of nucleotide monomers used in nucleic acid sequence representation. It will be understood that these monomers, when present in an oligonucleotide, are mutually linked by 5'-3'-phosphodiester bonds.

Abbreviation	Nucleotide(s)
A	adenosine
C	cytidine
G	guanosine
T	thymidine
U	uridine
N	any nucleotide (G, A, C, T or U)
a	2'-O-methyladenosine
c	2'-O-methylcytidine
g	2'-O-methylguanosine
u	2'-O-methyluridine

Abbreviation	Nucleotide(s)
dT	2'-deoxythymidine
s	phosphorothioate linkage

Example 2. TMPRSS6 siRNA Design

Transcripts

siRNAs targeting TMPRSS6 were designed and synthesized. The design used human transcript NM_153609.2 (SEQ ID NO:1, FIG.1) from the NCBI Refseq collection.

siRNA duplexes were designed with 100% identity to the TMPRSS6 gene.

A total of 655 sense and 655 antisense human TMPRSS6 derived siRNA oligos were designed. The oligos are presented in Table 2. Additional sense and antisense human TMPRSS6 derived siRNA oligos are presented in Table 3. Sense and antisense human TMPRSS6 derived siRNA oligos with modifications are presented in Table 4.

Table 2. Sense and antisense strand sequences of human TMPRSS6 dsRNAs

position of 5' base on transcript (NM_153609.2, SEQ ID NO:1)	Sense Sequence (5' to 3')	SEQ ID NO:	Antisense Sequence (5' to 3')	SEQ ID NO:
36	CUCUGGUGCGAGCUGACCU	9	AGGUCAGCUCGCACCAGAG	10
46	AGCUGACCUGAGAUGCACU	11	AGUGCAUCUCAGGUCAGCU	12
72	UCUGUGAGCUGUCUCGGCA	13	UGCCGAGACAGCUCACAGA	14
78	AGCUGUCUCGGCACCCACU	15	AGUGGGUGCCGAGACAGCU	16
79	GCUGUCUCGGCACCCACUU	17	AAGUGGGUGCCGAGACAGC	18
100	AGUCACUGCCGCCUGAUGU	19	ACAUCAGGCGGCAGUGACU	20
104	ACUGCCGCCUGAUGUUGUU	21	AACAACAUCAGGCGGCAGU	22
105	CUGCCGCCUGAUGUUGUUA	23	UAACAACAUCAGGCGGCAG	24
107	GCCGCCUGAUGUUGUUACU	25	AGUAACAACAUCAGGCGGC	26
110	GCCUGAUGUUGUUACUCUU	27	AAGAGUAACAACAUCAGGC	28
124	CUCUUCCACUCCAAAAGGA	29	UCCUUUUGGAGUGGAAGAG	30
131	ACUCCAAAAGGAUGCCCGU	31	ACGGGCAUCCUUUUGGAGU	32
233	GUGAGGACUCCAAGAGAAA	33	UUUCUCUUGGAGUCCUCAC	34
311	CUUCGGCGGGGGUGCUACU	35	AGUAGCACCCCCGCCGAAG	36
313	UCGGCGGGGGUGCUACUCU	37	AGAGUAGCACCCCCGCCGA	38
316	GCGGGGGUGCUACUCUGGU	39	ACCAGAGUAGCACCCCCGC	40
318	GGGGGUGCUACUCUGGUAU	41	AUACCAGAGUAGCACCCCC	42
319	GGGGUGCUACUCUGGUAUU	43	AAUACCAGAGUAGCACCCC	44
329	UCUGGUAUUUCCUAGGGUA	45	UACCCUAGGAAAUACCAGA	46
331	UGGUAUUUCCUAGGGUACA	47	UGUACCCUAGGAAAUACCA	48
332	GGUAUUUCCUAGGGUACAA	49	UUGUACCCUAGGAAAUACC	50
363	GGUCAGCCAGGUGUACUCA	51	UGAGUACACCUGGCUGACC	52

position of 5' base on transcript (NM_153609.2, SEQ ID NO:1)	Sense Sequence (5' to 3')	SEQ ID NO:	Antisense Sequence (5' to 3')	SEQ ID NO:
367	AGCCAGGUGUACUCAGGCA	53	UGCCUGAGUACACCUGGCU	54
375	GUACUCAGGCAGUCUGCGU	55	ACGCAGACUGCCUGAGUAC	56
377	ACUCAGGCAGUCUGCGUGU	57	ACACGCAGACUGCCUGAGU	58
380	CAGGCAGUCUGCGUGUACU	59	AGUACACGCAGACUGCCUG	60
382	GGCAGUCUGCGUGUACUCA	61	UGAGUACACGCAGACUGCC	62
383	GCAGUCUGCGUGUACUCAA	63	UUGAGUACACGCAGACUGC	64
384	CAGUCUGCGUGUACUCAAU	65	AUUGAGUACACGCAGACUG	66
389	UGCGUGUACUCAAUCGCCA	67	UGGCGAUUGAGUACACGCA	68
391	CGUGUACUCAAUCGCCACU	69	AGUGGCGAUUGAGUACACG	70
392	GUGUACUCAAUCGCCACUU	71	AAGUGGCGAUUGAGUACAC	72
394	GUACUCAAUCGCCACUUCU	73	AGAAGUGGCGAUUGAGUAC	74
406	CACUUCUCCCAGGAUCUUA	75	UAAGAUCCUGGGAGAAGUG	76
418	GAUCUUACCCGCCGGGAAU	77	AUUCCCGGCGGGUAAGAUC	78
420	UCUUACCCGCCGGGAAUCU	79	AGAUUCCCGGCGGGUAAGA	80
421	CUUACCCGCCGGGAAUCUA	81	UAGAUUCCCGGCGGGUAAG	82
423	UACCCGCCGGGAAUCUAGU	83	ACUAGAUUCCCGGCGGGUA	84
427	CGCCGGGAAUCUAGUGCCU	85	AGGCACUAGAUUCCCGGCG	86
428	GCCGGGAAUCUAGUGCCUU	87	AAGGCACUAGAUUCCCGGC	88
446	UCCGCAGUGAAACCGCCAA	89	UUGGCGGUUUCACUGCGGA	90
447	CCGCAGUGAAACCGCCAAA	91	UUUGGCGGUUUCACUGCGG	92
502	CGCCUGGGAACUUACUACA	93	UGUAGUAAGUUCCCAGGCG	94
503	GCCUGGGAACUUACUACAA	95	UUGUAGUAAGUUCCCAGGC	96
505	CUGGGAACUUACUACAACU	97	AGUUGUAGUAAGUUCCCAG	98
517	UACAACUCCAGCUCCGUCU	99	AGACGGAGCUGGAGUUGUA	100
518	ACAACUCCAGCUCCGUCUA	101	UAGACGGAGCUGGAGUUGU	102
520	AACUCCAGCUCCGUCUAUU	103	AAUAGACGGAGCUGGAGUU	104
541	UUUGGGGAGGGACCCCUCA	105	UGAGGGGUCCCUCCCCAAA	106
550	GGACCCCUCACCUGCUUCU	107	AGAAGCAGGUGAGGGGUCC	108
563	GCUUCUUCUGGUUCAUUCU	109	AGAAUGAACCAGAAGAAGC	110
566	UCUUCUGGUUCAUUCUCCA	111	UGGAGAAUGAACCAGAAGA	112
593	AGCACCGCCGGCUGAUGCU	113	AGCAUCAGCCGGCGGUGCU	114
680	UCCCCUACAGGGCCGAGUA	115	UACUCGGCCCUGUAGGGGA	116
683	CCUACAGGGCCGAGUACGA	117	UCGUACUCGGCCCUGUAGG	118
686	ACAGGGCCGAGUACGAAGU	119	ACUUCGUACUCGGCCCUGU	120
689	GGGCCGAGUACGAAGUGGA	121	UCCACUUCGUACUCGGCCC	122
710	CCGAGGGCCUAGUGAUCCU	123	AGGAUCACUAGGCCCUCGG	124
735	CAGUGUGAAAGACAUAGCU	125	AGCUAUGUCUUUCACACUG	126
759	GAAUUCCACGCUGGGUUGU	127	ACAACCCAGCGUGGAAUUC	128
760	AAUUCCACGCUGGGUUGUU	129	AACAACCCAGCGUGGAAUU	130
766	ACGCUGGGUUGUUACCGCU	131	AGCGGUAACAACCCAGCGU	132
767	CGCUGGGUUGUUACCGCUA	133	UAGCGGUAACAACCCAGCG	134
769	CUGGGUUGUUACCGCUACA	135	UGUAGCGGUAACAACCCAG	136
772	GGUUGUUACCGCUACAGCU	137	AGCUGUAGCGGUAACAACC	138
776	GUUACCGCUACAGCUACGU	139	ACGUAGCUGUAGCGGUAAC	140
872	AGGACCUCAUGCUCAAACU	141	AGUUUGAGCAUGAGGUCCU	142
878	UCAUGCUCAAACUCCGGCU	143	AGCCGGAGUUUGAGCAUGA	144
970	AUCACCUCGGUGUACGGCU	145	AGCCGUACACCGAGGUGAU	146
973	ACCUCGGUGUACGGCUGCA	147	UGCAGCCGUACACCGAGGU	148
1033	AUCAUGGCGGUCGUCUGGA	149	UCCAGACGACCGCCAUGAU	150
1034	UCAUGGCGGUCGUCUGGAA	151	UUCCAGACGACCGCCAUGA	152

position of 5' base on transcript (NM_153609.2, SEQ ID NO:1)	Sense Sequence (5' to 3')	SEQ ID NO:	Antisense Sequence (5' to 3')	SEQ ID NO:
1067	GCUACUACGACCCCUUCGU	153	ACGAAGGGGUCGUAGUAGC	154
1091	CCGUGCAGCCGGUGGUCUU	155	AAGACCACCGGCUGCACGG	156
1106	UCUUCCAGGCCUGUGAAGU	157	ACUUCACAGGCCUGGAAGA	158
1114	GCCUGUGAAGUGAACCUGA	159	UCAGGUUCACUUCACAGGC	160
1118	GUGAAGUGAACCUGACGCU	161	AGCGUCAGGUUCACUUCAC	162
1133	CGCUGGACAACAGGCUCGA	163	UCGAGCCUGUUGUCCAGCG	164
1135	CUGGACAACAGGCUCGACU	165	AGUCGAGCCUGUUGUCCAG	166
1162	GUCCUCAGCACCCCGUACU	167	AGUACGGGGUGCUGAGGAC	168
1163	UCCUCAGCACCCCGUACUU	169	AAGUACGGGGUGCUGAGGA	170
1168	AGCACCCCGUACUUCCCCA	171	UGGGGAAGUACGGGGUGCU	172
1185	CAGCUACUACUCGCCCCAA	173	UUGGGGCGAGUAGUAGCUG	174
1186	AGCUACUACUCGCCCCAAA	175	UUUGGGGCGAGUAGUAGCU	176
1190	ACUACUCGCCCCAAACCCA	177	UGGGUUUGGGGCGAGUAGU	178
1195	UCGCCCCAAACCCACUGCU	179	AGCAGUGGGUUUGGGGCGA	180
1211	GCUCCUGGCACCUCACGGU	181	ACCGUGAGGUGCCAGGAGC	182
1231	CCCUCUCUGGACUACGGCU	183	AGCCGUAGUCCAGAGAGGG	184
1244	ACGGCUUGGCCCUCUGGUU	185	AACCAGAGGGCCAAGCCGU	186
1245	CGGCTTGGCCCTCTGGTTT	187	AAACCAGAGGGCCAAGCCG	188
1247	GCUUGGCCCUCUGGUUUGA	189	UCAAACCAGAGGGCCAAGC	190
1254	CCUCUGGUUUGAUGCCUAU	191	AUAGGCAUCAAACCAGAGG	192
1285	CAGAAGUAUGAUUUGCCGU	193	ACGGCAAAUCAUACUUCUG	194
1288	AAGUAUGAUUUGCCGUGCA	195	UGCACGGCAAAUCAUACUU	196
1292	AUGAUUUGCCGUGCACCCA	197	UGGGUGCACGGCAAAUCAU	198
1306	ACCCAGGGCCAGUGGACGA	199	UCGUCCACUGGCCCUGGGU	200
1310	AGGGCCAGUGGACGAUCCA	201	UGGAUCGUCCACUGGCCCU	202
1312	GGCCAGUGGACGAUCCAGA	203	UCUGGAUCGUCCACUGGCC	204
1313	GCCAGUGGACGAUCCAGAA	205	UUCUGGAUCGUCCACUGGC	206
1360	CAGCCCUACGCCGAGAGGA	207	UCCUCUCGGCGUAGGGCUG	208
1443	CGGUGUGCGGGUGCACUAU	209	AUAGUGCACCCGCACACCG	210
1447	GUGCGGGUGCACUAUGGCU	211	AGCCAUAGUGCACCCGCAC	212
1448	UGCGGGUGCACUAUGGCUU	213	AAGCCAUAGUGCACCCGCA	214
1451	GGGUGCACUAUGGCUUGUA	215	UACAAGCCAUAGUGCACCC	216
1454	UGCACUAUGGCUUGUACAA	217	UUGUACAAGCCAUAGUGCA	218
1486	UGCCCUGGAGAGUUCCUCU	219	AGAGGAACUCUCCAGGGCA	220
1565	UGGAUGAGAGAAACUGCGU	221	ACGCAGUUUCUCUCAUCCA	222
1611	GGACAGCACAUGCAUCUCA	223	UGAGAUGCAUGUGCUGUCC	224
1613	ACAGCACAUGCAUCUCACU	225	AGUGAGAUGCAUGUGCUGU	226
1634	CCAAGGUCUGUGAUGGGCA	227	UGCCCAUCACAGACCUUGG	228
1646	AUGGGCAGCCUGAUUGUCU	229	AGACAAUCAGGCUGCCCAU	230
1649	GGCAGCCUGAUUGUCUCAA	231	UUGAGACAAUCAGGCUGCC	232
1654	CCUGAUUGUCUCAACGGCA	233	UGCCGUUGAGACAAUCAGG	234
1662	UCUCAACGGCAGCGACGAA	235	UUCGUCGCUGCCGUUGAGA	236
1687	UGCCAGGAAGGGGUGCCAU	237	AUGGCACCCCUUCCUGGCA	238
1696	GGGGUGCCAUGUGGGACAU	239	AUGUCCCACAUGGCACCCC	240
1699	GUGCCAUGUGGGACAUUCA	241	UGAAUGUCCCACAUGGCAC	242
1703	CAUGUGGGACAUUCACCUU	243	AAGGUGAAUGUCCCACAUG	244
1723	CAGUGUGAGGACCGGAGCU	245	AGCUCCGGUCCUCACACUG	246
1745	UGAAGAAGCCCAACCCGCA	247	UGCGGGUUGGGCUUCUUCA	248
1749	GAAGCCCAACCCGCAGUGU	249	ACACUGCGGGUUGGGCUUC	250
1830	CCCCUCCAGCCGCAUUGUU	251	AACAAUGCGGCUGGAGGGG	252

position of 5' base on transcript (NM_153609.2, SEQ ID NO:1)	Sense Sequence (5' to 3')	SEQ ID NO:	Antisense Sequence (5' to 3')	SEQ ID NO:
1897	CAGGUUCGGGGUCGACACA	253	UGUGUCGACCCCGAACCUG	254
1898	AGGUUCGGGGUCGACACAU	255	AUGUGUCGACCCCGAACCU	256
1900	GUUCGGGGUCGACACAUCU	257	AGAUGUGUCGACCCCGAAC	258
1935	CGCUGACCGCUGGGUGAUA	259	UAUCACCCAGCGGUCAGCG	260
1936	GCUGACCGCUGGGUGAUAA	261	UUAUCACCCAGCGGUCAGC	262
1938	UGACCGCUGGGUGAUAACA	263	UGUUAUCACCCAGCGGUCA	264
1941	CCGCUGGGUGAUAACAGCU	265	AGCUGUUAUCACCCAGCGG	266
1997	UGCUGUGGACCGUGUUCCU	267	AGGAACACGGUCCACAGCA	268
2023	GUGUGGCAGAACUCGCGCU	269	AGCGCGAGUUCUGCCACAC	270
2078	UCCUGCACCCGUACCACGA	271	UCGUGGUACGGGUGCAGGA	272
2079	CCUGCACCCGUACCACGAA	273	UUCGUGGUACGGGUGCAGG	274
2081	UGCACCCGUACCACGAAGA	275	UCUUCGUGGUACGGGUGCA	276
2186	CGCGCUCCCACUUCUUCGA	277	UCGAAGAAGUGGGAGCGCG	278
2209	GGCCUGCACUGCUGGAUUA	279	UAAUCCAGCAGUGCAGGCC	280
2215	CACUGCUGGAUUACGGGCU	281	AGCCCGUAAUCCAGCAGUG	282
2283	GGAUGUGCAGUUGAUCCCA	283	UGGGAUCAACUGCACAUCC	284
2311	UGCAGCGAGGUCUAUCGCU	285	AGCGAUAGACCUCGCUGCA	286
2312	GCAGCGAGGUCUAUCGCUA	287	UAGCGAUAGACCUCGCUGC	288
2315	GCGAGGUCUAUCGCUACCA	289	UGGUAGCGAUAGACCUCGC	290
2320	GUCUAUCGCUACCAGGUGA	291	UCACCUGGUAGCGAUAGAC	292
2333	AGGUGACGCCACGCAUGCU	293	AGCAUGCGUGGCGUCACCU	294
2335	GUGACGCCACGCAUGCUGU	295	ACAGCAUGCGUGGCGUCAC	296
2337	GACGCCACGCAUGCUGUGU	297	ACACAGCAUGCGUGGCGUC	298
2470	GGCUGUGGCCGGCCUAACU	299	AGUUAGGCCGGCCACAGCC	300
2471	GCUGUGGCCGGCCUAACUA	301	UAGUUAGGCCGGCCACAGC	302
2473	UGUGGCCGGCCUAACUACU	303	AGUAGUUAGGCCGGCCACA	304
2480	GGCCUAACUACUUCGGCGU	305	ACGCCGAAGUAGUUAGGCC	306
2482	CCUAACUACUUCGGCGUCU	307	AGACGCCGAAGUAGUUAGG	308
2483	CUAACUACUUCGGCGUCUA	309	UAGACGCCGAAGUAGUUAG	310
2485	AACUACUUCGGCGUCUACA	311	UGUAGACGCCGAAGUAGUU	312
2501	ACACCCGCAUCACAGGUGU	313	ACACCUGUGAUGCGGGUGU	314
2506	CGCAUCACAGGUGUGAUCA	315	UGAUCACACCUGUGAUGCG	316
2525	GCUGGAUCCAGCAAGUGGU	317	ACCACUUGCUGGAUCCAGC	318
2551	GGAACUGCCCCCCUGCAAA	319	UUUGCAGGGGGGCAGUUCC	320
2671	AGGAGGUGGCAUCUUGUCU	321	AGACAAGAUGCCACCUCCU	322
2674	AGGUGGCAUCUUGUCUCGU	323	ACGAGACAAGAUGCCACCU	324
2678	GGCAUCUUGUCUCGUCCCU	325	AGGGACGAGACAAGAUGCC	326
2680	CAUCUUGUCUCGUCCCUGA	327	UCAGGGACGAGACAAGAUG	328
2681	AUCUUGUCUCGUCCCUGAU	329	AUCAGGGACGAGACAAGAU	330
2739	CAGCUGGGGGUCAAGACGU	331	ACGUCUUGACCCCCAGCUG	332
2744	GGGGGUCAAGACGUCCCCU	333	AGGGGACGUCUUGACCCCC	334
2746	GGGUCAAGACGUCCCCUGA	335	UCAGGGGACGUCUUGACCC	336
2825	CCACUGCUGCCUAAUGCAA	337	UUGCAUUAGGCAGCAGUGG	338
2829	UGCUGCCUAAUGCAAGGCA	339	UGCCUUGCAUUAGGCAGCA	340
2835	CUAAUGCAAGGCAGUGGCU	341	AGCCACUGCCUUGCAUUAG	342
2857	CAGCAAGAAUGCUGGUUCU	343	AGAACCAGCAUUCUUGCUG	344
2894	GAGGUGCGCCCCACUCUGU	345	ACAGAGUGGGGCGCACCUC	346
2958	CUUCGGAAGCCCCUGGUCU	347	AGACCAGGGGCUUCCGAAG	348
2960	UCGGAAGCCCCUGGUCUAA	349	UUAGACCAGGGGCUUCCGA	350
2962	GGAAGCCCCUGGUCUAACU	351	AGUUAGACCAGGGGCUUCC	352

position of 5' base on transcript (NM_153609.2 , SEQ ID NO:1)	Sense Sequence (5' to 3')	SEQ ID NO:	Antisense Sequence (5' to 3')	SEQ ID NO:
2963	GAAGCCCCUGGUCUAACUU	353	AAGUUAGACCAGGGGCUUC	354
2968	CCCUGGUCUAACUUGGGAU	355	AUCCCAAGUUAGACCAGGG	356
2970	CUGGUCUAACUUGGGAUCU	357	AGAUCCCAAGUUAGACCAG	358
2975	CUAACUUGGGAUCUGGGAA	359	UUCCCAGAUCCCAAGUUAG	360
3006	CCAUCGGAGGGGACCCUCA	361	UGAGGGUCCCCUCCGAUGG	362
3045	UGGGCCUGCUGCCACUGUA	363	UACAGUGGCAGCAGGCCCA	364
3046	GGGCCUGCUGCCACUGUAA	365	UUACAGUGGCAGCAGGCCC	366
3052	GCUGCCACUGUAAGCCAAA	367	UUUGGCUUACAGUGGCAGC	368
3056	CCACUGUAAGCCAAAAGGU	369	ACCUUUUGGCUUACAGUGG	370
3071	AGGUGGGGAAGUCCUGACU	371	AGUCAGGACUUCCCCACCU	372
3174	GAAUAAAGCUGCCUGAUCA	373	UGAUCAGGCAGCUUUAUUC	374
3175	AAUAAAGCUGCCUGAUCAA	375	UUGAUCAGGCAGCUUUAUU	376
3180	AGCUGCCUGAUCAAAAAAA	377	UUUUUUUGAUCAGGCAGCU	378

Table 3. Unmodified sense and antisense strand sequences of human TMPRSS6 dsRNAs

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Duplex ID	SEQ ID NO.: (sense)	Sense Trans seq	Position in NM_153609.2	Position in NM_153609.2	SEQ ID NO.: (antisense)	Antisense Trans seq
AD-46230.1	43	GGGGUGCUACUCUGGUAUU	319	319-337	44	AAUACCAGAGUAGCACCCC
AD-46231.1	111	UCUUCUGGUUCAUUCUCCA	566	566-584	112	UGGAGAAUGAACCAGAAGA
AD-46232.1	131	ACGCUGGGUUGUUACCGCU	766	766-784	132	AGCGGUAACAACCCAGCGU
AD-46233.1	193	CAGAAGUAUGAUUUGCCGU	1285	1285-1303	194	ACGGCAAAUCAUACUUCUG
AD-46234.1	259	CGCUGACCGCUGGGUGAUA	1935	1935-1953	260	UAUCACCCAGCGGUCAGCG
AD-46235.1	45	UCUGGUAUUUCCUAGGGUA	329	329-347	46	UACCCUAGGAAAUACCAGA
AD-46236.1	117	CCUACAGGGCCGAGUACGA	683	683-701	118	UCGUACUCGGCCCUGUAGG
AD-46237.1	133	CGCUGGGUUGUUACCGCUA	767	767-785	134	UAGCGGUAACAACCCAGCG
AD-46238.1	203	GGCCAGUGGACGAUCCAGA	1312	1312-1330	204	UCUGGAUCGUCCACUGGCC
AD-46239.1	263	UGACCGCUGGGUGAUAACA	1938	1938-1956	264	UGUUAUCACCCAGCGGUCA
AD-46240.1	51	GGUCAGCCAGGUGUACUCA	363	363-381	52	UGAGUACACCUGGCUGACC
AD-46241.1	379	CUACAGGGCCGAGUACGAA	684	684-702	380	UUCGUACUCGGCCCUGUAG
AD-46242.1	135	CUGGGUUGUUACCGCUACA	769	769-787	136	UGUAGCGGUAACAACCCAG
AD-46243.1	217	UGCACUAUGGCUUGUACAA	1454	1454-1472	218	UUGUACAAGCCAUAGUGCA
AD-46244.1	604	CCUGGAGAGGUGUCCUUCA	2044	2044-2062	605	UGAAGGACACCUCUCCAGG
AD-46244.2	604	CCUGGAGAGGUGUCCUUCA	2044	2044-2062	605	UGAAGGACACCUCUCCAGG
AD-46245.1	53	AGCCAGGUGUACUCAGGCA	367	367-385	54	UGCCUGAGUACACCUGGCU
AD-46246.1	119	ACAGGGCCGAGUACGAAGU	686	686-704	120	ACUUCGUACUCGGCCCUGU
AD-46247.1	137	GGUUGUUACCGCUACAGCU	772	772-790	138	AGCUGUAGCGGUAACAACC
AD-46248.1	381	UGUGAUGGGGUCAAGGACU	1534	1534-1552	382	AGUCCUUGACCCCAUCACA

AD-46249.1	383	CUGGAGAGGUGUCCUUCAA	2045	2045-2063	384	UUGAAGGACACCUCUCCAG
AD-46250.1	89	UCCGCAGUGAAACCGCCAA	446	446-464	90	UUGGCGGUUUCACUGCGGA
AD-46251.1	121	GGGCCGAGUACGAAGUGGA	689	689-707	122	UCCACUUCGUACUCGGCCC
AD-46252.1	385	GGACCGACUGGCCAUGUAU	921	921-939	386	AUACAUGGCCAGUCGGUCC
AD-46253.1	606	CAACGGCCUGGAUGAGAGA	1557	1557-1575	607	UCUCUCAUCCAGGCCGUUG
AD-46253.2	606	CAACGGCCUGGAUGAGAGA	1557	1557-1575	607	UCUCUCAUCCAGGCCGUUG
AD-46254.1	387	AGUUGAUCCCACAGGACCU	2291	2291-2309	388	AGGUCCUGUGGGAUCAACU
AD-46255.1	91	CCGCAGUGAAACCGCCAAA	447	447-465	92	UUUGGCGGUUUCACUGCGG
AD-46256.1	123	CCGAGGGCCUAGUGAUCCU	710	710-728	124	AGGAUCACUAGGCCCUCGG
AD-46257.1	169	UCCUCAGCACCCCGUACUU	1163	1163-1181	170	AAGUACGGGGUGCUGAGGA
AD-46258.1	253	CAGGUUCGGGGUCGACACA	1897	1897-1915	254	UGUGUCGACCCCGAACCUG
AD-46259.1	293	AGGUGACGCCACGCAUGCU	2333	2333-2351	294	AGCAUGCGUGGCGUCACCU
AD-46260.1	389	AAACCGCCAAAGCCCAGAA	455	455-473	390	UUCUGGGCUUUGGCGGUUU
AD-46261.1	125	CAGUGUGAAAGACAUAGCU	735	735-753	126	AGCUAUGUCUUUCACACUG
AD-46262.1	183	CCCUCUCUGGACUACGGCU	1231	1231-1249	184	AGCCGUAGUCCAGAGAGGG
AD-46263.1	257	GUUCGGGGUCGACACAUCU	1900	1900-1918	258	AGAUGUGUCGACCCCGAAC
AD-46264.1	391	UGUGUGCCGGCUACCGCAA	2351	2351-2369	392	UUGCGGUAGCCGGCACACA
AD-46265.1	109	GCUUCUUCUGGUUCAUUCU	563	563-581	110	AGAAUGAACCAGAAGAAGC
AD-46266.1	393	AUUCCACGCUGGGUUGUUA	761	761-779	394	UAACAACCCAGCGUGGAAU
AD-46267.1	185	ACGGCUUGGCCCUCUGGUU	1244	1244-1262	186	AACCAGAGGGCCAAGCCGU
AD-46268.1	395	UCGCUGACCGCUGGGUGAU	1934	1934-1952	396	AUCACCCAGCGGUCAGCGA
AD-46269.1	608	AGUGGUGACCUGAGGAACU	2538	2538-2556	609	AGUUCCUCAGGUCACCACU
AD-46269.2	608	AGUGGUGACCUGAGGAACU	2538	2538-2556	609	AGUUCCUCAGGUCACCACU
AD-46270.1	397	CAAGCAGGGGGACAAGUAU	2612	2612-2630	398	AUACUUGUCCCCCUGCUUG
AD-46271.1	399	UGAUGUCUGCUCCAGUGAU	2696	2696-2714	400	AUCACUGGAGCAGACAUCA
AD-46272.1	359	CUAACUUGGGAUCUGGGAA	2975	2975-2993	360	UUCCCAGAUCCCAAGUUAG
AD-46273.1	47	UGGUAUUUCCUAGGGUACA	331	331-349	48	UGUACCCUAGGAAAUACCA
AD-46273.2	47	UGGUAUUUCCUAGGGUACA	331	331-349	48	UGUACCCUAGGAAAUACCA
AD-46273.3	47	UGGUAUUUCCUAGGGUACA	331	331-349	48	UGUACCCUAGGAAAUACCA
AD-46274.1	401	GAGGUGUCCUUCAAGGUGA	2050	2050-2068	402	UCACCUUGAAGGACACCUC
AD-46276.1	403	AAGCAGGGGGACAAGUAUU	2613	2613-2631	404	AAUACUUGUCCCCCUGCUU
AD-46277.1	331	CAGCUGGGGGUCAAGACGU	2739	2739-2757	332	ACGUCUUGACCCCCAGCUG
AD-46278.1	405	CUUGGGAUCUGGGAAUGGA	2979	2979-2997	406	UCCAUUCCCAGAUCCCAAG
AD-46279.1	407	GGUAUUUCCUAGGGUACAA	332	332-350	408	UUGUACCCUAGGAAAUACC
AD-46280.1	409	GGCUACCGCAAGGGCAAGA	2359	2359-2377	410	UCUUGCCCUUGCGGUAGCC
AD-46282.1	411	GCAGGGGGACAAGUAUUCU	2615	2615-2633	412	AGAAUACUUGUCCCCCUGC
AD-46283.1	413	GCUCAGCAGCAAGAAUGCU	2851	2851-2869	414	AGCAUUCUUGCUGCUGAGC
AD-46284.1	415	UUGGGAUCUGGGAAUGGAA	2980	2980-2998	416	UUCCAUUCCCAGAUCCCAA
AD-46285.1	417	CCAAAGCCCAGAAGAUGCU	461	461-479	418	AGCAUCUUCUGGGCUUUGG
AD-46286.1	419	GCUACCGCAAGGGCAAGAA	2360	2360-2378	420	UUCUUGCCCUUGCGGUAGC
AD-46286.2	419	GCUACCGCAAGGGCAAGAA	2360	2360-2378	420	UUCUUGCCCUUGCGGUAGC
AD-46288.1	423	UGGUGGCAGGAGGUGGCAU	2664	2664-2682	424	AUGCCACCUCCUGCCACCA

AD-46289.1	425	CCCACUCUGUACAGAGGCU	2903	2903-2921	426	AGCCUCUGUACAGAGUGGG
AD-46290.1	427	CUCACAGCCCAGACCCUCA	3128	3128-3146	428	UGAGGGUCUGGGCUGUGAG
AD-46291.1	429	CCUCUCUGGACUACGGCUU	1232	1232-1250	430	AAGCCGUAGUCCAGAGAGG
AD-46293.1	431	GUGGCAGGAGGUGGCAUCU	2666	2666-2684	432	AGAUGCCACCUCCUGCCAC
AD-46294.1	433	UUCGGAAGCCCCUGGUCUA	2959	2959-2977	434	UAGACCAGGGGCUUCCGAA
AD-46295.1	435	AGCUCAGCUGCCCUUUGGA	3157	3157-3175	436	UCCAAAGGGCAGCUGAGCU
AD-46296.1	437	GGCCUGGAUGAGAGAAACU	1561	1561-1579	438	AGUUUCUCUCAUCCAGGCC
AD-46297.1	439	UGGCAGGAGGUGGCAUCUU	2667	2667-2685	440	AAGAUGCCACCUCCUGCCA
AD-46298.1	349	UCGGAAGCCCCUGGUCUAA	2960	2960-2978	350	UUAGACCAGGGGCUUCCGA
AD-46299.1	421	GCUCAGCUGCCCUUUGGAA	3158	3158-3176	422	UUCCAAAGGGCAGCUGAGC
AD-46299.2	421	GCUCAGCUGCCCUUUGGAA	3158	3158-3176	422	UUCCAAAGGGCAGCUGAGC
AD-46300.1	441	ACUGUGACUGUGGCCUCCA	1808	1808-1826	442	UGGAGGCCACAGUCACAGU
AD-46301.1	321	AGGAGGUGGCAUCUUGUCU	2671	2671-2689	322	AGACAAGAUGCCACCUCCU
AD-46302.1	443	CCCCUGGUCUAACUUGGGA	2967	2967-2985	444	UCCCAAGUUAGACCAGGGG
AD-46303.1	445	UCAGCUGCCCUUUGGAAUA	3160	3160-3178	446	UAUUCCAAAGGGCAGCUGA
AD-46304.1	447	UCGGGGUCGACACAUCUGU	1902	1902-1920	448	ACAGAUGUGUCGACCCCGA
AD-46305.1	449	GUCCCUGAUGUCUGCUCCA	2691	2691-2709	450	UGGAGCAGACAUCAGGGAC
AD-46306.1	355	CCCUGGUCUAACUUGGGAU	2968	2968-2986	356	AUCCCAAGUUAGACCAGGG
AD-46307.1	610	CAGCUGCCCUUUGGAAUAA	3161	3161-3179	611	UUAUUCCAAAGGGCAGCUG
AD-46307.2	610	CAGCUGCCCUUUGGAAUAA	3161	3161-3179	611	UUAUUCCAAAGGGCAGCUG
AD-46308.1	451	UCAUCGCUGACCGCUGGGU	1931	1931-1949	452	ACCCAGCGGUCAGCGAUGA

Table 4. Modified sense and antisense strand sequences of human TMPRSS6 dsRNAs

Duplex ID	SEQ ID NO.: (sense)	Sense sequence	Position in NM_153609.2	Position in NM_153609.2	SEQ ID NO.: (antisense)	Antisense sequence
AD-46230.1	453	GGGGuGcuAcucuGGuAuudTsdT	319	319-337	454	AAuACcAGAGuAGcACCCCdTsdT
AD-46231.1	455	ucuucuGGuucAuucuccAdTsdT	566	566-584	456	UGGAGAAUGAACcAGAAGAdTsdT
AD-46232.1	457	AcGcuGGGuuGuuAccGcudTsdT	766	766-784	458	AGCGGuAAcAACCcAGCGUdTsdT
AD-46233.1	459	cAGAAGuAuGAuuuGccGudTsdT	1285	1285-1303	460	ACGGcAAAUcAuACUUCUGdTsdT
AD-46234.1	461	cGcuGAccGcuGGGuGAuAdTsdT	1935	1935-1953	462	uAUcACCcAGCGGUcAGCGdTsdT
AD-46235.1	463	ucuGGuAuuuccuAGGGuAdTsdT	329	329-347	464	uACCCuAGGAAAuACcAGAdTsdT
AD-46236.1	465	ccuAcAGGGccGAGuAcGAdTsdT	683	683-701	466	UCGuACUCGGCCCUGuAGGdTsdT
AD-46237.1	467	cGcuGGGuuGuuAccGcuAdTsdT	767	767-785	468	uAGCGGuAAcAACCcAGCGdTsdT
AD-46238.1	469	GGccAGuGGAcGAuccAGAdTsdT	1312	1312-1330	470	UCUGGAUCGUCcACUGGCCdTsdT
AD-46239.1	471	uGAccGcuGGGuGAuAAcAdTsdT	1938	1938-1956	472	UGUuAUcACCcAGCGGUcAdTsdT
AD-46240.1	473	GGucAGccAGGuGuAcucAdTsdT	363	363-381	474	UGAGuAcACCUGGCUGACCdTsdT
AD-46241.1	475	cuAcAGGGccGAGuAcGAAdTsdT	684	684-702	476	UUCGuACUCGGCCCUGuAGdTsdT
AD-46242.1	478	cuGGGuuGuuAccGcuAcAdTsdT	769	769-787	479	UGuAGCGGuAAcAACCcAGdTsdT
AD-46243.1	480	uGcAcuAuGGcuuGuAcAAdTsdT	1454	1454-1472	481	UUGuAcAAGCcAuAGUGcAdTsdT
AD-46244.1	482	ccuGGAGAGGuGuccuucAdTsdT	2044	2044-2062	483	UGAAGGAcACCUCUCcAGGdTsdT
AD-46244.2	482	ccuGGAGAGGuGuccuucAdTsdT	2044	2044-2062	483	UGAAGGAcACCUCUCcAGGdTsdT

AD-46245.1	484	AGccAGGuGuAcucAGGcAdTsdT	367	367-385	485	UGCCUGAGuAcACCUGGCUdTsdT
AD-46246.1	486	AcAGGGccGAGuAcGAAGudTsdT	686	686-704	487	ACUUCGuACUCGGCCCUGUdTsdT
AD-46247.1	488	GGuuGuuAccGcuAcAGcudTsdT	772	772-790	489	AGCUGuAGCGGuAAcAACCdTsdT
AD-46248.1	490	uGuGAuGGGGucAAGGAcudTsdT	1534	1534-1552	491	AGUCCUUGACCCcAUcAcAdTsdT
AD-46249.1	492	cuGGAGAGGuGuccuucAAdTsdT	2045	2045-2063	493	UUGAAGGAcACCUCUCcAGdTsdT
AD-46250.1	494	uccGcAGuGAAAccGccAAdTsdT	446	446-464	495	UUGGCGGUUUcACUGCGGAdTsdT
AD-46251.1	496	GGGccGAGuAcGAAGuGGAdTsdT	689	689-707	497	UCcACUUCGuACUCGGCCCdTsdT
AD-46252.1	498	GGAccGAcuGGccAuGuAudTsdT	921	921-939	499	AuAcAUGGCcAGUCGGUCCdTsdT
AD-46253.1	500	cAAcGGccuGGAuGAGAGAdTsdT	1557	1557-1575	501	UCUCUcAUCcAGGCCGUUGdTsdT
AD-46253.2	500	cAAcGGccuGGAuGAGAGAdTsdT	1557	1557-1575	501	UCUCUcAUCcAGGCCGUUGdTsdT
AD-46254.1	502	AGuuGAucccAcAGGAccudTsdT	2291	2291-2309	503	AGGUCCUGUGGGAUcAACUdTsdT
AD-46255.1	504	ccGcAGuGAAAccGccAAAdTsdT	447	447-465	505	UUUGGCGGUUUcACUGCGGdTsdT
AD-46256.1	506	ccGAGGGccuAGuGAuccudTsdT	710	710-728	507	AGGAUcACuAGGCCCUCGGdTsdT
AD-46257.1	508	uccucAGcAccccGuAcuudTsdT	1163	1163-1181	509	AAGuACGGGGUGCUGAGGAdTsdT
AD-46258.1	510	cAGGuucGGGGucGAcAcAdTsdT	1897	1897-1915	511	UGUGUCGACCCCGAACCUGdTsdT
AD-46259.1	512	AGGuGAcGccAcGcAuGcudTsdT	2333	2333-2351	513	AGcAUGCGUGGCGUcACCUdTsdT
AD-46260.1	514	AAAccGccAAAGcccAGAAdTsdT	455	455-473	515	UUCUGGGCUUUGGCGGUUUdTsdT
AD-46261.1	516	cAGuGuGAAAGAcAuAGcudTsdT	735	735-753	517	AGCuAUGUCUUUcAcACUGdTsdT
AD-46262.1	518	cccucucuGGAcuAcGGcudTsdT	1231	1231-1249	519	AGCCGuAGUCcAGAGAGGGdTsdT
AD-46263.1	520	GuucGGGGucGAcAcAucudTsdT	1900	1900-1918	521	AGAUGUGUCGACCCCGAACdTsdT
AD-46264.1	522	uGuGuGccGGcuAccGcAAdTsdT	2351	2351-2369	523	UUGCGGuAGCCGGcAcAcAdTsdT
AD-46265.1	524	GcuucuucuGGuucAuucudTsdT	563	563-581	525	AGAAUGAACcAGAAGAAGCdTsdT
AD-46266.1	526	AuuccAcGcuGGGuuGuuAdTsdT	761	761-779	527	uAAcAACCcAGCGUGGAAUdTsdT
AD-46267.1	528	AcGGcuuGGcccucuGGuudTsdT	1244	1244-1262	529	AACcAGAGGGCcAAGCCGUdTsdT
AD-46268.1	530	ucGcuGAccGcuGGGuGAudTsdT	1934	1934-1952	531	AUcACCcAGCGGUcAGCGAdTsdT
AD-46269.1	532	AGuGGuGAccuGAGGAAcudTsdT	2538	2538-2556	533	AGUUCCUcAGGUcACcACUdTsdT
AD-46269.2	532	AGuGGuGAccuGAGGAAcudTsdT	2538	2538-2556	533	AGUUCCUcAGGUcACcACUdTsdT
AD-46270.1	534	cAAGcAGGGGGAcAAGuAudTsdT	2612	2612-2630	535	AuACUUGUCCCCCUGCUUGdTsdT
AD-46271.1	536	uGAuGucuGcuccAGuGAudTsdT	2696	2696-2714	537	AUcACUGGAGcAGAcAUcAdTsdT
AD-46272.1	538	cuAAcuuGGGAucuGGGAAdTsdT	2975	2975-2993	539	UUCCcAGAUCCcAAGUuAGdTsdT
AD-46273.1	540	uGGuAuuuccuAGGGuAcAdTsdT	331	331-349	541	UGuACCCuAGGAAAuACcAdTsdT
AD-46273.2	540	uGGuAuuuccuAGGGuAcAdTsdT	331	331-349	541	UGuACCCuAGGAAAuACcAdTsdT
AD-46273.3	540	uGGuAuuuccuAGGGuAcAdTsdT	331	331-349	541	UGuACCCuAGGAAAuACcAdTsdT
AD-46274.1	542	GAGGuGuccuucAAGGuGAdTsdT	2050	2050-2068	543	UcACCUUGAAGGAcACCUCdTsdT
AD-46276.1	544	AAGcAGGGGGAcAAGuAuudTsdT	2613	2613-2631	545	AAuACUUGUCCCCCUGCUUdTsdT
AD-46277.1	546	cAGcuGGGGGucAAGAcGudTsdT	2739	2739-2757	547	ACGUCUUGACCCCcAGCUGdTsdT
AD-46278.1	548	cuuGGGAucuGGGAAuGGAdTsdT	2979	2979-2997	549	UCcAUUCCcAGAUCCcAAGdTsdT
AD-46279.1	550	GGuAuuuccuAGGGuAcAAdTsdT	332	332-350	551	UUGuACCCuAGGAAAuACCdTsdT
AD-46280.1	552	GGcuAccGcAAGGGcAAGAdTsdT	2359	2359-2377	553	UCUUGCCCUUGCGGuAGCCdTsdT
AD-46282.1	554	GcAGGGGGAcAAGuAuucudTsdT	2615	2615-2633	555	AGAAuACUUGUCCCCCUGCdTsdT
AD-46283.1	556	GcucAGcAGcAAGAAuGcudTsdT	2851	2851-2869	557	AGcAUUCUUGCUGCUGAGCdTsdT
AD-46284.1	558	uuGGGAucuGGGAAuGGAAdTsdT	2980	2980-2998	559	UUCcAUUCCcAGAUCCcAAdTsdT

AD-46285.1	560	ccAAAGcccAGAAGAuGcudTsdT	461	461-479	561	AGcAUCUUCUGGGCUUUGGdTsdT
AD-46286.1	562	GcuAccGcAAGGGcAAGAAdTsdT	2360	2360-2378	563	UUCUUGCCCUUGCGGuAGCdTsdT
AD-46286.2	562	GcuAccGcAAGGGcAAGAAdTsdT	2360	2360-2378	563	UUCUUGCCCUUGCGGuAGCdTsdT
AD-46288.1	564	uGGuGGcAGGAGGuGGcAudTsdT	2664	2664-2682	565	AUGCcACCUCCUGCcACcAdTsdT
AD-46289.1	566	cccAcucuGuAcAGAGGcudTsdT	2903	2903-2921	567	AGCCUCUGuAcAGAGUGGGdTsdT
AD-46290.1	568	cucAcAGcccAGAcccucAdTsdT	3128	3128-3146	569	UGAGGGUCUGGGCUGUGAGdTsdT
AD-46291.1	570	ccucucuGGAcuAcGGcuudTsdT	1232	1232-1250	571	AAGCCGuAGUCcAGAGAGGdTsdT
AD-46293.1	572	GuGGcAGGAGGuGGcAucudTsdT	2666	2666-2684	573	AGAUGCcACCUCCUGCcACdTsdT
AD-46294.1	574	uucGGAAGccccuGGucuAdTsdT	2959	2959-2977	575	uAGACcAGGGGCUUCCGAAdTsdT
AD-46295.1	576	AGcucAGcuGcccuuuGGAdTsdT	3157	3157-3175	577	UCcAAAGGGcAGCUGAGCUdTsdT
AD-46296.1	578	GGccuGGAuGAGAGAAAcudTsdT	1561	1561-1579	579	AGUUUCUCUcAUCcAGGCCdTsdT
AD-46297.1	580	uGGcAGGAGGuGGcAucuudTsdT	2667	2667-2685	581	AAGAUGCcACCUCCUGCcAdTsdT
AD-46298.1	582	ucGGAAGccccuGGucuAAdTsdT	2960	2960-2978	583	UuAGACcAGGGGCUUCCGAdTsdT
AD-46299.1	584	GcucAGcuGcccuuuGGAAdTsdT	3158	3158-3176	585	UUCcAAAGGGcAGCUGAGCdTsdT
AD-46299.2	584	GcucAGcuGcccuuuGGAAdTsdT	3158	3158-3176	585	UUCcAAAGGGcAGCUGAGCdTsdT
AD-46300.1	586	AcuGuGAcuGuGGccuccAdTsdT	1808	1808-1826	587	UGGAGGCcAcAGUcAcAGUdTsdT
AD-46301.1	588	AGGAGGuGGcAucuuGucudTsdT	2671	2671-2689	589	AGAcAAGAUGCcACCUCCUdTsdT
AD-46302.1	590	ccccuGGucuAAcuuGGGAdTsdT	2967	2967-2985	591	UCCcAAGUuAGACcAGGGGdTsdT
AD-46303.1	592	ucAGcuGcccuuuGGAAuAdTsdT	3160	3160-3178	593	uAUUCcAAAGGGcAGCUGAdTsdT
AD-46304.1	594	ucGGGGucGAcAcAucuGudTsdT	1902	1902-1920	595	AcAGAUGUGUCGACCCCGAdTsdT
AD-46305.1	596	GucccuGAuGucuGcuccAdTsdT	2691	2691-2709	597	UGGAGcAGAcAUcAGGGACdTsdT
AD-46306.1	598	cccuGGucuAAcuuGGGAudTsdT	2968	2968-2986	599	AUCCcAAGUuAGACcAGGGdTsdT
AD-46307.1	600	cAGcuGcccuuuGGAAuAAdTsdT	3161	3161-3179	601	UuAUUCcAAAGGGcAGCUGdTsdT
AD-46307.2	600	cAGcuGcccuuuGGAAuAAdTsdT	3161	3161-3179	201	UuAUUCcAAAGGGcAGCUGdTsdT
AD-46308.1	602	ucAucGcuGAccGcuGGGudTsdT	1931	1931-1949	603	ACCcAGCGGUcAGCGAUGAdTsdT

Synthesis of TMPRSS6 Sequences

TMPRSS6 iRNA sequences can be synthesized on a MerMade 192 synthesizer at 1μmol scale.

Endolight chemistry can be applied as detailed below.

All pyrimidines (cytosine and uridine) in the sense strand contained 2'-O-Methyl bases (2' O-Methyl C and 2'-O-Methyl U)

In the antisense strand, pyrimidines adjacent to (towards 5' position) ribo A nucleoside can be replaced with their corresponding 2-O-Methyl nucleosides

A two base dTsdT extension at 3' end of both sense and anti sense sequences can be introduced

The sequence file can be converted to a text file to make it compatible for loading in the MerMade 192 synthesis software

Synthesis, Cleavage and deprotection

The synthesis of TMPRSS6 sequences use solid supported oligonucleotide synthesis using phosphoramidite chemistry.

The synthesis of the above sequences can be performed at 1 μm scale in 96 well plates. The amidite solutions can be prepared at 0.1M concentration and ethyl thio tetrazole (0.6M in Acetonitrile) can be used as activator.

The synthesized sequences can be cleaved and deprotected in 96 well plates, using methylamine in the first step and fluoride reagent in the second step. The crude sequences can be precipitated using acetone: ethanol (80:20) mix and the pellets re-suspended in 0.02M sodium acetate buffer. Samples from each sequence can be analyzed by LC-MS to confirm the identity, and by UV for quantification. A selected set of samples can also be analyzed by IEX chromatography to determine purity.

Purification and desalting

All sequences can be purified on AKTA explorer purification system using Source 15Q column. Sample injection and collection can be performed in 96 well (1.8mL -deep well) plates. A single peak corresponding to the full length sequence can be collected in the eluent. The purified sequences can be desalted on a Sephadex G25 column using AKTA purifier. The desalted TMPRSS6 sequences can be analyzed for concentration (by UV measurement at A260) and purity (by ion exchange HPLC). The single strands can then be submitted for annealing.

Example 3. *In vitro* screening of TMPRSS6 siRNA duplexes for TMPRSS6 knockdown activity.

TMPRSS6 siRNA duplexes were screened for the ability to knockdown TMPRSS6 expression *in vitro*. Single dose screening, dose response screening, and viability of host cells were evaluated.

***In vitro* screening**:

Cell culture and transfections for single dose and dose response studies:

HeLa or Hep3B cells (ATCC, Manassas, VA) were grown to near confluence at 37°C in an atmosphere of 5% CO_2 in X (ATCC) supplemented with 10% FBS, streptomycin, and glutamine (ATCC) before being released from the plate by trypsinization. Transfection was carried out by adding 14.8µl of Opti-MEM plus 0.2µl of Lipofectamine RNAiMax per well (Invitrogen, Carlsbad CA. cat # 13778-150) to 5µl of siRNA duplexes per well into a 96-well plate and incubated at room temperature for 15 minutes. 80µl of complete growth media without antibiotic containing ~2 $x10^4$ HeLa or Hep3B cells were then added to the siRNA mixture. Cells were incubated for either 24 or 120 hours prior to RNA purification. Single dose experiments were performed at 10nM and 0.1nM final duplex concentration and dose response experiments were done at 10, 1, 0.5, 0.1, 0.05, 0.01, 0.005, 0.001, 0.0005, 0.0001, 0.00005, 0.00001 nM final duplex concentration.

Total RNA isolation using DYNABEADS® mRNA Isolation Kit (Invitrogen, part #: 610-12):

Cells were harvested and lysed in 150µl of Lysis/Binding Buffer then mixed for 5 minutes at 850rpm using an Eppendorf ® Thermomixer (the mixing speed was the same throughout the process). Ten microliters of magnetic beads and 80µl of Lysis/Binding Buffer mixture were added to a round bottom plate and mixed for 1 minute. Magnetic beads were captured using a magnetic stand and the supernatant was removed without disturbing the beads. After removing the supernatant, the lysed cells were added to the remaining beads and mixed for 5 minutes. After removing the supernatant, the magnetic beads were washed twice with 150µl of Wash Buffer A and mixed for one minute. The beads were captured again and the supernatant was removed. The beads were then washed with 150µl Wash Buffer B, captured and the supernatant was removed. Beads were next washed with 150µl Elution Buffer, captured and supernatant removed. The beads were then allowed to dry for two minutes. After drying, 50µl of Elution Buffer was added and mixed for five minutes at 70°C. The beads were captured on a magnet for five minutes. 40µl of supernatant was removed and added to another 96 well plate.

cDNA synthesis using ABI High capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, Cat #4368813):

A master mix of 2µl of 10X Buffer, 0.8µl of 25X dNTPs, 2µl of Random primers, 1µl of Reverse Transcriptase, 1µl of RNase inhibitor and 3.2µl of H2O per reaction were added into 10µl total RNA. cDNA was generated using a Bio-Rad C-1000 or S-1000 thermal cycler (Hercules, CA) through the following steps: 25°C 10 min, 37°C 120 min, 85°C 5 sec, 4°C hold.

Real time PCR:

2µl of cDNA were added to a master mix containing 0.5µl GAPDH TaqMan Probe (Applied Biosystems Cat #4326317E), 0.5µl TMPRSS6 TaqMan probe (Applied Biosystems cat # Hs00542184_m1) and 5µl Lightcycler 480 probe master mix (Roche Cat #04887301001) per well in a 384 well 50 plates (Roche cat # 04887301001). Real time PCR was done in an ABI 7900HT Real Time PCR system (Applied Biosystems) using the ΔΔCt(RQ) assay. Each duplex was tested in two independent transfections and each transfection was assayed in duplicate, unless otherwise noted in the summary tables.

To calculate relative fold change, real time data were analyzed using the ΔΔCt method and normalized to assays performed with cells transfected with 10nM AD-1955, or mock transfected cells. IC50s were calculated using a 4 parameter fit model using XLFit and normalized to cells transfected with AD-1955 over the same dose range, or to its own lowest dose.

Viability screens. HeLa or Hep3B cells (ATCC, Manassas, VA) were grown to near confluence at 37°C in an atmosphere of 5% CO_2 in X (ATCC) supplemented with 10% FBS, streptomycin, and glutamine (ATCC) before being released from the plate by trypsinization. Cell viability was measured on days 3 and 5 in HeLa and Hep3B cells following transfection with 100, 10, 1, 0.1, 0.01 and 0.0001nM siRNA. Cells were plated at a density of $2.5X10^3$-$5X10^3$ cells per well in 96 well plates. Each siRNA was assayed in triplicate and the data averaged. siRNAs targeting PLK1 and AD-19200 were included as positive controls for loss of viability and AD-1955 as a negative control. PLK1 and AD-19200 result in a dose dependant loss of viability. To measure viability, 20ul of CellTiter Blue (Promega) was added to each well of the 96 well plates after 3 and 5 days and incubated at 37°C for 2 hours. Plates were then read in a Spectrophotometer (Molecular Devices) at $560_{Ex}/590_{Em}$. Viability was expressed as the average value of light units from three replicate transfections +/- standard deviation.

***In vitro* knockdown of TMPRSS6 expression by TMPRSS6 siRNA duplexes.**

Table 5 presents data indicating the knockdown of TMPRSS6 in Hep3B cells transfected with siRNAs targeting TMPRSS6. The data is expressed as a fraction of TMPRSS6 message remaining in cells transfected with siRNAs targeting TMPRSS6, relative to cells transfected with a negative control siRNA, AD-1955. Cells that were not treated ("naïve" cells) served as a second negative control. All siRNAs were tested at least twice, and qPCR reactions were also performed in duplicate. Single dose experiments were performed at 10nM and 0.1nM final siRNA duplex concentration.

Table 5. TMPRSS6 expression in single dose screen *in vitro*.

Duplex ID	10nM Ave	0.1nM Ave	10nM SD	0.1nM SD
AD-46230.1	0.89	1.14	0.036	0.145
AD-46230.1	0.85	1.22	0.039	0.063
AD-46231.1	0.11	0.29	0.017	0.007
AD-46232.1	0.78	0.87	0.03	0.023
AD-46233.1	0.6	0.98	0.033	0.046
AD-46234.1	0.79	1.06	0.082	0.068
AD-46235.1	0.18	0.87	0.009	0.066
AD-46235.1	0.18	0.96	0.009	0.132
AD-46236.1	0.15	1.06	0.007	0.036
AD-46237.1	0.81	0.98	0.043	0.027
AD-46238.1	0.71	0.99	0.069	0.031
AD-46239.1	0.83	1.3	0.035	0.073
AD-46240.1	0.89	0.99	0.027	0.079
AD-46240.1	0.88	1	0.009	0.034
AD-46241.1	0.6	0.9	0.029	0.029
AD-46242.1	0.81	0.91	0.016	0.049
AD-46243.1	0.82	0.87	0.029	0.066
AD-46244.1	0.19	0.43	0.018	0.028
AD-46245.1	0.48	0.79	0.148	0.016
AD-46245.1	0.51	0.82	0.147	0.028
AD-46246.1	0.39	0.89	0.012	0.043
AD-46247.1	0.84	0.9	0.047	0.019
AD-46248.1	0.68	0.95	0.059	0.075
AD-46249.1	0.17	0.29	0.005	0.152
AD-46250.1	0.19	0.53	0.017	0.011
AD-46251.1	0.16	0.47	0.007	0.005

AD-46252.1	1.04	1.08	0.031	0.038
AD-46253.1	0.27	0.45	0.02	0.031
AD-46254.1	1.03	1.08	0.221	0.021
AD-46255.1	0.52	0.84	0.029	0.036
AD-46256.1	0.81	1.02	0.025	0.015
AD-46257.1	0.64	0.97	0.016	0.076
AD-46258.1	0.91	0.98	0.054	0.059
AD-46259.1	0.77	1.03	0.052	0.067
AD-46260.1	1.24	1	0.634	0.031
AD-46261.1	0.12	0.19	0.007	0.006
AD-46262.1	0.58	1.27	0.016	0.024
AD-46263.1	0.79	0.95	0.03	0.021
AD-46264.1	0.93	1.16	0.052	0.095
AD-46265.1	0.09	0.47	0.007	0.017
AD-46266.1	0.25	0.8	0.024	0.018
AD-46267.1	0.65	0.84	0.058	0.02
AD-46268.1	0.92	1	0.008	0.048
AD-46269.1	0.37	0.52	0.037	0.024
AD-46270.1	0.26	0.55	0.01	0.03
AD-46271.1	0.35	0.8	0.044	0.029
AD-46272.1	0.62	0.91	0.015	0.061
AD-46273.1	0.18	0.3	0.02	0.012
AD-46274.1	0.88	0.85	0.04	0.016
AD-46276.1	0.33	0.64	0.024	0.024
AD-46277.1	0.85	0.89	0.12	0.026
AD-46278.1	0.24	0.7	0.019	0.059
AD-46279.1	0.55	0.79	0.008	0.025
AD-46280.1	0.96	0.84	0.059	0.042
AD-46282.1	0.21	0.47	0.017	0.004
AD-46283.1	0.62	1.01	0.05	0.03
AD-46284.1	0.42	0.78	0.016	0.019
AD-46285.1	0.37	0.86	0.014	0.042
AD-46286.1	0.19	0.49	0.019	0.027
AD-46288.1	0.65	0.88	0.052	0.032
AD-46289.1	0.89	0.92	0.062	0.032
AD-46290.1	0.83	0.9	0.035	0.029
AD-46291.1	0.65	0.87	0.014	0.014
AD-46293.1	0.31	0.68	0.012	0.054
AD-46294.1	0.25	0.7	0.015	0.031
AD-46295.1	0.2	0.42	0.004	0.029
AD-46296.1	0.43	0.83	0.012	0.043

AD-46297.1	0.3	0.6	0.009	0.017
AD-46298.1	0.91	0.91	0.08	0.008
AD-46299.1	0.26	0.57	0.018	0.052
AD-46300.1	0.98	0.91	0.037	0.024
AD-46301.1	0.65	0.87	0.018	0.051
AD-46302.1	0.92	1.01	0.021	0.048
AD-46303.1	0.13	0.43	0.008	0.019
AD-46304.1	1.11	1.01	0.016	0.056
AD-46305.1	0.21	0.73	0.029	0.011
AD-46306.1	0.84	0.96	0.114	0.092
AD-46307.1	0.27	0.49	0.007	0.019
AD-46308.1	0.69	0.83	0.02	0.024
Naïve	1.04	1.06	0.021	0.018
Naïve	1.07	1.29	0.065	0.059
AD-1955	0.85	0.85	0.055	0.071
AD-1955	1.1	0.97	0.034	0.04
AD-1955	1	0.98	0.036	0.058
AD-1955	1.04	0.98	0.053	0.049
AD-1955	1.04	1.08	0.021	0.039
AD-1955	0.98	1.19	0.049	0.058

IC_{50} of select TMPRSS6 siRNA duplexes in *in vitro* dose response screen.

Table 6 presents the IC_{50} values of select TMPRSS6 siRNA duplexes determined from *in vitro* dose response screens. TMPRSS6 siRNA duplexes that were efficacious in the 10 nM and 0.1 nM single dose screen (Table 5), were tested for TMPRSS6 knockdown activity in a dose response at 1 and 5 days following transfection in Hep3B cells. Dose response experiments were conducted at 10, 1, 0.5, 0.1, 0.05, 0.01, 0.005, 0.001, 0.0005, 0.0001, 0.00005, 0.00001 nM final siRNA duplex concentration. For normalization, knockdown of TMPRSS6 was measured relative to the non-targeting control, AD-1955, or the value obtained at the lowest siRNA concentration for each duplex tested.

Table 6. IC_{50} of select TMPRSS6 siRNA duplexes in *in vitro* dose response screen.

	Normalized to low dose		Normalized to AD-1955	
Duplex ID	Day 1 (nM)	Day 5 (nM)	Day 1 (nM)	Day 5 (nM)
AD-46250.1	0.57	0.08	0.22	0.04
AD-46265.1	0.14	0.07	0.2	0.03

AD-46231.1	0.07	0.04	0.06	0.02
AD-46251.1	0.27	0.1	0.37	0.07
AD-46261.1	0.04	0.09	0.08	0.05
AD-46253.1	0.78	0.07	0.35	0.13
AD-46244.1	0.14	0.13	0.2	0.32
AD-46269.1	0.06	0.57	0.07	1.16
AD-46270.1	0.94	No IC50	0	No IC50
AD-46282.1	1.16	No IC50	0.02	No IC50
AD-46297.1	0.05	No IC50	0.08	No IC50
AD-46299.1	0.01	3.89	0.03	0.69
AD-46303.1	0.01	2.47	0.03	0.04
AD-46307.1	1.02	0.02	2.68	0.15
AD-46273.1	0.23	0.03	0.72	0.1
AD-46286.1	0.22	0.46	0.53	0.46
AD-46249.1	0.27	1.96	0.31	5.87
AD-46295.1	0.76	0.31	0.24	0.1

In vitro viability screening of HeLa and HEP3B cell lines transfected with TMPRSS6 siRNA duplexes.

Table 7 presents viability data of HeLa and HEP3B cell lines transfected with TMPRSS6 siRNA duplexes. Viability data are expressed as average raw fluorescence units, where smaller values represent lower viability. Error is expressed as standard deviation from three replicate transfections.

Table 7. Viability of HeLa and HEP3B cell lines transfected with TMPRSS6 siRNA duplexes.

	HeLa Day 3 10nM	HeLa Day 3 1nM	HeLa Day 3 0.1nM	HeLa Day 3 0.01nM	HeLa Day 3 0.001nM	HeLa Day 3 0.0001nM		HeLa Day 3 10nM SD	HeLa Day 3 1nM SD	HeLa Day 3 0.1nM SD	HeLa Day 3 0.01n M SD	HeLa Day 3 0.001 nM SD	HeLa Day 3 0.0001 nM SD
AD-46250.1	5260	13504	29520	30542	30924	30956		150	62	272	220	799	751
AD-46265.1	12234	29940	32497	33323	32124	32882		968	884	1071	946	707	595
AD-46231.1	25177	28407	32021	32650	33375	32704		710	420	127	1697	356	667
AD-46251.1	29528	30151	30215	32163	31743	31726		416	102	31	1588	518	1091
AD-46261.1	16677	26331	30594	31681	32847	31544		390	277	431	1375	681	583
AD-46253.1	21580	28887	30953	31684	32457	31491		1158	437	524	944	229	455
AD-46244.1	13230	16369	26545	31359	32753	32280		197	165	255	357	589	1318
AD-46269.1	9978	19514	29290	30839	31529	31173		597	360	1406	400	743	626

AD-46270.1	17543	17834	31180	31087	32793	31314		370	1026	771	552	391	1293
AD-46282.1	29055	32421	31840	31006	34287	32185		446	618	430	855	323	133
AD-46297.1	8126	16696	28128	33928	33955	32322		193	598	733	895	1266	392
AD-46299.1	31922	30196	30880	30447	31900	32608		1459	617	58	194	773	964
AD-46303.1	27309	28325	27975	29319	30310	30935		1363	572	421	295	306	95
AD-46307.1	33156	33240	32059	33072	32135	33307		667	258	775	1164	102	286
AD-46273.1	24465	29130	30417	33043	34639	31876		142	768	271	261	853	800
AD-46286.1	3640	9590	29713	33138	32877	30814		34	631	371	1185	1641	599
AD-46249.1	17315	25591	30443	31599	32719	29855		981	258	578	482	1412	886
AD-46295.1	30565	31730	30772	31777	32874	30916		403	261	1223	1880	981	441
AD-19200	9727	15752	31352	32521	30110	30650		648	699	763	1543	55	9
PLK	1166	1626	27849	29902	30512	30273		23	44	91	299	362	563
AD-1955	26502	30164	30267	31906	33309	30906		5669	134	353	645	233	696
Naïve	32821	32311	30805	31683	33238	31470		1455	631	555	557	288	164
Naïve	33594	32373	32005	34024	35629	33401		554	253	754	899	55	649
Naïve	30695	30651	29956	31377	32734	32527		304	299	807	874	646	225

	HeLa Day 5 10nM	HeLa Day 5 1nM	HeLa Day 5 0.1nM	HeLa Day 5 0.01nM	HeLa Day 5 0.001nM	HeLa Day 5 0.0001nM		HeLa Day 5 10nM SD	HeLa Day 5 1nM SD	HeLa Day 5 0.1nM SD	HeLa Day 5 0.01nM SD	HeLa Day 5 0.001 nM SD	HeLa Day 5 0.0001 nM SD
AD-46250.1	2344	25502	46627	44986	46479	46070		44	1916	157	913	598	2016
AD-46265.1	10411	46611	48725	47425	47238	47942		300	327	602	1479	2145	1690
AD-46231.1	41079	46963	48575	48060	47467	48500		1645	319	243	998	1821	1203
AD-46251.1	42551	47044	49088	48269	47755	48719		1597	420	162	1105	1434	1232
AD-46261.1	37500	46441	48702	47953	47776	48878		689	441	451	1447	1614	1159
AD-46253.1	31772	45899	48606	47801	47693	49237		1310	65	648	1550	1365	789
AD-46244.1	11597	28046	46020	47413	47670	49430		967	527	395	1336	937	869
AD-46269.1	10704	37735	47496	47629	47496	49194		317	161	198	1359	1502	986
AD-46270.1	16356	26284	48520	48011	48016	49358		382	663	497	1121	1024	681
AD-46282.1	22372	42327	47297	47478	47450	49349		656	715	343	1513	2057	883
AD-46297.1	4228	26993	47037	47269	46961	48993		41	657	593	1847	1574	639
AD-46299.1	45283	45485	46334	43966	42922	46772		1088	908	382	2057	3580	1131
AD-46303.1	42669	46358	46240	45624	46920	46764		849	183	791	890	90	539
AD-46307.1	47710	47466	47974	47671	47911	48505		273	539	680	399	238	309
AD-46273.1	36834	45018	47522	47912	48316	48804		680	432	104	619	308	248
AD-46286.1	2970	31215	47504	47883	48062	48999		515	1262	1093	826	87	541
AD-46249.1	20356	44959	48534	47988	48053	49145		884	1033	1238	1045	619	530
AD-46295.1	46448	48014	49195	48654	48432	49355		685	1021	746	1183	645	407
AD-19200	26444	35772	39724	48377	48373	49509		725	1009	1246	540	762	408

PLK	1105	1804	37258	47955	47893	49416		44	95	1110	781	474	515
AD-1955	44857	47272	48354	48050	48668	49721		322	388	756	880	585	490
Naïve	48734	48454	48549	47246	48004	49067		850	303	743	1166	349	102
Naïve	48318	47839	45252	47098	47128	48914		969	527	223	797	548	526
Naïve	45189	45096	45508	44334	45177	47004		1327	938	579	1342	930	350

	Hep3B Day 3 10nM	Hep3 B Day 3 1nM	Hep3B Day 3 0.1nM	Hep3B Day 3 0.01nM	Hep3B Day 3 0.001nM	Hep3B Day 3 0.0001nM		Hep3B Day 3 10nM SD	Hep3 B Day 3 1nM SD	Hep3B Day 3 0.1nM SD	Hep3B Day 3 0.01n M SD	Hep3 B Day 3 0.001 nM SD	Hep3B Day 3 0.0001 nM SD
AD-46250.1	4495	4905	6786	7022	6122	6033		225	105	56	85	151	49
AD-46265.1	6453	6990	6917	6685	6165	5974		187	79	103	70	121	21
AD-46231.1	6478	7042	6808	6444	6173	5987		97	19	35	66	131	69
AD-46251.1	5663	5990	6084	6241	5869	6298		445	38	73	69	63	88
AD-46261.1	5380	6025	5824	6325	5801	6076		376	14	29	67	81	65
AD-46253.1	5417	6078	5840	6113	5568	6503		549	29	103	81	20	72
AD-46244.1	4743	5479	5884	6078	6170	6593		29	43	51	168	60	70
AD-46269.1	2788	2958	5479	5878	5899	5739		64	14	97	215	80	22
AD-46270.1	4378	4720	5579	6127	6066	6522		235	94	167	17	43	260
AD-46282.1	5096	5932	6258	5988	6068	6724		101	34	32	107	59	20
AD-46297.1	1134	1325	4477	6051	6199	6626		40	64	80	101	134	55
AD-46299.1	5875	5836	6251	5872	6016	6726		47	64	39	54	81	104
AD-46303.1	6879	7060	6801	6793	6306	6827		43	32	59	60	126	65
AD-46307.1	6951	6826	6613	6511	6119	7093		97	148	46	82	97	91
AD-46273.1	6628	6749	6711	6839	6237	6958		122	24	59	59	48	109
AD-46286.1	5384	5405	5755	6469	6299	6207		81	5	45	33	95	58
AD-46249.1	3955	4239	5214	6549	6171	6537		141	70	134	37	35	27
AD-46295.1	6186	6535	5776	6500	6247	6252		96	34	141	35	41	35
AD-19200	2304	3860	5592	6634	6063	6111		95	24	43	41	67	74
PLK	1484	1668	3385	6283	5714	6015		36	52	130	94	112	143
AD-1955	5718	5826	5633	6356	6369	6460		27	60	16	80	108	122
Naïve	5799	6503	6350	6351	6002	6449		69	98	44	40	72	66
Naïve	5623	6550	5950	6103	5574	6489		23	49	37	82	59	93
Naïve	5895	6021	5550	5908	5573	6769		72	27	55	90	64	42

	Hep3B Day 5 10nM	Hep3 B Day 5 1nM	Hep3B Day 5 0.1nM	Hep3B Day 5 0.01nM	Hep3B Day 5 0.001nM	Hep3B Day 5 0.0001nM		Hep3B Day 5 10nM SD	Hep3 B Day 5 1nM SD	Hep3B Day 5 0.1nM SD	Hep3B Day 5 0.01n M SD	Hep3 B Day 5 0.001 nM	Hep3B Day 5 0.0001 nM SD

												SD	
AD-46250.1	4758	5572	9636	12294	11079	10674		311	285	180	901	575	403
AD-46265.1	9937	12543	10822	13430	12967	12089		323	1714	1094	107	1407	704
AD-46231.1	12650	13786	11763	13765	14003	13857		1002	422	1551	177	213	320
AD-46251.1	8543	9397	10581	13642	12990	13568		518	1054	707	289	1247	475
AD-46261.1	10459	11700	11735	13764	13738	13210		148	1308	459	277	712	210
AD-46253.1	11125	12124	11533	14213	13967	11946		473	1531	772	262	679	1015
AD-46244.1	7330	7939	10428	12695	13584	11852		451	416	1104	61	358	1473
AD-46269.1	2316	2442	11476	13621	12821	11090		507	623	574	299	831	298
AD-46270.1	6643	5235	11774	12788	13487	11895		179	386	709	1032	635	760
AD-46282.1	7767	10214	12650	12859	12980	11175		214	1116	569	1282	925	169
AD-46297.1	1012	1124	9438	12403	12063	11599		47	96	162	990	1118	83
AD-46299.1	13643	13396	12404	12113	12782	12913		1585	2086	202	896	1040	1209
AD-46303.1	10567	12918	10617	11203	11189	11260		456	1263	106	309	310	153
AD-46307.1	13787	14089	11830	13512	13489	12773		208	467	900	60	504	203
AD-46273.1	13801	13484	12719	14212	14305	12499		386	219	1250	382	128	176
AD-46286.1	5783	6472	10990	14352	14424	12234		93	78	472	632	103	649
AD-46249.1	3763	5086	10729	14293	14283	12608		269	124	453	570	443	633
AD-46295.1	14870	15096	11289	14697	14336	12000		539	224	453	698	689	903
AD-19200	1546	6337	10310	14261	13551	11486		132	379	456	250	646	754
PLK	1337	1636	6996	14661	13860	12555		31	79	759	740	423	296
AD-1955	11717	12560	12164	14504	13008	11077		1146	1210	1289	392	1405	56
Naïve	13989	14873	11512	14022	13458	11399		404	316	267	412	635	114
Naïve	14167	14550	11269	14247	13793	11771		197	426	230	640	664	888
Naïve	13857	14632	10432	13485	14164	12808		231	1150	474	546	177	1028

Example 4. TMPRSS6 siRNA duplex lead Selection.

To select specific TMPRSS6 siRNAs for use in further *in vivo* experimentation, chemically modified siRNAs were screened by transfection in HEP3B human hepatoma cells for TMPRSS6 gene silencing activity. Two highly potent siRNAs with minimal predicted off-target potential and with multi-species reactivity, including human cynomolgus monkey, rat, and mouse, were selected for evaluation *in vivo*. Potency of the two selected TMPRSS6 siRNAs was also confirmed in primary mouse hepatocytes, wherein both TMPRSS6 siRNA-1 (AD-46273) and TMPRSS6 siRNA-2 (AD-46286) demonstrated strong TMPRSS6 gene silencing activity, with TMPRSS6 siRNA-1 (AD-46273) demonstrating an IC_{50} of 70 pM (FIG. 2A) and TMPRSS6 siRNA-2 (AD-46286) demonstrating an IC_{50} of 140pM (FIG. 2B).

Example 5. The effect of TMPRSS6 siRNA mediated silencing of TMPRSS6 in WT C57BL/6 Mice.

The effect of TMPRSS6 siRNA on TMPRSS6 and HAMP1 mRNA expression in WT C57BL/6 Mice.

In order to evaluate the effect of LNP-TMPRSS6 siRNA-1 (AD-46273) and LNP-TMPRSS6 siRNA-2 (AD-46286) *in vivo*, eight week old female WT C57BL/6 mice were dosed via tail vein IV injection with 1mg/kg LNP-TMPRSS6 siRNA-1 (AD-46273) or LNP-TMPRSS6 siRNA-2 (AD-46286) or LNP-AD-19551 (siRNA targeting the non-mammalian gene LUCIFERASE). The TMPRSS6 siRNAs were formulated with LNP11 (MC3). The mice were sacrificed 24 hours post dosing, and livers removed, flash frozen, and ground into powder. A small amount (~20mg) of liver powder was disrupted in lysis buffer and used for mRNA analysis by TaqMan®. A total of five mice were used per group. The data are expressed as a percent of LNP-Luc control ratios of target TMPRSS6 mRNA relative to B-actin mRNA. As shown in FIG. 3A, there was a specific and potent dose dependent inhibition of liver TMPRSS6 mRNA expression by LNP-TMPRSS6 siRNA-1 (AD-46273) and LNP-TMPRSS6 siRNA-2 (AD-46286) (data represent mean +/-standard deviation), with an ED_{50} of 0.035mg/kg, and an ED_{50} of 0.18 mg/kg, respectively. As shown in FIG. 3B, there was also a dose dependent inhibition of liver HAMP1 mRNA expression by LNP-TMPRSS6 siRNA-1 (AD-46273) and LNP-TMPRSS6 siRNA-2 (AD-46286).

The duration of TMPRSS6 siRNA mediated silencing of TMPRSS6 and HAMP1 gene expression in WT C57BL/6 Mice.

In order to evaluate the duration of the TMPRSS6 siRNA mediated knockdown of TMPRSS6 and HAMP1 gene expression, eight week old WT C57BL/6 mice were administered a single 1mg/kg dose via tail vein IV injection with LNP-TMPRSS6 siRNA-1 (AD-46273), or LNP-Luc control (LNP-AD-1955), or PBS; all siRNA agents were delivered as LNP11 formulations. The mice were sacrificed at 6 hours, 24 hours, 48 hours, 3 days, 7 days, and 14 days. The mRNA expression level of TMPRSS6 and HAMP1 in the liver was analyzed using TaqMan® assay and normalized to B-actin. Five mice were used per group, and the data is represented in FIG. 4 as mean +/- standard deviation. As shown in FIG. 4, 1 mg/kg single dose of LNP-TMPRSS6 siRNA-1 (AD-46273) knocked down TMPRSS6 mRNA expression as early as six hours post dosing, and reduced TMPRSS6 mRNA

expression to approximately 90% of LNP-Luc control or PBS control for the duration of the two week time period. HAMP1 gene expression was increased starting 24 hours post dosing and was maintained for the duration of the two week time period, with a maximum increase of 200% of control on day 14 post dosing (FIG. 4). In addition, serum iron levels were assayed as the percentage of transferrin (Tf) saturation using an Olympus AU 400. The level of transferrin saturation was calculated as the ratio of serum iron to total iron binding capacity (TIBC) and is expressed as a percent of transferrin saturation. The percent of transferrin saturation was reduced by approximately 50% starting 24 hours post dosing and maintained over the two week time period, indicating that the circulating iron levels in the serum were decreased (FIG. 4). Level of TMPRSS6 siRNA mediated silencing of TMPRSS6 necessary to maintain the TMPRSS6 siRNA mediated effects on HAMP1 gene expression and serum iron levels in WT C57BL/6 Mice.

In order to evaluate the level of TMPRSS6 siRNA mediated silencing of TMPRSS6 necessary to maintain the TMPRSS6 siRNA mediated effects on HAMP1 gene expression and serum iron levels in WT C57BL/6 mice; C57BL/6 mice were dosed with 0.3 mg/kg LNP-TMPRSS6 siRNA-1 (AD-46273), or LNP-Luc control, or PBS; all siRNA agents were delivered as LNP11 formulations. The mice were sacrificed at 5 hours, 24 hours, 48 hours, 3 days, 7 days, 14 days, 21, days, and 28 days post dosing. The mRNA expression level of TMPRSS6 and HAMP1 was analyzed using TaqMan® assay and normalized to B-actin. Five mice were used per group, and the data is represented in FIG. 5 as mean +/- standard deviation. As shown in FIG. 5, the maximal reduction of TMPRSS6 gene expression of 90% was achieved 24 hours post dosing and maintained up until day three post dosing. At day seven post dosing, TMPRSS6 gene expression was reduced by approximately 85%; HAMP1 gene expression was induced to approximately 250% of control; and transferrin saturation (%) was reduced by approximately 50% (FIG. 5). At day 21 post dosing, TMPRSS6 gene expression was reduced by approximately 40%; HAMP1 gene expression had normalized; and serum iron levels, as measured by transferrin saturation (%), began to return to normal levels (FIG. 5). In summary, maximal knockdown of TMPRSS6 mRNA expression was achieved at 24 hours post dosing and returned to approximately 50% of normal expression levels by 3 weeks post dosing; hepcidin mRNA levels were increased as early as 24 hours and maintained up to seven days post dosing; hepcidin levels returned to control levels on day fourteen post dosing; and transferrin saturation, as an indicator of circulating iron levels, was reduced by 50% of control levels as early as 24 hours post dosing, and was normalized

towards week four. Thus the data presented in FIG. 5 illustrates that more than 50% TMPRSS6 silencing is required to maintain the LNP-TMPRSS6 siRNA-1 (AD-46273) mediated effects on HAMP1 gene expression and serum iron levels.

The effect of TMPRSS6 siRNA mediated silencing of TMPRSS6 on hematological parameters in WT C57BL/6 Mice.

In order to evaluate the effect of TMPRSS6 siRNA mediated silencing of TMPRSS6 on hematological parameters, including hemoglobin (HGB) and hematocrit; WT C57BL/6 mice were dosed with 1 mg/kg single dose of TMPRSS6 siRNA-1 (AD-46273) or LNP-Luc control, or PBS; and subsequently sacrificed at different time points up to two weeks post dosing. Hematological parameters including, hemoglobin (HGB), hematocrit, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), and the reticulocyte hemoglobin content (Chr) were assayed using Advia 120 analyzer. As shown in FIGs. 6A and 6B, silencing of TMPRSS6 in Th3/+ mice led to a decrease HGB (FIG. 6A), and a decrease in hematocrit (FIG. 6B) in WT C57BL/6 mice. There was a similar effect on mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), and the reticulocyte hemoglobin content (Chr).

Example 6. The effect of TMPRSS6 siRNA mediated silencing of TMPRSS6 in Thalassemic Mice (Th3/+).

The effect of TMPRSS6 siRNA mediated silencing of TMPRSS6 on serum iron parameters in Thalassemic Mice (Th3/+).

To evaluate the effect of TMPRSS6 siRNA mediated silencing of TMPRSS6 on serum iron parameters, including iron levels, unsaturated iron-binding capacity (UIBC), and Tf saturation in thalassemic mice (Th3/+), six week old Th3/+ mice were dosed via tail vein injection with 1 mg/kg LNP-TMPRSS6 siRNA-1 (AD-46273), or LNP-Luc control, or PBS, and the mice were sacrificed two weeks post dosing. Five mice were used per group, and data represented in FIG. 7 as mean +/- standard deviation, with ** denoting a P-value<0.01 and *** denoting a P-value<0.001. As shown in FIG. 7, silencing of TMPRSS6 in Th3/+ mice led to a significant reduction of serum iron, UIBC, and Tf saturation compared to the control PBS group.

The effect of TMPRSS6 siRNA mediated silencing of TMPRSS6 on reticulocyte and erthyrocyte parameters in Thalassemic Mice (Th3/+).

To evaluate the effect of TMPRSS6 siRNA mediated silencing of TMPRSS6 on reticulocyte and erythrocyte parameters, including reticulocyte number, reticulocyte hemoglobin content (CHr), and erythrocyte number (RBC), in thalassemic mice (Th3/+); six week old Th3/+ mice were dosed via tail vein injection, with 1 mg/kg LNP-TMPRSS6 siRNA-1 (AD-46273), or LNP-Luc control, or PBS, and the mice were sacrificed two weeks post dosing. Reticulocyte and erythrocyte parameters, including reticulocyte number, reticulocyte hemoglobin content (CHr), and erythrocyte number (RBC) were assayed using Advia 120 analyzer. Five mice were used per group, and the data is represented in FIGs. 8A-8C as mean +/- standard deviation, with ** denoting a P-value<0.01 and *** denoting a P-value<0.001. As shown in FIGs. 8A and 8B, respectively, silencing of TMPRSS6 in Th3/+ mice led to a significant reduction in the number of reticulocytes as well as the hemoglobin content of reticulocytes (Chr). In addition, silencing of TMPRSS6 in Th3/+ mice led to a significant increase in the number of mature erythrocytes (RBC) (FIG. 8C), demonstrating a significant improvement in ineffective erythropoiesis, extramedullary hematopoiesis, and red blood cell production.

The effect of TMPRSS6 siRNA mediated silencing of TMPRSS6 on hematological parameters in Thalassemic Mice (Th3/+).

To evaluate the effect of TMPRSS6 siRNA mediated silencing of TMPRSS6 on hematological parameters, including hematocrit (HCT), hemoglobin (HGB), red blood cell distribution width (RDW), and mean corpuscle value (MCV) in thalassemic mice (Th3/+); six week old Th3/+ mice were dosed via tail vein injection, with 1 mg/kg LNP-TMPRSS6 siRNA-1 (AD-46273), or LNP-Luc control, or PBS, and the mice were sacrificed two weeks post dosing. Hematological paramters including hematocrit (HCT), hemoglobin (HGB), red blood cell distribution width (RDW), and mean corpuscle value (MCV); were assayed using Advia 120 analyzer. Five mice were used per group, and the data is represented in FIG. 9 as mean +/- standard deviation, with ** denoting a P-value<0.01 and *** denoting a P-value<0.001. Silencing of TMPRSS6 in Th3/+ mice led to a significant increase in HCT (FIG. 9A), a significant increase in HGB (FIG. 9B), a significant decrease in RDW (FIG. 9C), and a significant decrease in MCV (FIG. 9D). The data presented in FIG. 9 illustrates a

normalization of the β-thalassemia phenotype in these hematological parameters post administration of the LNP-TMPRSS6 siRNA-1 (AD-46273).

The effect of TMPRSS6 siRNA mediated silencing of TMPRSS6 on peripheral blood morphology in Thalassemic Mice (Th3/+).

To evaluate the effect of TMPRSS6 siRNA mediated silencing of TMPRSS6 on peripheral blood morphology in thalassemic mice (Th3/+); six week old Th3/+ mice were dosed via tail vein injection with, 1 mg/kg LNP-TMPRSS6 siRNA-1 (AD-46273) or LNP-Luc control, and the mice were sacrificed two weeks post dosing. May-Grunwald/Gimsa stain at 10X magnification showed a marked decrease in polychromasia in the Th3/+ mice treated with the TMPRSS6 siRNA compared to control, representative of the decreased reticulocyte number as well as an overall trend toward normalization of the mature red blood cell morphology. May-Grunwald/Gimsa stain at 10X magnification also showed slight anisocytosis was induced by the WT TMPRSS6 siRNA animal when compared to WT control animal.

The effect of TMPRSS6 siRNA mediated silencing of TMPRSS6 on splenic architecture in Thalassemic Mice (Th3/+).

To evaluate the effect of TMPRSS6 siRNA mediated silencing of TMPRSS6 on splenic architecture in thalassemic mice (Th3/+); six week old Th3/+ mice were dosed via tail vein injection, with 1 mg/kg LNP-TMPRSS6 siRNA-1 (AD-46273), or LNP-Luc control, or PBS, and mice were sacrificed two weeks post dosing. Hematoxylin and eosin (H&E) stain at 10X magnification showed Th3/+ mice treated with the TMPRSS6 siRNA compared to control had a normalization of splenic architecture, including a reduction in sinusoidal extramedullary erythropoiesis and the reappearance of white pulp nodules.

The effect of TMPRSS6 siRNA mediated silencing of TMPRSS6 on spleen and liver iron content in Thalassemic Mice (Th3/+).

To evaluate the effect of TMPRSS6 siRNA mediated silencing of TMPRSS6 on spleen and liver iron content in thalassemic mice (Th3/+); six week old Th3/+ mice were dosed via tail vein injection, with 1 mg/kg LNP-TMPRSS6 siRNA-1 (AD-46273), or LNP-Luc control, or PBS, and the mice were sacrificed two weeks post dosing. Five mice were used per group, and the data is represented in FIGs. 10A-10C as mean +/- standard deviation, with **

denoting a P-value<0.01 and *** denoting a P-value<0.001. Silencing of TMPRSS6 in Th3/+ mice led to a significant reduction of spleen iron content and spleen weight (FIG. 10A and FIG. 10B, respectively), indicating a normalization of extramedullary hematopoiesis. A trend towards a reduction liver iron content was also observed, but was not statistically significant (FIG. 10C).

The above results demonstrate that silencing of TMPRSS6 by systemic administration of formulated siRNAs increases HAMP expression to levels sufficient to ameliorate the phenotype in a mouse model of β-thalassemia intermedia. Therefore, LNP-TMPRSS6-siRNAs are being developed for congenital iron overload disorders characterized by abnormally low hepcidin levels, (*e.g.*, β-thalassemia intermedia and hereditary hemochromatosis).

EQUIVALENTS

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

We Claim:

1. A double-stranded ribonucleic acid (dsRNA) for inhibiting expression of TMPRSS6, wherein said dsRNA comprises a sense strand and an antisense strand, the antisense strand comprising a region of complementarity to a TMPRSS6 transcript which comprises at least 15 contiguous nucleotides differing by no more than 3 nucleotides from one of the antisense sequences listed in Tables 2, 3 or 4.

2. The dsRNA of claim 1, wherein said dsRNA comprises at least one modified nucleotide.

3. The dsRNA of claim 2, wherein at least one of said modified nucleotides is chosen from the group consisting of: a 2'-O-methyl modified nucleotide, a nucleotide comprising a 5'-phosphorothioate group, and a terminal nucleotide linked to a cholesteryl derivative or dodecanoic acid bisdecylamide group.

4. The dsRNA of claim 2, wherein said modified nucleotide is chosen from the group consisting of: a 2'-deoxy-2'-fluoro modified nucleotide, a 2'-deoxy-modified nucleotide, a locked nucleotide, an abasic nucleotide, 2'-amino-modified nucleotide, 2'-alkyl-modified nucleotide, morpholino nucleotide, a phosphoramidate, and a non-natural base comprising nucleotide.

5. The dsRNA of claim 1, wherein the region of complementarity is at least 17 nucleotides in length.

6. The dsRNA of claim 1, wherein the region of complementarity is between 19 and 21 nucleotides in length.

7. The dsRNA of claim 1, wherein the region of complementarity is 19 nucleotides in length.

8. The dsRNA of claim 1, wherein each strand is no more than 30 nucleotides in length.

9. The dsRNA of claim 1, wherein at least one strand comprises a 3' overhang of at least 1 nucleotide.

10. The dsRNA of claim 1, wherein at least one strand comprises a 3' overhang of at least 2 nucleotides.

11. The dsRNA of claim 1, further comprising a ligand.

12. The dsRNA of claim 11, wherein the ligand is conjugated to the 3' end of the sense strand of the dsRNA.

13. The dsRNA of claim 1, wherein the region of complementarity consists of one of the antisense sequences of Tables 2, 3 or 4.

14. The dsRNA of claim 1, wherein the dsRNA comprises a sense strand consisting of a sense strand sequence selected from Tables 2, 3 or 4 and an antisense strand consisting of an antisense sequence selected from Tables 2, 3 or 4.

15. A cell containing the dsRNA of claim 1.

16. A pharmaceutical composition for inhibiting expression of a TMPRSS6 gene comprising the dsRNA of claim 1.

17. The pharmaceutical composition of claim 16, further comprising a lipid formulation.

18. The pharmaceutical composition of claim 17, wherein the lipid formulation is a SNALP, or XTC formulation

19. A method of inhibiting TMPRSS6 expression in a cell, the method comprising:

(a) introducing into the cell the dsRNA of claim 1; and

(b) maintaining the cell produced in step (a) for a time sufficient to obtain degradation of the mRNA transcript of a TMPRSS6 gene, thereby inhibiting expression of the TMPRSS6 gene in the cell.

20. The method of claim 19, wherein the TMPRSS6 expression is inhibited by at least 30%.

21. A method of treating a disorder mediated by TMPRSS6 expression comprising administering to a human in need of such treatment a therapeutically effective amount of the dsRNA of claim 1 or a pharmaceutical composition of claims 16-18.

22. The method of claim 21, wherein the human has a disorder associated with hemochromatosis.

23. The method of claim 21, wherein the human has a β-thalassemia.

24. The method of claim 21, wherein the human has β-thalassemia intermedia.

25. The method of claim 23, wherein the administration of the dsRNA to the subject causes a decrease in iron in the serum of the subject by at least 10%.

26. The method of claim 21, wherein the dsRNA is administered at a concentration of 0.01 mg/kg-5 mg/kg bodyweight of the subject.

27. A vector encoding at least one strand of a dsRNA, wherein said dsRNA comprises a region of complementarity to at least a part of an mRNA encoding TMPRSS6, wherein said dsRNA is 30 base pairs or less in length, and wherein said dsRNA targets said mRNA for cleavage.

28. The vector of claim 27, wherein the region of complementarity is at least 15 nucleotides in length.

29. The vector of claim 27, wherein the region of complementarity is 19 to 21 nucleotides in length.

30. A cell comprising the vector of claim 27.

31. The dsRNA of claim 1, wherein the dsRNA comprises a sense strand consisting of a sequence selected from the group consisting of SEQ ID NO:111, SEQ ID NO:455, SEQ ID NO:109, SEQ ID NO:524, SEQ ID NO:89, SEQ ID NO:494, SEQ ID NO:445, SEQ ID NO:592, SEQ ID NO:47, and SEQ ID NO:540; and an antisense strand consisting of a sequence selected from the group consisting of SEQ ID NO:112, SEQ ID NO:456, SEQ ID NO:110, SEQ ID NO:525, SEQ ID NO:90, SEQ ID NO:495, SEQ ID NO:446, SEQ ID NO:593, SEQ ID NO:48 and SEQ ID NO:541.