COMPOSITIONS AND METHODS FOR INHIBITING EXPRESSION OF THE ALAS1 GENE

Related Applications

This application claims priority to U.S. provisional application number 61/887288 filed on October 4, 2013 and to U.S. provisional application number 61/983720 filed on April 24, 2014. The entire content of each of the foregoing applications is hereby incorporated herein by reference.

Sequence Listing

The instant application contains a Sequence Listing which has been submitted electronically in ASCII format and is hereby incorporated by reference in its entirety. Said ASCII copy, created on October 2, 2014, is named A2038-7202WO_SL.txt and is 1,107,486 bytes in size.

Field of the Invention

The invention relates to the specific inhibition of the expression of the ALAS1 gene.

Background of the Invention

The inherited porphyrias are a family of disorders resulting from the deficient activity of specific enzymes in the heme biosynthetic pathway, also referred to herein as the porphyrin pathway. Deficiency in the enzymes of the porphyrin pathway leads to insufficient heme production and to an accumulation of porphyrin precursors and porphyrins, which are toxic to tissue in high concentrations.

Of the inherited porphyrias, acute intermittent porphyria (AIP, e.g., autosomal dominant AIP), variegate porphyria (VP, e.g., autosomal dominant VP), hereditary coproporphyria (copropophyria or HCP, e.g., autosomal dominant HCP), and 5' aminolevulinic acid (also known as δ- aminolevulinic acid or ALA) dehydratase deficiency porphyria (ADP, e.g., autosomal recessive ADP) are classified as acute hepatic porphyrias and are manifested by acute neurological attacks that can be life threatening. The acute attacks are characterized by

autonomic, peripheral, and central nervous symptoms, including severe abdominal pain, hypertension, tachycardias, constipation, motor weakness, paralysis, and seizures. If not treated properly, quadriplegia, respiratory impairment, and death may ensue. Various factors, including cytrochrome P450-inducing drugs, dieting, and hormonoal changes can precipitate acute attacks by increasing the activity of hepatic 5'-aminolevulinic acid synthase 1 (ALAS1) , the first and rate-limiting enzyme of the heme biosynthetic pathway. In the acute porphyrias, e.g., AIP, VP, HCP and ADP, the respective enzyme deficiencies result in hepatic production and accumulation of one or more substances (e.g., porphyrins and/or porphyrin precursors, e.g., ALA and/or PBG) that can be neurotoxic and can result in the occurrence of acute attacks. See, e.g., Balwani, M and Desnick, R.J., *Blood*, 120:4496-4504, 2012.

The current therapy for the acute neurologic attacks is the intravenous administration of hemin (Panhematin®, Lundbeck or Normosang®, Orphan Europe), which provides exogenous heme for the negative feedback inhibition of ALAS1, and thereby, decreases production of ALA and PBG. Hemin is used for the treatment during an acute attack and for prevention of attacks, particularly in women with the actue porphyrias who experience frequent attacks with the hormonal changes during their menstrual cycles. While patients generally respond well, its effect is slow, typically taking two to four days or longer to normalize urinary ALA and PBG concentrations towards normal levels. As the intravenous hemin is rapidly metabolized, three to four infusions are usually necessary to effectively treat or prevent an acute attack. In addition, repeated infusions may cause iron overload and phlebitis, which may compromise peripheral venous access. Although orthotrophic liver transplantation is curative, this procedure has significant morbidity and mortality and the availability of liver donors is limited. Therefore, an alternative therapeutic approach that is more effective, fast-acting, and safe is needed. It would be particularly advantageous if such treatment could be delivered by subcutaneous administration, as this would preclude the need for infusions and prolonged hospitalization.

AIP, also referred to as porphobilinogen deaminase (PBGD) deficiency, or hydroxymethylbilane synthase (HMBS) deficiency, is the most common of the acute hepatic prophyrias. The prevalence of AIP is estimated to be 5-10 in 100,000, with about 5-10% of patients being symptomatic. AIP is an autosomal dominant disorder caused by mutations in the *HMBS* gene that result in reduced, e.g., half-normal activity of the enzyme. Previously, a mouse

model of AIP that has ~30% of wildtype HMBS activity was generated by homologous recombination. Like human patients, these mice increase hepatic ALAS1 activity and accumulate large quantities of plasma and urinary ALA and PBG when administered porphyrinogenic drugs, such as phenobarbital. Thus, they serve as an excellent model to evaluate the efficacy of novel therapeutics for the acute hepatic porphyrias.

Summary of the Invention

The present invention describes methods and iRNA compositions for modulating the expression of an ALAS1 gene. In certain embodiments, expression of an ALAS1 gene is reduced or inhibited using an ALAS1-specific iRNA. Such inhibition can be useful in treating disorders related to ALAS1 expression, such as porphyrias.

Accordingly, described herein are compositions and methods that effect the RNA-induced silencing complex (RISC)-mediated cleavage of RNA transcripts of the ALAS1 gene, such as in a cell or in a subject (*e.g.*, in a mammal, such as a human subject). Also described are compositions and methods for treating a disorder related to expression of an ALAS1 gene, such as a porphyria, *e.g.*, X-linked sideroblastic anemia (XLSA), ALA deyhdratase deficiency porphyria (Doss porphyria or ADP), acute intermittent porphyria (AIP), congenital erythropoietic porphyria (CEP), prophyria cutanea tarda (PCT), hereditary coproporphyria (coproporphyria, or HCP), variegate porphyria (VP), erythropoietic protoporphyria (EPP), or transient erythroporphyria of infancy. In some embodiments, the disorder is an acute hepatic porphyria, *e.g.*, ALA deyhdratase deficiency porphyria (ADP), AIP, HCP, or VP. In certain embodiments, the disorder is ALA deyhdratase deficiency porphyria (ADP) or AIP.

In embodiments, the porphyria is a hepatic porphyria, e.g., a porphyria selected from acute intermittent porphyria (AIP) hereditary coproporphyria (HCP), variegate porphyria (VP), ALA deyhdratase deficiency porphyria (ADP), and hepatoerythropoietic porphyria. In embodiments, the porphyria is a homozygous dominant hepatic porphyria (e.g., homozygous dominant AIP, HCP, or VP) or hepatoerythropoietic porphyria, In embodiments, the porphyria is a dual porphyria.

As used herein, the term "iRNA," "RNAi", "iRNA agent," "RNAi agent," or "iRNA molecule," refers to an agent that contains RNA as that term is defined herein, and which

mediates the targeted cleavage of an RNA transcript, e.g., via an RNA-induced silencing complex (RISC) pathway. In one embodiment, an iRNA as described herein effects inhibition of ALAS1 expression in a cell or mammal.

The iRNAs included in the compositions featured herein encompass a dsRNA having an RNA strand (the antisense strand) having a region, e.g., 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 an ALAS1 gene (*e.g.*, a mouse or human ALAS1 gene) (also referred to herein as an "ALAS1-specific iRNA"). Alternatively, or in combination, iRNAs encompass a 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 an ALAS1 gene (*e.g.*, a human variant 1 or 2 of an ALAS1 gene) (also referred to herein as a "ALAS1-specific iRNA").

In embodiments, the iRNA (e.g, dsRNA) described herein comprises an antisense strand having a region that is substantially complementary to a region of a human ALAS1. In embodiments, the human ALAS1 has the sequence of NM_000688.4 (SEQ ID NO:1) or NM_000688.5 (SEQ ID NO:382). In embodiments, the human ALAS1 has the sequence of NM_199166.1.

In embodiments, the antisense sequence of the iRNA (e.g., dsRNA) targets within the region 871 to 895 (plus or minus 5, 4, 3, 2, or 1 nucleotides in either or both directions on the 5' and/or 3' end) on the ALAS1 transcript NM_000688.4. In embodiments, the antisense sequence targets the nucleotides 871 to 893, 871 to 892, or 873 to 895 on the ALAS1 transcript NM_000688.4. In embodiments, the antisense sequence comprises or consists of a sequence that is fully complementary or substantially complementary to nucleotides 871 to 893, 871 to 892, or 873 to 895 on the ALAS1 transcript NM_000688.4.

In one aspect, a double-stranded ribonucleic acid (dsRNA) for inhibiting expression of ALAS1 is provided, wherein said dsRNA comprises a sense strand and an antisense strand, the antisense strand comprising a region of complementarity to an ALAS1 RNA transcript, which antisense strand comprises at least 15 (e.g., at least 16, 17, 18, 19, 20, 21, 22, or 23) contiguous nucleotides differing by no more than 3, 2 or 1 nucleotides from the sequence of

UAAGAUGAGACACUCUUUCUGGU (SEQ ID NO: 4153) or UAAGAUGAGACACUCTUUCUGGU (SEQ ID NO: 4154). In embodiments, the antisense strand comprises the sequence of UAAGAUGAGACACUCUUUCUGGU (SEQ ID NO: 4153) or UAAGAUGAGACACUCTUUCUGGU (SEQ ID NO: 4154). In embodiments, the sense strand comprises the sequence of CAGAAAGAGUGUCUCAUCUUA (SEQ ID NO: 4155). In embodiments, one or more nucleotides of the antisense strand and/or sense strand are modified as described herein. In embodiments, the dsRNA comprises (i) an antisense strand that comprises, or consists of, the antisense sequence of AD-60489, AD-60519, or AD-61193 and/or (ii) a sense strand that comprises, or consists of, the sense sequence of AD-60489, AD-60519, or AD-61193 (including one or more (*e.g.*, all) of the modifications of the antisense strand and/or antisense strand of AD-60489, AD-60519, or AD-61193).

In one aspect, a double-stranded ribonucleic acid (dsRNA) for inhibiting expression of ALAS1 is provided, wherein said dsRNA comprises a sense strand and an antisense strand, the antisense strand comprising a region of complementarity to an ALAS1 RNA transcript, which antisense strand comprises at least 15 (e.g., at least 16, 17, 18, 19, 20, 21, 22, or 23) contiguous nucleotides differing by no more than 3 (e.g., by no more than 0, 1 or 2) nucleotides from an antisense sequence listed in any one of Tables 21 to 40, or an unmodified version of an antisense sequence (e.g., a version having the same nucleotide sequence except that some or all of the nucleotides are unmodified) listed in any one of Tables 21 to 40. In one embodiment, the antisense sequence comprises at least 15 (e.g., at least 16, 17, 18, 19, 20, 21, 22, or 23) contiguous nucleotides differing by no more than 3 (e.g., by no more than 0, 1 or 2) nucleotides from (i) the antisense sequence of AD-60489, AD-60519, or AD-61193 or (ii) an unmodified version of any one of these sequences. In embodiments, the antisense strand comprises at least 15 (e.g., at least 16, 17, 18, 19, 20, 21, 22, or 23) contiguous nucleotides differing by no more than 3 (e.g., by no more than 0, 1 or 2) nucleotides from the sequence of UAAGAUGAGACACUCUUUCUGGU (SEQ ID NO: 4153) or UAAGAUGAGACACUCTUUCUGGU (SEQ ID NO: 4154). In an embodiment, the antisense sequence targets positions 871-893 of NM_000688.4 (SEQ ID NO:1). In embodiments, the sense strand comprises the sequence of CAGAAAGAGUGUCUCAUCUUA (SEQ ID NO:

4155). In embodiments, one or more nucleotides of the antisense strand and/or sense strand are modified as described herein.

In some embodiments, the dsRNA is not a sense and/or antisense sequence listed in any one of Tables 2, 3, 6, 7, 8, 9, 14, 15, 18 or 20.

In one embodiment, a double-stranded ribonucleic acid (dsRNA) for inhibiting expression of ALAS1 is provided, wherein said dsRNA comprises a sense strand and an antisense strand, the antisense strand comprising a region of complementarity to an ALAS1 RNA transcript, which antisense strand comprises at least 15 (e.g., at least 16, 17, 18, 19, 20, 21, 22, or 23) contiguous nucleotides differing by no more than 3 nucleotides, no more than 2 nucleotides, or no more than one nucleotide, from the antisense sequence of AD-60519. In embodiments, one or more nucleotides are modified as described herein.

In one embodiment, a double-stranded ribonucleic acid (dsRNA) for inhibiting expression of ALAS1 is provided, wherein said dsRNA comprises a sense strand and an antisense strand, the antisense strand comprising a region of complementarity to an ALAS1 RNA transcript, which antisense strand comprises at least 15 (e.g., at least 16, 17, 18, 19, 20, 21, 22, or 23) contiguous nucleotides differing by no more than 3 (e.g., by no more than 0, 1 or 2) nucleotides from the antisense sequence of AD-60489, or a derivative of AD-60489 as described herein. In embodiments, one or more nucleotides are modified as described herein, *e.g.*, one or more (or all) nucleotides of AD-60489 are modified as described herein. In embodiments, the derivative of AD-60489 is AD-60501, AD-60519, AD-60901, AD-60495, AD-60900, AD-60935, AD-60879, AD-61190, AD-61191, AD-60865, AD-60861, AD-60876, AD-61193, AD-60519, AD-60519, or AD-60901. In embodiments, the derivative of AD-60489 is AD-60519. In embodiments, the derivative of AD-60489 is AD-61193.

In one embodiment, a double-stranded ribonucleic acid (dsRNA) for inhibiting expression of ALAS1 is provided, wherein said dsRNA comprises a sense strand and an antisense strand, the antisense strand comprising a region of complementarity to an ALAS1 RNA transcript, which antisense strand comprises at least 15 (e.g., at least 16, 17, 18, 19, 20, 21, 22, or 23) contiguous nucleotides differing by no more than 3 (e.g., by no more than 0, 1 or 2) nucleotides from a derivative of AD-58632 described herein. In embodiments, one or more nucleotides are modified as described herein, *e.g.*, one or more (or all) nucleotides of AD-58632

are modified as described herein. In embodiments, the derivative of AD-58632 is AD-60405, AD-60887, AD-60923, AD-60434, AD-60892, AD-60419, AD-60924, AD-60445, AD-60925, and AD-60926, AD-60820, AD-60843, AD-60819, AD-61140, AD-61141, AD-61142, AD-60835, AD-60839, AD-61143, AD-61144, AD-61145, AD-61146, AD-60892, or AD-60419. In embodiments, the derivative of AD-58632 is AD-60819.

In some embodiments, the dsRNA has an IC_{50} of less than 1nM. In some embodiments, the dsRNA has an IC_{50} in the range of 0.01-1nM. In embodiments, the dsRNA has an IC_{50} of less than 0.05 nM. In embodiments, the dsRNA has an IC_{50} of less than 0.02 nM. In embodiments, the dsRNA has an IC_{50} of less than 0.01 nM. In embodiments, the IC_{50} is determined as described herein in the Examples.

In some embodiments, the dsRNA has a single dose ED50 of less than about 10 mg/kg. In some embodiments, the dsRNA has a single dose ED50 of less than about 5 mg/kg. In embodiments, the EC50 is determined as described herein in the Examples.

In some embodiments, the dsRNA shows improved activity compared with AD-58632. In some embodiments, the dsRNA shows improved activity compared with AD-60489. In some embodiments, the dsRNA shows improved activity compared with AD-58632 and AD-60489.

In embodiments, the dsRNA is AD-60501, AD-60519, AD-60901, AD-60495, AD-60900, AD-60935, AD-60879, AD-61190, AD-61191, AD-60865, AD-60861, AD-60876, AD-61193, AD-60519, AD-60519, AD-60901, AD-60405, AD-60887, AD-60923, AD-60434, AD-60892, AD-60419, AD-60924, AD-60445, AD-60925, AD-60926, AD-60820, AD-60843, AD-60819, AD-61140, AD-61141, AD-61142, AD-60835, AD-60839, AD-61143, AD-61144, AD-61145, AD-61146, AD-60892, or AD-60419 (*e.g.*, including the nucleotide sequence and/or one or more (*e.g.*, all) of the modifications of the aforesaid dsRNAs). In embodiments, the dsRNA comprises an antisense strand that comprises, or consists of, an antisense sequence (and/or one or more (*e.g.*, all) of the modifications)) selected from AD-60501, AD-60519, AD-60901, AD-60495, AD-60900, AD-60935, AD-60879, AD-61190, AD-61191, AD-60865, AD-60861, AD-60876, AD-61193, AD-60519, AD-60519, AD-60901, AD-60405, AD-60887, AD-60923, AD-60434, AD-60892, AD-60419, AD-60924, AD-60445, AD-60925, AD-60926, AD-60820, AD-60843, AD-60819, AD-61140, AD-61141, AD-61142, AD-60835, AD-60839, AD-61143, AD-61144, AD-61145, AD-61146, AD-60892, or AD-60419. In embodiments, the dsRNA

comprises a sense strand that comprises, or consists of, a sense sequence (and/or one or more (*e.g.*, all) of the modifications)) selected from AD-60501, AD-60519, AD-60901, AD-60495, AD-60900, AD-60935, AD-60879, AD-61190, AD-61191, AD-60865, AD-60861, AD-60876, AD-61193, AD-60519, AD-60519, AD-60901, AD-60405, AD-60887, AD-60923, AD-60434, AD-60892, AD-60419, AD-60924, AD-60445, AD-60925, AD-60926, AD-60820, AD-60843, AD-60819, AD-61140, AD-61141, AD-61142, AD-60835, AD-60839, AD-61143, AD-61144, AD-61145, AD-61146, AD-60892, or AD-60419.

In embodiments, a double-stranded ribonucleic acid (dsRNA) for inhibiting expression of ALAS1 is provided, wherein the dsRNA comprises (i) an antisense strand that comprises, or consists of, the sequence of UAAGAUGAGACACUCUUUCUGGU (SEQ ID NO: 4153) or UAAGAUGAGACACUCTUUCUGGU (SEQ ID NO: 4154) and/or (ii) a sense strand that comprises, or consists of, the sequence of CAGAAAGAGUGUCUCAUCUUA (SEQ ID NO: 4155). In embodiments, one or more nucleotides of the antisense strand and/or sense strand are modified as described herein.

In embodiments, a double-stranded ribonucleic acid (dsRNA) for inhibiting expression of ALAS1 is provided, wherein the dsRNA comprises (i) an antisense strand that comprises, or consists of, the antisense sequence of AD-60489 and/or (ii) a sense strand that comprises, or consists of, the sense sequence of AD-60489 (wherein the sense and/or antisense sequence includes one or more (*e.g.*, all) of the modifications of the sense strand and/or antisense strand of AD-60489).

In embodiments, a double-stranded ribonucleic acid (dsRNA) for inhibiting expression of ALAS1 is provided, wherein the dsRNA comprises (i) an antisense strand that comprises, or consists of, the antisense sequence of AD-60519 and/or (ii) a sense strand that comprises, or consists of, the sense sequence of AD-60519 (wherein the sense and/or antisense sequence includes one or more (*e.g.*, all) of the modifications of the sense strand and/or antisense strand of AD-60519).

In embodiments, a double-stranded ribonucleic acid (dsRNA) for inhibiting expression of ALAS1 is provided, wherein the dsRNA comprises (i) an antisense strand that comprises, or consists of, the antisense sequence of AD-61193 and/or (ii) a sense strand that comprises, or consists of, the sense sequence of AD-61193 (wherein the sense and/or antisense sequence

includes one or more (*e.g.*, all) of the modifications of the sense strand and/or antisense strand of AD-61193).

In embodiments, a double-stranded ribonucleic acid (dsRNA) for inhibiting expression of ALAS1 is provided, wherein the dsRNA comprises (i) an antisense strand that comprises, or consists of, the antisense sequence of AD-60819 and/or (ii) a sense sequence that comprises, or consists of, the sense sequence of AD-60819 (wherein the sense and/or antisense sequence includes one or more (*e.g.*, all) of the modifications of the sense strand and/or antisense strand of AD-60819).

In embodiments, a dsRNA for inhibiting expression of ALAS1 is provided, wherein the dsRNA comprises (i) an antisense strand that comprises, or consists of, the antisense sequence of AD-60489, AD-60519, AD-61193, or AD-60819 (or a corresponding unmodified antisense sequence) and/or (ii) a sense strand that comprises, or consists of, the sense sequence of AD-60489, AD-60519, AD-61193, or AD-60819 (or a corresponding unmodified antisense sequence). In embodiments, the dsRNA comprises (i) an antisense strand that consists of the antisense sequence of AD-60489, AD-60519, AD-61193, or AD-60819 and/or (ii) a sense strand that consists of the sense sequence of AD-60489, AD-60519, AD-61193, or AD-60819, except that the antisense strand and/or sense strand of the dsRNA differs by 1, 2, or 3 nucleotides from the corresponding antisense and/or sense sequence of AD-60489, AD-60519, AD-61193, or AD-60819.

The sequences and modifications of AD-60489, AD-60519, AD-61193, and AD-60819 are shown in Table 44 below.

Table 44: Sequences and Modifications of AD-60489, AD-60519, AD-61193, AD-60819

Target sites of antisense sequence on NM_000688.4	Duplex Name	Sense Sequence (5'-3')	Antisense Sequence (5'-3')	Corresponding unmodified sense sequence	Corresponding unmodified antisense sequence
871-893	AD-60489	CfsasGfaAfaGfaGfUfGfuCfuCfaUfcUfuAfL96 (SEQ ID NO: 4156)	usAfsaGfaUfgAfgAfcacUfcUfuUfcUfgsgsu (SEQ ID NO: 4157)	CAGAAAGAGUGUCUCAUCUUA (SEQ ID NO: 4158)	UAAGAUGAGACACUCUUUCUGGU (SEQ ID NO: 4159)
871-893	AD-60519	csasgaaaGfaGfuGfuCfuCfaucuuaL96 (SEQ ID NO: 4160)	usAfsAfGfaUfgAfgAfcAfcUfcUfuUfcUfgsgsu (SEQ ID NO: 4161)	CAGAAAGAGUGUCUCAUCUUA (SEQ ID NO: 4162)	UAAGAUGAGACACUCUUUCUGGU (SEQ ID NO: 4163)
871-893	AD-61193	csasgaaaGfaGfuGfuCfuCfaucuuaL96 (SEQ ID NO: 4164)	usAfsaGfaUfgAfgAfcacUfcdTuUfcUfgsgsu (SEQ ID NO: 4165)	CAGAAAGAGUGUCUCAUCUUA (SEQ ID NO: 4166)	UAAGAUGAGACACUCTUUCUGGU (SEQ ID NO: 4167)
873-895	AD-60819	GfsasAfaGfaGfuGfuCfuCfaucuuCfuuL96 (SEQ ID NO: 4168)	asAfsgAfaGfaugAfgAfcAfcucuuucsusg (SEQ ID NO: 4169)	GAAAGAGUGUCUCAUCUUCUU (SEQ ID NO: 4170)	AAGAAGAUGAGACACUCUUUCUG (SEQ ID NO: 4171)

wherein c, a, g, u = 2'-OMe ribonucleosides; Af, Cf, G, Uf = 2'F ribonucleosides; S = phosphorothioate; L96 has the structure depicted in Table 1.

In embodiments, a double-stranded ribonucleic acid (dsRNA) for inhibiting expression of ALAS1 is provided, wherein the dsRNA comprises (i) an antisense strand that comprises, or consists of, the antisense sequence of AD-60489, AD-60519, or AD-61193 and/or (ii) a sense strand that comprises, or consists of, the sense sequence of AD-60489, AD-60519, or AD-61193 (including the nucleotide sequence and one or more (*e.g.*, all) of the modifications of the sense strand and/or antisense strand of AD-60489, AD-60519, or AD-61193).

In embodiments, a double-stranded ribonucleic acid (dsRNA) for inhibiting expression of ALAS1 is provided, wherein the dsRNA is AD-60489, AD-60519, AD-61193, or AD-60819. In embodiments, a double-stranded ribonucleic acid (dsRNA) for inhibiting expression of ALAS1 is provided, wherein the dsRNA is AD-60489, AD-60519, or AD-61193 (*e.g.*, including the nucleotide sequence and/or one or more (*e.g.*, all) of the modifications of AD-60489, AD-60519, or AD-61193).

In embodiments, the dsRNA is, comprises, or consists of, AD-60489 (*e.g.*, including the nucleotide sequence and/or one or more (*e.g.*, all) of the modifications of AD-60489).

In embodiments, the dsRNA is, comprises, or consists of, AD-60519 (*e.g.*, including the nucleotide sequence and/or one or more (*e.g.*, all) of the modifications of AD-60519).

In embodiments, the dsRNA is, comprises, or consists of, AD-61193 (*e.g.*, including the nucleotide sequence and/or one or more (*e.g.*, all) of the modifications of AD-61193).

In embodiments, the dsRNA is, comprises, or consists of, AD-60819 (*e.g.*, including the nucleotide sequence and/or one or more (*e.g.*, all) of the modifications of AD-60819).

In embodiments, the dsRNA (e.g., AD-60489, AD-60519, AD-61193, AD-60819, or another dsRNA disclosed herein in any one of Tables 21 to 40) is effective to suppress the liver level of ALAS1 mRNA, e.g., to achieve silencing of at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, or 80% (e.g., such that ALAS1 mRNA levels are decreased to 90% or less, 80% or less, 70% or less, 60% or less, 50% or less, 40% or less, 30% or less, or 20% or less of a control level of liver ALAS1 mRNA, e.g., the level in an untreated individual or group of individuals, e.g., an individual or group of individuals treated with PBS only). In embodiments, the effectiveness of the dsRNA in suppressing the liver level of ALAS1 mRNA is assessed using a non-human primate model, e.g., as described herein in the Examples.

In embodiments, the dsRNA (e.g., AD-60489, AD-60519, AD-61193, AD-60819, or another dsRNA disclosed herein in any one of Tables 21 to 40) is effective to suppress the circulating level of ALAS1 mRNA, e.g., to achieve silencing of at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, or 80% (e.g., such that ALAS1 mRNA levels are decreased to 90% or less, 80% or less, 70% or less, 60% or less, 50% or less, 40% or less, 30% or less, or 20% or less of a control level of circulating ALAS1 mRNA, e.g., the level prior to treatment with the dsRNA, or the level in an untreated individual or group of individuals). In embodiments, the effectiveness of the dsRNA in suppressing the circulating level of ALAS1 mRNA is assessed using a non-human primate model, e.g., as described herein in the Examples. In embodiments, the circulating level of ALAS1 mRNA is assessed using a circulating extracellular RNA detection (cERD) assay, *e.g.*, as described herein or in Sehgal, A. et al. Quantitation of tissue-specific target gene modulation using circulating RNA (Poster presented on February 9, 2012 at the Keystone Gene Silencing by small RNAs symposium (Vancouver, February 7-12, 2012) or

Sehgal, A. et al. Tissue-specific gene silencing monitored in circulating RNA, *RNA*, 20: 1-7, published online December 19, 2013.

The cERD method can be applied to any appropriate biological sample. In embodiments, the circulating level of ALAS1 mRNA is assessed using a blood sample, e.g., a serum sample. In embodiments, the circulating level of ALAS1 mRNA is assessed using a urine sample.

In embodiments, the dsRNA is a derivative of AD-60489 that is disclosed herein, e.g., in any one of the tables herein. In embodiments, the dsRNA shows improved activity compared with AD-60489. In some such embodiments, the dsRNA is AD-60519.

In embodiments, the dsRNA is a derivative of AD-58632 that is disclosed herein, e.g., in any one of the tables herein. In embodiments, the dsRNA shows improved activity compared with AD-58632.

In embodiments, improved activity is indicated by a lower IC50, e.g., as determined based on in vitro assays, e.g., as described herein, e.g., in the Examples.

In embodiments, improved activity is indicated by a lower effective dose. The effective dose may be determined based on the administration of a single dose or multiple repeated doses. In embodiments, the effective dose is determined based on the single dose ED50. In embodiments, the effective dose or the single dose ED50 is determined based on an in vivo assay. In embodiments, the in vivo assay is conducted in a non-human animal, e.g., in a rat, in a non-human primate, or in a mouse.

In embodiments, the effective dose is determined based on the dose required to obtain a reduction of in a level of ALAS1 mRNA (e.g., a liver level of ALAS1 mRNA and/or a circulating level of ALAS1 mRNA), e.g., as described herein in the Examples. In embodiments, circulating mRNA is assessed using the cERD assay.

In embodiments, the effective dose is determined based on the dose required to obtain a reduction of a level (e.g., a urine and/or plasma level) of ALA and/or PBG.

In embodiments, the effective dose is determined based on the dose required to obtain a particular treatment effect described herein, e.g., prevention or reduction of symptoms associated with a porphyria.

In embodiments, improved activity is indicated by the achievement of a higher liver level of the dsRNA. In embodiments, a higher liver level is obtained after a single dose of dsRNA

(e.g., a dose of 1, 2.5, 3, 5, or 10 mg/kg). In embodiments, a higher liver level is obtained after multiple doses of dsRNA have been administered (e.g., 2-10 daily or weekly doses of 1, 2.5, 3, 5, or 10 mg/kg).

In one embodiment, the iRNA encompasses a dsRNA having an RNA strand (the antisense strand) having a region that is substantially complementary to a portion of an ALAS1 mRNA, *e.g.*, a human ALAS1 mRNA (*e.g.*, a human ALAS1 mRNA as provided in SEQ ID NO:1 or SEQ ID NO:382).

In one embodiment, an iRNA for inhibiting expression of an ALAS1 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 an ALAS1 transcript, and the region of complementarity is 30 nucleotides or less, and at least 15 nucleotides in length. Generally, the iRNA is 19 to 24 nucleotides in length.

In some embodiments, the iRNA is 19-21 nucleotides in length. In some embodiments, the iRNA is 19-21 nucleotides in length and is in a lipid formulation, e.g. a lipid nanoparticle (LNP) formulation (e.g., an LNP11 formulation).

In some embodiments, the iRNA is 21-23 nucleotides in length. In some embodiments, the iRNA is 21-23 nucleotides in length and is in the form of a conjugate, e.g., conjugated to one or more GalNAc derivatives as described herein.

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. An iRNA targeting ALAS1, upon contact with a cell expressing ALAS1, inhibits the expression of an ALAS1 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 iRNA targeting ALAS1 is formulated in a stable nucleic acid lipid particle (SNALP).

In one embodiment, an iRNA (e.g., a dsRNA) featured herein includes a first sequence of a dsRNA that is selected from the group consisting of the sense sequences of Tables 21 to 40 and a second sequence that is selected from the group consisting of the corresponding antisense sequences of Tables 21 to 40.

The iRNA molecules featured herein can include naturally occurring nucleotides or can include at least one modified nucleotide. In embodiments, the at least one modified nucleotide include one or more of a modification on the nucleotide chosen from the group consisting of a locked nucleic acid (LNA), an acyclic nucleotide, a hexitol or hexose nucleic acid (HNA), a cyclohexene nucleic acid (CeNA), 2′-methoxyethyl, 2′-O-alkyl, 2′-O-allyl, 2′-C- allyl, 2′-fluoro, 2′-deoxy, 2'-hydroxyl, or any combination thereof. In one embodiment, the at least one modified nucleotide includes, but is not limited to a 2'-O-methyl modified nucleotide, 2′-fluoro modified nucleotide, a nucleotide having a 5'-phosphorothioate group, and a terminal nucleotide linked to a ligand, *e.g.*, an N-acetylgalactosamine (GalNAc) or 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 acyclic nucleotide, an abasic nucleotide, 2'-amino-modified nucleotide, 2'-alkyl-modified nucleotide, morpholino nucleotide, a phosphoramidate, and a non-natural base comprising nucleotide. Such a modified sequence can be based, e.g., on a first sequence of said iRNA selected from the group consisting of the sense sequences of disclosed in Tables 21-40, and a second sequence selected from the group consisting of the corresponding antisense sequences disclosed in Tables 21-40.

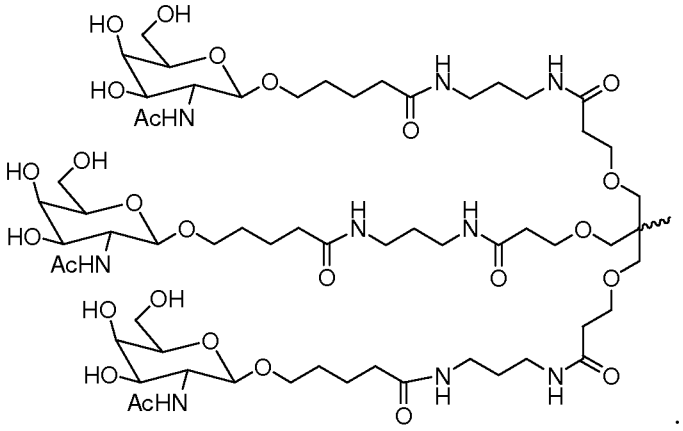
In one embodiment, an iRNA as described herein targets a wildtype ALAS1 RNA transcript variant, and in another embodiment, the iRNA targets a mutant transcript (*e.g.*, an ALAS1 RNA carrying an allelic variant). For example, an iRNA featured in the invention can target a polymorphic variant, such as a single nucleotide polymorphism (SNP), of ALAS1. In another embodiment, the iRNA targets both a wildtype and a mutant ALAS1 transcript. In yet another embodiment, the iRNA targets a particular transcript variant of ALAS1 (*e.g.*, human ALAS1 variant 1). In yet another embodiment, the iRNA agent targets multiple transcript variants (*e.g.*, both variant 1 and variant 2 of human ALAS1).

In one embodiment, an iRNA featured in the invention targets a non-coding region of an ALAS1 RNA transcript, such as the 5' or 3' untranslated region of a transcript.

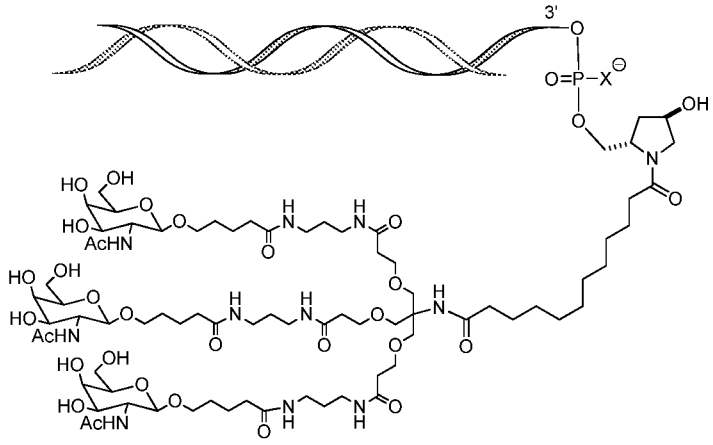
In some embodiments, an iRNA as described herein is in the form of a conjugate, e.g., a carbohydrate conjugate, which may serve as a targeting moiety and/or ligand, as described herein. In one embodiment, the conjugate is attached to the 3' end of the sense strand of the

dsRNA. In some embodiments, the conjugate is attached via a linker, e.g., via a bivalent or trivalent branched linker.

In some embodiments, the conjugate comprises one or more N-acetylgalactosamine (GalNAc) derivatives. Such a conjugate is also referred to herein as a GalNAc conjugate. In some embodiments, the conjugate targets the RNAi agent to a particular cell, e.g., a liver cell, e.g., a hepatocyte. The GalNAc derivatives can be attached via a linker, e.g., a bivalent or trivalent branched linker. In particular embodiments, the conjugate is

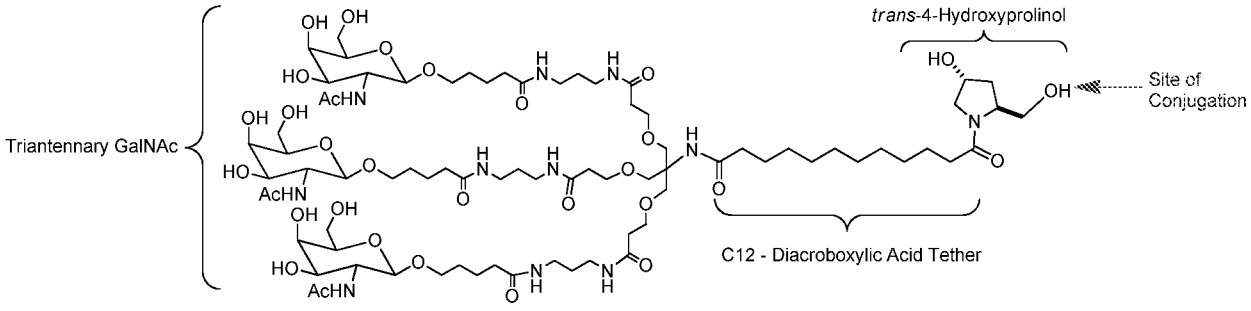


In some embodiments, the RNAi agent is attached to the carbohydrate conjugate via a linker, e.g., a linker as shown in the following schematic, wherein X is O or S



In some embodiments, X is O. In some embodiments, X is S.

In some embodiments, the RNAi agent is conjugated to L96 as defined in Table 1 and shown below



In one embodiment, the dsRNA has one, two, three, four, five, six, seven, eight, nine, ten, eleven, twelve, thirteen, fourteen, fifteen, sixteen or all of the following:

(i) is chemically synthesized, *e.g.*, is synthesized by solid phase oligonucleotide synthesis;

(ii) all the nucleotides in the dsRNA are modified, *e.g.*, all the nucleotides are 2'-OMe or 2'-F modified, or a combination of 2'-OMe and 2'-F modified;

(iii) all nucleotides are connected through 3'-5' phosphodiester linkages;

(iv) the sense strand comprises or consists of 21 nucleotides;

(v) the antisense sense strand comprises or consists of 23 nucleotides;

(vi) has a blunt-end at the 3'-end of sense strand;

(vii) has a 3'-overhang, *e.g.*, has a two-nucleotide overhang, at the 3'-end of the antisense strand;

(viii) is covalently attached to a ligand containing three N-acetylgalactosamine (GalNAc) moieties;

(ix) the 3'-end of the sense strand is conjugated to the triantennary GalNAc moiety (*e.g.*, referred to herein as L96 as defined in Table 1). In one embodiment, the 3'-end is linked to the triantennary GalNAc moiety through a phosphodiester linkage;

(x) has an antisense strand that comprises one or more (*e.g.*, four) phosphorothioate linkages. In one embodiment, the phosphorothioate linkages are located at the 3' end and at the 5' end of the antisense strand. In one embodiment, two phosphorothioate linkages are located at the 3' end and two phosphorothioate linkages are located at the 5' end of the antisense strand;

(xi) has a sense strand that comprises one or more (*e.g.*, two) phosphorothioate linkages. In one embodiment, the one or more (*e.g.*, two) phosphorothioate linkages are located at the 5' end of the sense strand;

(xii) 21 nucleotides of the sense strand hybridize to the complementary 21 nucleotides of the antisense strand;

(xiii) forms 21 nucleotide base pairs and a two-base overhang at the 3'-end of the antisense strand;

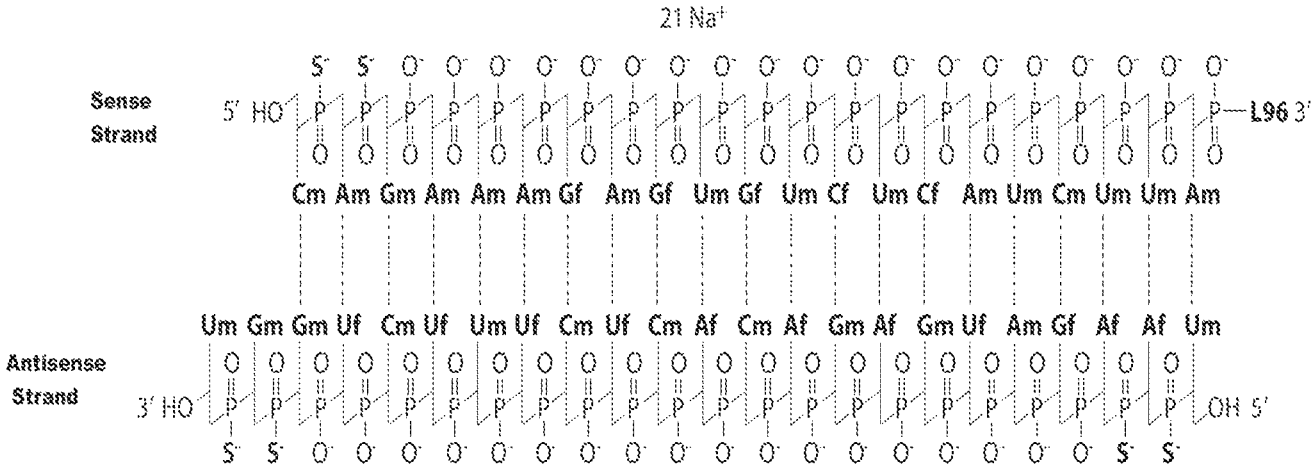
(xiv) comprises, or consists of, a sense and antisense strand having the sequence of AD-60519;

(xv) has a sense strand with 10, 12, 14, 16, 18, 19, 20 or all of the modifications of the sense strand of AD-60519;

(xvi) has an antisense strand with 10, 12, 14, 16, 18, 19, 20 or all of the modifications of the antisense strand of AD-60519; or

(xvii) has the duplex sequence and all the modifications of AD-60519.

In embodiments, the dsRNA is in the form of a conjugate having the following structure (also referred to herein as AD-60519 or ALN-60519) (SEQ ID NOS 5238-5239, respectively, in order of appearance):



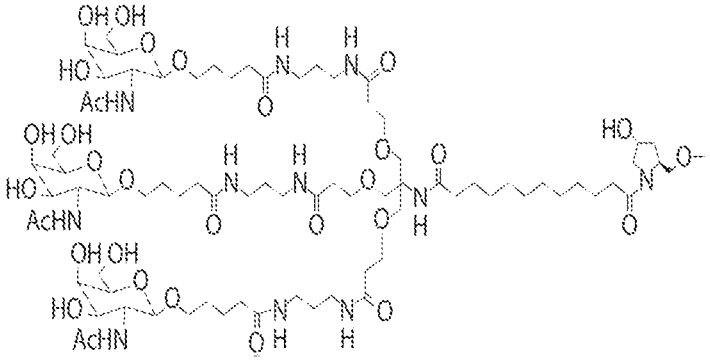
ALN-60519

Af, Cf, Gf, Uf = 2'-F ribonucleosides

Am, Cm, Gm, Um = 2'-OMe ribonucleosides

S = phosphorothioate

L96 =



In an aspect provided herein is a composition, e.g., a pharmaceutical composition, that 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 inhibiting the expression of an

ALAS1 gene in an organism, generally a human subject. In one embodiment, the composition is used for treating a porphyria, *e.g.*, AIP.

In one aspect, an iRNA provided herein is a double-stranded ribonucleic acid (dsRNA) for inhibiting expression of ALAS1, wherein said dsRNA comprises a sense strand and an antisense strand 15-30 base pairs in length and the antisense strand is complementary to at least 15 contiguous nucleotides of SEQ ID NO: 1 or 382.

In a further aspect, an iRNA provided herein is a double stranded RNAi (dsRNA) comprising a sense strand complementary to an antisense strand, wherein said antisense strand comprises a region of complementarity to an ALAS1 RNA transcript, wherein each strand has about 14 to about 30 nucleotides, wherein said double stranded RNAi agent is represented by formula (III):

sense: $5'\ n_p\text{-}N_a\text{-}(X\ X\ X)_i\text{-}N_b\text{-}Y\ Y\ Y\text{-}N_b\text{-}(Z\ Z\ Z)_j\text{-}N_a\text{-}n_q\ 3'$

antisense: $3'\ n_p'\text{-}N_a'\text{-}(X'X'X')_k\text{-}N_b'\text{-}Y'Y'Y'\text{-}N_b'\text{-}(Z'Z'Z')_l\text{-}N_a'\text{-}n_q'\ 5'$

(III)

wherein:

i, j, k, and l are each independently 0 or 1;

p, p', q, and q′ are each independently 0-6;

each N_a and N_a' independently represents an oligonucleotide sequence comprising 0-25 nucleotides which are either modified or unmodified or combinations thereof, each sequence comprising at least two differently modified nucleotides;

each N_b and N_b' independently represents an oligonucleotide sequence comprising 0-10 nucleotides which are either modified or unmodified or combinations thereof;

each n_p, n_p', n_q, and n_q' independently represents an overhang nucleotide;

XXX, YYY, ZZZ, X′X′X′, Y′Y′Y′, and Z′Z′Z′ each independently represent one motif of three identical modifications on three consecutive nucleotides;

modifications on N_b differ from the modification on Y and modifications on N_b' differ from the modification on Y′.

In embodiments, the sense strand is conjugated to at least one ligand.

In embodiments, i is 1; j is 1; or both i and j are 1.

In embodiments, k is 1; l is 1; or both k and l are 1.

In embodiments, XXX is complementary to X′X′X′, YYY is complementary to Y′Y′Y′, and ZZZ is complementary to Z′Z′Z′.

In embodiments, the Y′Y′Y′ motif occurs at the 11, 12 and 13 positions of the antisense strand from the 5'-end.

In embodiments, the Y′ is 2′-O-methyl.

In embodiments, the duplex region is 15-30 nucleotide pairs in length.

In embodiments, the duplex region is 17-23 nucleotide pairs in length.

In embodiments, the duplex region is 19-21 nucleotide pairs in length.

In embodiments, the duplex region is 21-23 nucleotide pairs in length.

In embodiments, the modification on the nucleotide is selected from the group consisting of a locked nucleic acid (LNA), an acyclic nucleotide, a hexitol or hexose nucleic acid (HNA), a cyclohexene nucleic acid (CeNA), 2′-methoxyethyl, 2′-O-alkyl, 2′-O-allyl, 2′-C- allyl, 2′-fluoro, 2′-deoxy, 2’-hydroxyl, and any combination thereof.

In embodiments, the modifications on the nucleotides are selected from the group consisting of LNA, HNA, CeNA, 2′-methoxyethyl, 2′-O-alkyl, 2′-O-allyl, 2′-C- allyl, 2′-fluoro, 2′-deoxy, 2’-hydroxyl, and combinations thereof.

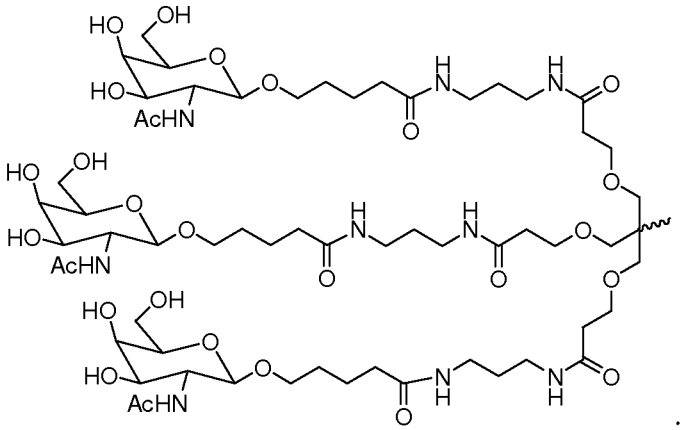
In embodiments, the modifications on the nucleotides are 2′-O-methyl, 2′-fluoro or both.

In embodiments, the ligand comprises a carbohydrate.

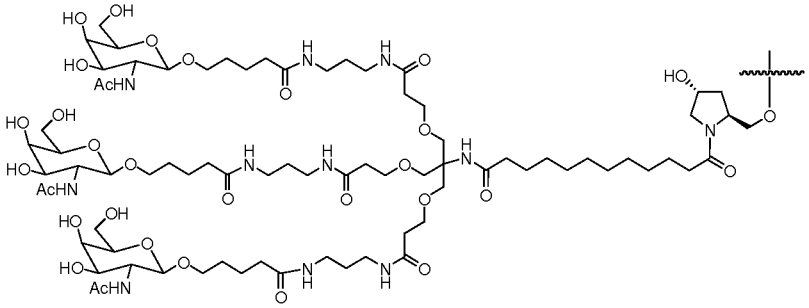
In embodiments, the ligand is attached via a linker.

In embodiments, the linker is a bivalent or trivalent branched linker.

In embodiments, the ligand is



In embodiments, the ligand and linker are as shown in Formula XXIV:



In embodiments, the ligand is attached to the 3’ end of the sense strand.

In embodiments, the dsRNA consists of or comprises a nucleotide sequence selected from the group of sequences provided in Tables 21-40.

In a further aspect, an iRNA provided herein is a double-stranded ribonucleic acid (dsRNA) for inhibiting expression of ALAS1, wherein said dsRNA comprises a sense strand and an antisense strand, the antisense strand comprising a region of complementarity to an ALAS1 RNA transcript, which antisense strand comprises at least 15 contiguous nucleotides differing by no more than 3 nucleotides from one of the antisense sequences listed in any one of Tables 21-40. In embodiments, the nucleotides of the antisense strand have fewer modifications, more modifications, or different modifications compared with the antisense sequences listed in any one of Tables 21-40.

In embodiments, the sense and antisense sequences are those of a duplex disclosed herein that suppresses ALAS1 mRNA expression by at least 50%, 60%, 70%, 80%, 85% or 90%, e.g., as assessed using an assay disclosed in the Examples provided herein.

In embodiments, ALAS1 mRNA expression is assessed based on an ALAS1 mRNA level in the liver, e.g., as assessed using a liver biopsy sample. In embodiments, ALAS1 mRNA expression is assessed based on an ALAS1 mRNA level in a biological fluid, e.g., blood, serum, plasma, cerebrospinal fluid, or urine. In embodiments, ALAS1 mRNA expression is assessed using a circulating extracellular RNA detection (cERD) assay, e.g., a cERD assay as described herein or in Sehgal, A. et al. Quantitation of tissue-specific target gene modulation using circulating RNA (Poster presented on February 9, 2012 at the Keystone Gene Silencing by small RNAs symposium (Vancouver, February 7-12, 2012) or Sehgal, A. et al. Tissue-specific gene silencing monitored in circulating RNA, *RNA*, 20: 1-7, published online December 19, 2013.

In some embodiments, the dsRNA comprises at least one modified nucleotide.

In some embodiments, at least one of the 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.

In some embodiments, the 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

acyclic nucleotide, an abasic nucleotide, 2'-amino-modified nucleotide, 2'-alkyl-modified nucleotide, morpholino nucleotide, a phosphoramidate, and a non-natural base comprising nucleotide.

In some embodiments, the region of complementarity is at least 17 nucleotides in length.

In some embodiments, the region of complementarity is between 19 and 21 nucleotides in length.

In some embodiments, the region of complementarity is 19 nucleotides in length.

In some embodiments, each strand is no more than 30 nucleotides in length.

In some embodiments, at least one strand comprises a 3' overhang of at least 1 nucleotide. In embodiments, the antisense strand comprises a 3' overhang of at least 1 nucleotide.

In some embodiments, at least one strand comprises a 3' overhang of at least 2 nucleotides. In embodiments, the antisense strand comprises a 3' overhang.of at least 2 nucleotides. In embodiments, the antisense strand comprises a 3' overhang.of 2 nucleotides.

In some embodiments, a dsRNA described herein further comprises a ligand.

In some embodiments, the ligand is a GalNAc ligand.

In some embodiments, the ligand targets the dsRNA to hepatocytes.

In some embodiments, the ligand is conjugated to the 3' end of the sense strand of the dsRNA.

In some embodiments, the region of complementarity consists of an antisense sequence selected from the antisense sequences listed in Tables 21-40, or a corresponding antisense sequence in which some or all of the nucleotides are unmodified. In embodiments, the region of complementarity consists of the sequence UAAGAUGAGACACUCUUUCUGGU (SEQ ID NO: 4153) or UAAGAUGAGACACUCTUUCUGGU (SEQ ID NO: 4154). In some embodiments, the region of complementarity consists of the antisense sequence of the duplex AD-60489. In some embodiments, the region of complementarity consists of the antisense sequence of the duplex AD-60519.

In embodiments, the region of complementarity consists of an antisense sequence selected from a duplex disclosed herein that suppresses ALAS1 mRNA expression by at least 50%, 60%, 70%, 80%, 85% or 90%, e.g., as assessed using an assay disclosed in the Examples provided herein.

In some embodiments, the dsRNA comprises a sense strand consisting of a sense strand sequence selected from Tables 21-40, and an antisense strand consisting of an antisense sequence selected from Tables 21-40. In embodiments, the dsRNA comprises a pair of corresponding sense and antisense sequences selected from those of the duplexes disclosed in Tables 21-40.

In one aspect, the invention provides a cell containing at least one of the iRNAs (e.g., dsRNAs) featured herein. The cell is generally a mammalian cell, such as a human cell. In some embodiments, the cell is an erythroid cell. In other embodiments, the cell is a liver cell (*e.g.*, a hepatocyte).

In an aspect provided herein is a pharmaceutical composition for inhibiting expression of an ALAS1 gene, the composition comprising an iRNA (e.g., a dsRNA) described herein.

In embodiments of the pharmaceutical compositions described herein, the iRNA (e.g., dsRNA) is administered in an unbuffered solution. In embodiments, the unbuffered solution is saline or water, e.g., water for injection.

In embodiments, the pharmaceutical composition comprises AD-60519 and water for injection. In embodiments, the composition comprises about 100 to 300 mg/mL, *e.g.*, 200 mg/mL, of AD-60519. In embodiments, the composition has a pH of 6.0-7.5, e.g., about 7.0. In embodiments, the composition is for subcutaneous injection. In embodiments, the pharmaceutical composition is packaged in a container (e.g., a glass vial, e.g., a 2 mL glass vial,) at a volume of about 0.3 to 1 mL, *e.g.*, 0.55 mL. In embodiments, the pharmaceutical composition is ALN-AS1 as described herein in the examples.

In embodiments of the pharmaceutical compositions described herein, the iRNA (e.g., dsRNA is administered with a buffer solution. In embodiments, the buffer solution comprises acetate, citrate, prolamine, carbonate, or phosphate or any combination thereof. In embodiments, the buffer solution is phosphate buffered saline (PBS).

In embodiments of the pharmaceutical compositions described herein, the iRNA (e.g., dsRNA) is targeted to hepatocytes.

In embodiments of the pharmaceutical compositions described herein, the composition is administered intravenously.

In embodiments of the pharmaceutical compositions described herein, the composition is administered subcutaneously.

In embodiments, a pharmaceutical composition comprises an iRNA (e.g., a dsRNA) described herein that comprises a ligand (e.g., a GalNAc ligand) that targets the iRNA (e.g., dsRNA) to hepatocytes.

In embodiments, a pharmaceutical composition comprises an iRNA (e.g., a dsRNA) described herein that comprises a ligand (e.g., a GalNAc ligand), and the pharmaceutical composition is administered subcutaneously. In embodiments, the ligand targets the iRNA (e.g., dsRNA) to hepatocytes.

In certain embodiments, a pharmaceutical composition, e.g., a composition described herein, includes a lipid formulation. In some embodiments, the RNAi agent is in a LNP formulation, e.g., a MC3 formulation. In some embodiments, the LNP formulation targets the RNAi agent to a particular cell, e.g., a liver cell, e.g., a hepatocyte. In embodiments, the lipid formulation is a LNP11 formulation. In embodiments, the composition is administered intravenously.

In another embodiment, the pharmaceutical composition is formulated for administration according to a dosage regimen described herein, *e.g.*, 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, the 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 longer.

In another embodiment, a composition containing an iRNA featured in the invention, *e.g.*, a dsRNA targeting ALAS1, is administered with a non-iRNA therapeutic agent, such as an agent known to treat a porphyria (e.g., AIP), or a symptom of a porphyria (e.g., pain). In another embodiment, a composition containing an iRNA featured in the invention, *e.g.*, a dsRNA targeting AIP, is administered along with a non-iRNA therapeutic regimen, such as hemin or glucose (*e.g.*, glucose infusion (e.g., IV glucose)). For example, an iRNA featured in the invention can be administered before, after, or concurrent with glucose, dextrose, or a similar treatment that serves to restore energy balance (*e.g.*, total parenteral nutrition). An iRNA

featured in the invention can also be administered before, after, or concurrent with the administration of a heme product (*e.g.*, hemin, heme arginate, or heme albumin), and optionally also in combination with a glucose (e.g. IV glucose) or the like.

Typically, glucose administered for the treatment of a porphyria is administered intravenously (IV). Administration of glucose intravenously is referred to herein as "IV glucose." However, alternative embodiments in which glucose is administered by other means are also encompassed.

In one embodiment, an ALAS1 iRNA is administered to a patient, and then the non-iRNA agent or therapeutic regimen (*e.g.*, glucose and/or a heme product) is administered to the patient (or vice versa). In another embodiment, an ALAS1 iRNA and the non-iRNA therapeutic agent or therapeutic regimen are administered at the same time.

In an aspect provided herein is a method of inhibiting ALAS1 expression in a cell, the method comprising: (a) introducing into the cell an iRNA (e.g. a dsRNA) described herein and (b) maintaining the cell of step (a) for a time sufficient to obtain degradation of the mRNA transcript of an ALAS1 gene, thereby inhibiting expression of the ALAS1 gene in the cell.

In an aspect provided herein is a method for reducing or inhibiting the expression of an ALAS1 gene in a cell (*e.g.*, an erythroid cell or a liver cell, such as, *e.g.*, a hepatocyte). The method includes:

(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 ALAS1, 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 ALAS1, inhibits expression of an ALAS1 gene by at least 10%, *e.g.*, at least 20%, at least 30%, at least 40% or more; and

(b) maintaining the cell of step (a) for a time sufficient to obtain degradation of the mRNA transcript of the ALAS1 gene, thereby reducing or inhibiting expression of an ALAS1gene in the cell.

In embodiments of the foregoing methods of inhibiting ALAS1 expression in a cell, the cell is treated *ex vivo*, *in vitro*, or *in vivo*. In embodiments, the cell is a hepatocyte.

In embodiments, the cell is present in a subject in need of treatment, prevention and/or management of a disorder related to ALAS1 expression.

In embodiments, the disorder is a porphyria. In embodiments, the porphyria is acute intermittent porphyria or ALA-dehydratase deficiency porphyria.

In embodiments, the porphyria is a hepatic porphyria, e.g., a porphyria selected from acute intermittent porphyria (AIP) hereditary coproporphyria (HCP), variegate porphyria (VP), ALA deyhdratase deficiency porphyria (ADP), and hepatoerythropoietic porphyria. In embodiments, the porphyria is a homozygous dominant hepatic porphyria (e.g., homozygous dominant AIP, HCP, or VP) or hepatoerythropoietic porphyria. In embodiments, the porphyria is a dual porphyria.

In embodiments, the expression of ALAS1 is inhibited by at least 30%.

In embodiments, the iRNA (e.g., dsRNA) has an IC_{50} in the range of 0.01-1nM.

In certain embodiments, the cell (*e.g.*, the hepatocyte) is a mammalian cell (*e.g.*, a human, non-human primate, or rodent cell).

In one embodiment, the cell is treated *ex vivo*, *in vitro*, or *in vivo* (*e.g.*, the cell is present in a subject (*e.g.*, a patient in need of treatment, prevention and/or management of a disorder related to ALAS1 expression).

In one embodiment, the subject is a mammal (*e.g.*, a human) at risk, or diagnosed with a porphyria, *e.g.*, X-linked sideroblastic anemia (XLSA), ALA deyhdratase deficiency porphyria (ADP or Doss porphyria), acute intermittent porphyria (AIP), congenital erythropoietic porphyria (CEP), prophyria cutanea tarda (PCT), hereditary coproporphyria (coproporphyria, or HCP), variegate porphyria (VP), erythropoietic protoporphyria (EPP), or transient erythroporphyria of infancy. In some embodiments, the disorder is an acute hepatic porphyria, *e.g.*, ALA deyhdratase deficiency porphyria (ADP), AIP, HCP, or VP. In specific embodiments, the disorder is ALA deyhdratase deficiency porphyria (ADP) or AIP.

In embodiments, the porphyria is a hepatic porphyria, e.g., a porphyria selected from acute intermittent porphyria (AIP) hereditary coproporphyria (HCP), variegate porphyria (VP), ALA deyhdratase deficiency porphyria (ADP), and hepatoerythropoietic porphyria. In embodiments, the porphyria is a homozygous dominant hepatic porphyria (e.g., homozygous dominant AIP, HCP, or VP) or hepatoerythropoietic porphyria, In embodiments, the porphyria is a dual porphyria.

In one embodiment, the dsRNA introduced reduces or inhibits expression of an ALAS1 gene in the cell.

In one embodiment, the dsRNA introduced reduces or inhibits expression of an ALAS1 gene, or the level of one or more porphyrins or porphyrin precursors (e.g., δ-aminolevulinic acid (ALA), porphopilinogen (PBG), hydroxymethylbilane (HMB), uroporphyrinogen I or III, coproporphyrinogen I or III, protoporphrinogen IX, and protoporphyrin IX) or porphyrin products or metabolites, by at least 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50% or more compared to a reference, (*e.g.*, an untreated cell or a cell treated with a non-targeting control dsRNA). Without being bound by theory, ALAS1 is the first enzyme of the porphyrin pathway. Thus, reducing expression of the ALAS1 gene is likely to reduce the level of one or more porphyrin precursors, porphyrins or porphyrin products or metabolites.

In other aspects, the invention provides methods for treating, preventing or managing pathological processes related to ALAS1 expression (*e.g.*, pathological processes involving porphyrins, porphyrin precuorsors, or defects in the porphyrin pathway, such as, for example, porphyrias). In one embodiment, the method includes administering to a subject, *e.g.,* a patient in need of such treatment, prevention or management, an effective (*e.g.*, a therapeutically or prophylactically effective) amount of one or more of the iRNAs featured herein.

In an aspect provided herein is a method of treating and/or preventing a disorder related to ALAS1 expression comprising administering to a subject in need of such treatment a therapeutically effective amount of an iRNA (e.g., a dsRNA) described herein, or a composition comprising an iRNA (e.g., a dsRNA) described herein.

In an aspect provided herein is a method of treating and/or preventing a porphyria comprising administering to a subject in need of such treatment a double-stranded ribonucleic acid (dsRNA), wherein said dsRNA comprises a sense strand and an antisense strand 15-30 base

pairs in length and the antisense strand is complementary to at least 15 contiguous nucleotides of SEQ ID NO:1 or SEQ ID NO:382.

In one embodiment, subject (e.g., the patient) has a porphyria. In another embodiment, the subject (e.g., patient) is at risk for developing a porphyria. In some embodiments, administration of the iRNA targeting ALAS1 alleviates or relieves the severity of at least one symptom of a disorder related to ALAS1 in the patient.

In one embodiment, the subject is a mammal (*e.g.*, a human) at risk, or that has been diagnosed with, a disorder related to ALAS1 expression, e.g., a porphyria, *e.g.*, X-linked sideroblastic anemia (XLSA), ALA deyhdratase deficiency porphyria (Doss porphyria), acute intermittent porphyria (AIP), congenital erythropoietic porphyria (CEP), prophyria cutanea tarda (PCT), hereditary coproporphyria (coproporphyria, or HCP), variegate porphyria (VP), erythropoietic protoporphyria (EPP), or transient erythroporphyria of infancy. In a further embodiment, the porphyria is an acute hepatic porphyria, *e.g.*, ALA deyhdratase deficiency porphyria (ADP), AIP, HCP, or VP. In some such embodiments, the disorder is ALA deyhdratase deficiency porphyria (ADP) or AIP.

In embodiments the subject has, or is at risk for developing, a porphyria. In embodiments, the porphyria is a hepatic porphyria, e.g., a porphyria selected from acute intermittent porphyria (AIP) hereditary coproporphyria (HCP), variegate porphyria (VP), ALA deyhdratase deficiency porphyria (ADP), and hepatoerythropoietic porphyria. In embodiments, the porphyria is a homozygous dominant hepatic porphyria (e.g., homozygous dominant AIP, HCP, or VP) or hepatoerythropoietic porphyria, In embodiments, the porphyria is a dual porphyria.

In embodiments, a porphyria, a symptom of porphyria, a prodrome, or an attack of porphyria is induced by exposure to a precipitating factor, as described herein. In some embodiments, the precipitating factor is a chemical exposure. In some embodiments, the precipitating factor is a drug, e.g., a prescription drug or an over the counter drug. In some embodiments, the precipitating factor is the menstrual cycle, e.g., a particular phase of the menstrual cycle, e.g., the luteal phase.

In embodiments, the iRNA (e.g., dsRNA) or composition comprising the iRNA is administered after an acute attack of porphyria.

In embodiments, the iRNA (e.g., dsRNA) or composition comprising the iRNA is administered during an acute attack of porphyria.

In embodiments, the iRNA (e.g., dsRNA) or composition comprising the iRNA is administered prophylactically to prevent an acute attack of porphyria.

In embodiments, the iRNA (e.g., dsRNA) is formulated as an LNP formulation.

In emtodiments, the iRNA (e.g., dsRNA) is in the form of a GalNAc conjugate.

In embodiments, iRNA (e.g., dsRNA) is administered at a dose of 0.05-50 mg/kg.

In embodiments, the iRNA (e.g., dsRNA) is administered at a concentration of 0.01 mg/kg-5 mg/kg bodyweight of the subject.

In embodiments, the iRNA (e.g., dsRNA) is formulated as an LNP formulation and is administered at a dose of 0.05-5 mg/kg.

In embodiments, the iRNA (e.g., dsRNA) is in the form of a GalNAc conjugate and is administered at a dose of 0.5-50 mg/kg. In certain embodiments, the iRNA in the GalNAc conjugate is administered at a dose of less than 10 mg/kg (e.g., 5 mg/kg or less) e.g., once per week; e.g., a dose of 1 mg/kg or less, 2.5 mg/kg or less, or 5 mg/kg or less, e.g., once per week. In one embodiment, iRNA in the GalNAc conjugate is administered at a dose of about 2.5 mg/kg or less, e.g., once per week. In one embodiment, the administration of the iRNA in the GalNAc conjugate is subcutaneous.

In embodiments, the iRNA (e.g., dsRNA) is in the form of a GalNAc conjugate and is administered, e.g., subcutaneously, at a dose of 0-5 mg/kg, e.g. 0-2.5 mg/kg or 1-2.5 mg/kg. In embodiments, the iRNA is administered weekly. In embodiments, the iRNA is administered as a composition comprising the iRNA and water for injection. In embodiments, the iRNA is AD-60519. In embodiments, the composition comprises the iRNA, e.g., AD-60519, at a concentration of about 200 mg/mL.

In embodiments, the method decreases a level of a porphyrin or a porphyrin precursor in the subject.

In embodiments, the level is decreased by at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, or 90%. In an embodiment, the level is decreased by at least 30%.

In embodiments, the porphyrin precursor is δ-aminolevulinic acid (ALA) or porphopilinogen (PBG).

In embodiments, the iRNA (e.g., dsRNA) has an IC_{50} in the range of 0.01-1nM.

In embodiments, a method described herein

(i) ameliorates a symptom associated with an ALAS1 related disorder (e.g., a porphyria)

(ii) inhibits ALAS1 expression in the subject (e.g., as assessed using the cERD assay),

(iii) decreases a level of a porphyrin precursor (e.g., ALA or PBG) or a porphyrin in the subject,

(iv) decreases frequency of acute attacks of symptoms associated with a porphyria in the subject, or

(v) decreases incidence of acute attacks of symptoms associated with a porphyria in the subject when the subject is exposed to a precipitating factor (e.g., the premenstrual phase or the luteal phase).

In embodiments, the method ameliorates pain and/or progressive neuropathy.

In embodiments, the iRNA (e.g., dsRNA) or composition comprising the iRNA is administered according to a dosing regimen.

In some embodiments, the iRNA (e.g., dsRNA) or composition comprising the iRNA is administered before or during an acute attack of porphyria. In some embodiments, the iRNA is administered before an acute attack of porphyria.

In some embodiments, the iRNA (e.g., dsRNA) or composition comprising the iRNA is administered during a prodrome. In embodiments, the prodrome is characterized by abdominal pain, nausea, psychological symptoms (e.g., anxiety), restlessness and/or insomnia.

In embodiments, the iRNA (e.g., dsRNA) or composition comprising the iRNA is administered during a particular phase of the menstrual cycle, e.g., during the luteal phase.

In embodiments, the method ameliorates or prevents cyclical attacks of porphyria, e.g., by reducing the severity, duration, or frequency of attacks. In embodiments, the cyclical attacks are associated with a precipitating factor. In embodiments, the precipitating factor is the menstrual cycle, e.g., a particular phase of the menstrual cycle, e.g., the luteal phase.

In embodiments, the subject has an elevated level of ALA and/or PBG. In embodiments, the level of ALA and/or PBG is elevated in plasma or urine from the subject. In embodiments,

the subject has or is at risk for developing a porphyria, e.g., a hepatic porphyria. In embodiments, the subject is asymptomatic. In embodiments, the subject carries a genetic alteration (e.g., a gene mutation) associated with a porphyria, as described herein. In embodiments, the subject has or is at risk for developing a porphyria and suffers from pain (e.g., chronic pain, e.g., chronic neuropathic pain) and/or neuropathy (e.g., progressive neuropathy). In embodiments, the subject does not suffer from acute attacks but suffers from pain (e.g., chronic pain, e.g., chronic neuropathic pain) and/or neuropathy (e.g., progressive neuropathy). In embodiments, the pain is abdominal pain.

In embodiments, the subject (a) has an elevated level of ALA and/or PBG and (b) suffers from pain (e.g., chronic pain, e.g., chronic neuropathic pain) and/or neuropathy (e.g., progressive neuropathy). In embodiments, the pain is abdominal pain.

In embodiments, the subject has a plasma level and/ or a urine level of ALA and/or PBG that is elevated. In embodiments, the elevated level of ALA and/or PBG is accompanied by other symptoms, e.g., pain (e.g., chronic pain, e.g., chronic neuropathic pain) or neuropathy (e.g., progressive neuropathy). In embodiments, the pain is abdominal pain. In embodiments, the subject is asymptomatic. In embodiments, the subject has a genetic mutation associated with a porphyria, e.g., a mutation as described herein.

In embodiments, the subject has a level (e.g., a plasma level or a urine level) of a porphyrin precursor, e.g., ALA and/or PBG, that is elevated, e.g., the level is greater than, or greater than or equal to, a reference value. In embodiments, the level is greater than the reference value. In embodiments, the reference value is two standard deviations above the mean level in a sample of healthy individuals. In embodiments, the reference value is an upper reference limit.

In embodiments, the subject has a plasma level and/or a urine level of ALA and/or PBG that is greater than, or greater than or or equal to, 2 times, 3 times, 4 times, or 5 times that of an upper reference limit. As used herein, an "upper reference limit" refers to a level that is the upper limit of the 95% confidence interval for a reference sample, e.g., a sample of normal (e.g., wild type) or healthy individuals, e.g., individuals who do not carry a genetic mutation associated with a porphyria and/or individuals who do not suffer from a porphyria. In embodiments, the subject has a urine level of ALA and/or PBG that is greater than 2 to 4 times that of an upper

reference limit. In embodiments, the subject has a urine level of ALA and/or PBG that is greater than 4 times that of an upper reference limit.

In embodiments, the reference value for plasma PBG is 0.12 μmol/L. In embodiments, the subject is a human and has a plasma PBG level that is greater than, or greater than or equal to, 0.12 μmol/L, 0.24 μmol/L, 0.36 μmol/L, 0.48 μmol/L, or 0.60 μmol/L. In embodiments, the subject is a human and has a plasma level of PBG that is greater than, or greater than or equal to, 0.48 μmol/L.

In embodiments, the reference value for urine PBG is 1.2 mmol/mol creatinine. In embodiments, the subject is a human and has a urine PBG level that is greater than, or greater than or equal to, 1.2 mmol/mol creatinine, 2.4 mmol/mol creatinine, 3.6 mmol/mol creatinine, 4.8 mmol/mol creatinine, or 6.0 mmol/mol creatinine. In embodiments, the subject is a human and has a urine level of PBG that is greater than, or greater than or equal to, 4.8 mmol/mol creatinine.

In embodiments, the reference value for plasma ALA is 0.12 μmol/L. In embodiments, the subject is a human and has a plasma ALA level that is greater than, or greater than or equal to, 0.12 μmol/L, 0.24 μmol/L, 0.36 μmol/L, 0.48 μmol/L, or 0.60 μmol/L. In embodiments, the subject is a human and has a plasma ALA level that is greater than, or greater than or equal to 0.48 μmol/L.

In embodiments, the reference value for urine ALA is 3.1 mmol/mol creatinine. In embodiments, the subject is a human and has a urine ALA level that is greater than, or greater than or equal to, 3.1 mmol/mol creatinine, 6.2 mmol/mol creatinine, 9.3 mmol/mol creatinine, 12.4 mmol/mol creatinine, or 15.5 mmol/mol creatinine.

In embodiments, the method decreases one or more signs or symptoms of a porphyria. In embodiments, the method decreases an elevated level of ALA and/or PBG. In embodiments, the method decreases pain (e.g., chronic pain, e.g. chronic neuropathic pain) and/or neuropathy (e.g., progressive neuropathy). In embodiments, the pain is abdominal pain. In embodiments, the pain is neuropathic pain (e.g., pain associated with the progressive neuropathy of acute porphyrias). The decrease in pain can include, e.g., prevention of pain, delay in the onset of pain, reduction in the frequency of pain, and/or reduction in severity of pain. In embodiments, the decrease in pain is assessed based on the subject's use of pain medication.

In embodiments, the method ameliorates or prevents acute attacks of porphyria, e.g., by reducing the severity, duration, or frequency of attacks.

In embodiments, the method decreases or prevents nerve damage.

In embodiments, the method prevents deterioration (e.g., prevents development of abnormalities) of or results in an improvement of clinical measures, e.g., clinical measures of muscle and/or nerve function, e.g., EMG and/or nerve conduction velocities.

In embodiments, the method decreases heme use by the subject.

In embodiments, the method decreases pain medication use by the subject.

In embodiments, the method reduces hospitalization.

In embodiments, the method is effective to reduce a level of ALA and/or PBG (e.g., a plasma or urine level of ALA and/or PBG). In embodiments, the method is effective to produce a predetermined reduction in the elevated level of ALA and/or PBG.

In embodiments, the predetermined reduction is a reduction to a value that is less than or equal to a reference value. In some embodiments, the reference value is an upper reference limit. In some embodiments, the reference value is the value that is two standard deviations above the mean level in a reference sample.

In embodiments, the method is effective to reduce the level of ALA and/or PBG in a subject to a level that is below two times the upper reference limit. In embodiments, the method is effective to reduce the level of ALA to below two times the upper reference limit. In embodiments, the method is effective to reduce the level of PBG to below two times the upper reference limit.

In embodiments, the iRNA (e.g., dsRNA) or composition comprising the iRNA is administered as a single dose or at multiple doses, *e.g.*, according to a dosing regimen.

In embodiments, the iRNA (e.g., dsRNA) or composition comprising the iRNA is administered prophylactically to a subject who is at risk for developing a porphyria. In embodiments, the iRNA (e.g., dsRNA) or composition comprising the iRNA is administered prophylactically beginning at puberty. In embodiments, the subject carries a genetic mutation associated with a porphyria and/or has an elevated level of ALA and/or PBG (e.g., an elevated plasma or urine level of ALA and/or PBG). In embodiments, the mutation makes an individual susceptible to an acute attack (e.g., upon exposure to a precipitating factor, e.g., a drug, dieting or

other precipitating factor, e.g., a precipitating factor as disclosed herein). In embodiments, the mutation is associated with elevated levels of a porphyrin or a porphyrin precursor (e.g., ALA and/or PBG). In embodiments, the mutation is associated with chronic pain (e.g., chronic neuropathic pain) and/or neuropathy (e.g., progressive neuropathy).

In embodiments, the mutation is a mutation in the ALAS1 gene. In embodiments, the mutation is a mutation in the ALAS1 gene promoter, or in regions upstream or downstream from the ALAS1 gene. In embodiments, the mutation is a mutation in transcription factors or other genes that interact with ALAS1. In embodiments, the mutation is a mutation in a gene that encodes an enzyme in the heme biosynthetic pathway.

In embodiments, the iRNA (e.g., dsRNA or a conjugate thereof) or composition comprising the iRNA is administered subcutaneously. In embodiments, the iRNA is in the form of a GalNAc conjugate. In embodiments, the iRNA (e.g., the dsRNA) is administered at a dose of 0.5-50 mg/kg. In certain embodiments, the iRNA is administered at a dose of less than 10 mg/kg (e.g., 5 mg/kg or less) once per week; e.g., a dose of 1 mg/kg or less, 2.5 mg/kg or less, or 5 mg/kg or less, e.g., once per week. In one embodiment, iRNA is administered at a dose of about 2.5 mg/kg or less, e.g., once per week.

In embodiments, the subject to be treated is asymptomatic and has an elevated level of ALA and/or PBG. In embodiments, the subject has a porphyria, e.g., AIP. In embodiments, the patient suffers from recurrent porphyric attacks.

In embodiments, the iRNA (*e.g.*, AD-60519) is administered at a dose of less than 5 mg/kg, *e.g.*, at 0.1, 0.35, 1.0, or 2.5 mg/kg. In embodiments, the iRNA (*e.g.*, AD-60519) is administered in repeated doses, e.g., weekly doses.

In one embodiment, the subject is asymptomatic and has an elevated level of ALA and/or PBG, and the iRNA (*e.g.*, AD-60519) is administered at single doses, *e.g.*, at 0.1, 0.35, 1.0, or 2.5 mg/kg; or in repeatedly weekly dosages, *e.g.*, of 1 and 2.5 mg/kg for several weeks (e.g., for 4 weeks).

In one embodiment, the subject has AIP, *e.g.*, is an AIP patient, the iRNA (*e.g.*, AD-60519) is administered at a dose of 1-2.5 mg/kg weekly.

In embodiments, a treatment regimen is employed in which the iRNA is initially administered more frequently, followed by less frequent administration. In embodiments, the

iRNA is initially administered once per day for multiple days (e.g., for 2-14 days, e.g., for 2, 3, 4, 5, 6, or 7 days). In embodiments, the iRNA is subsequently administered once per week. In embodiments, the iRNA is subsequently administered once every two weeks. In embodiments, the iRNA is subsequently administered at a frequency that is effective to reduce one or more signs or symptoms of a porphyria.

In one aspect provided herein is a method of treating a subject with an elevated level of ALA and/or PBG, the method comprising administering to the subject a double-stranded ribonucleic acid (dsRNA), wherein said dsRNA comprises a sense strand and an antisense strand 15-30 base pairs in length and the antisense strand is complementary to at least 15 contiguous nucleotides of SEQ ID NO:1 or SEQ ID NO:382.

In one aspect provided herein is a method of treating a subject with an elevated level of ALA and/or PBG, the method comprising administering to the subject a therapeutically effective amount of an dsRNA or a composition comprising a dsRNA, as described herein.

In some embodiments, the methods described herein are effective to decrease the level of ALA and/or PBG. In some embodiments, the level of ALA and/or PBG is decreased such that it is less than, or less than or equal to, a reference value, e.g., an upper reference limit.

In embodiments, the subject to be treated is asymptomatic and has an elevated level of ALA and/or PBG. In embodiments, the subject has a porphyria, e.g., AIP.

In embodiments, the iRNA is administered at a dose of less than 5 mg/kg, e.g., at 0.1, 0.35 1.0, or 2.5 mg/kg. In embodiments, the iRNA is administered in repeated doses, e.g., weekly doses.

In another aspect, the invention provides methods for decreasing a level of a porphyrin or a porphyrin precursor in a cell (*e.g.*, an erythroid cell or a liver cell, such as, *e.g.*, a hepatocyte). In one embodiment, the cell is treated *ex vivo*, *in vitro*, or *in vivo* (*e.g.*, the cell is present in a subject (*e.g.*, a patient in need of treatment, prevention and/or management of a disorder related to ALAS1 expression). The method includes contacting the cell with an effective amount of one or more of the iRNAs targeting ALAS1, *e.g.*, one or more of the iRNAs disclosed herein, thereby decreasing the level of a porphyrin or a porphyrin precursor in the cell; or decreasing the level of a porphyrin or a porphyrin precursor in other cells, tissues, or fluids within a subject in which the

cell is located; relative to the level prior to contacting. Such methods can be used to treat (*e.g.*, ameliorate the severity) of disorders related to ALAS1 expression, such as porphyrias, e.g., AIP or ALA dehydratase deficiency porphyria.

In one embodiment, the contacting step is effected *ex vivo*, *in vitro*, or *in vivo*. For example, the cell can be present in a subject, *e.g.*, a mammal (*e.g.*, a human) at risk, or that has been diagnosed with, a porphyria. In an embodiment, the porphyria is an acute hepatic porphyria. In embodiments, the porphyria is a hepatic porphyria, e.g., a porphyria selected from acute intermittent porphyria (AIP), hereditary coproporphyria (HCP), variegate porphyria (VP), ALA deyhdratase deficiency porphyria (ADP), and hepatoerythropoietic porphyria. In embodiments, the porphyria is a homozygous dominant hepatic porphyria (e.g., homozygous dominant AIP, HCP, or VP) or hepatoerythropoietic porphyria, In embodiments, the porphyria is a dual porphyria.

In an aspect provided herein is a method for decreasing a level of a porphyrin or a porphyrin precursor (e.g., ALA or PBG) in a cell, comprising contacting the cell with an iRNA (e.g. a dsRNA), as described herein, in an amount effective to decrease the level of the porphyrin or the porphyrin precursor in the cell.

In embodiments, the cell is a hepatocyte. In embodiments, the porphyrin or porphyrin precursor is δ-aminolevulinic acid (ALA), porphopilinogen (PBG), hydroxymethylbilane (HMB), uroporphyrinogen I or III, coproporphyrinogen I or III, protoporphrinogen IX, or protoporphyrin IX. In embodiments, the porphyrin precursor is ALA or PBG.

In one embodiment, the cell is an erythroid cell. In a further embodiment, the cell is a liver cell (*e.g.*, a hepatocyte).

In an aspect provided herein is a vector encoding at least one strand of an iRNA (e.g., a dsRNA) as described herein.

In an aspect provided herein is 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 ALAS1, wherein said dsRNA is 30 base pairs or less in length, and wherein said dsRNA targets said mRNA for cleavage.

In embodiments, the region of complementarity is at least 15 nucleotides in length.

In embodiments, the region of complementarity is 19 to 21 nucleotides in length.In one aspect, the invention provides a vector for inhibiting the expression of an ALAS1 gene in a cell. In one embodiment, the vector comprises an iRNA as described herein. 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 one embodiment the vector comprises at least one strand of an ALAS1 iRNA.

In an aspect provided herein is a cell comprising a vector as described herein.In an aspect provided herein is a cell containing a vector for inhibiting the expression of an ALAS1 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 one embodiment, the cell is a liver cell (*e.g.*, a hepatocyte). In another embodiment, the cell is an erythroid cell.

In another aspect, a method is provided for assaying the level of circulating extracellular ALAS1 mRNA in a subject, said method comprising detecting (*e.g.*, measuring) the level of ALAS1 mRNA in a biological fluid sample (*e.g.*, a blood sample (e.g., a serum or plasma sample), a cerebrospinal fluid sample, or a urine from the subject, said biological fluid sample comprising the ALAS1 mRNA, thereby assaying the level of circulating extracellular ALAS1 mRNA in the subject.

In another aspect, a method is provided for assaying the level of circulating extracellular ALAS1 mRNA in a subject, said method comprising (i) providing RNA (*e.g.*, extracellular RNA) from a biological fluid sample (e.g., blood or plasma sample) from the subject, said biological fluid sample comprising the ALAS1 mRNA; (ii) obtaining an ALAS1 cDNA from the ALAS1 mRNA; (iii) contacting the ALAS1 cDNA with a nucleic acid complementary (e.g., probe and/or primer) to the ALAS1 cDNA or a portion thereof, thereby producing a reaction mix; and (iv) detecting (e.g., measuring) the level of ALAS1 cDNA in the reaction mix, wherein the ALAS1 cDNA level is indicative of the ALAS1 mRNA level, thereby assaying the level of circulating extracellular ALAS1 mRNA in the subject.

In embodiments, said biological fluid sample is a blood sample. In embodiments, said biological fluid sample is a serum sample. In embodiments, said biological fluid sample is a urine sample.

In embodiments, the the method comprises PCR, qPCR or 5'-RACE.

In embodiments, said nucleic acid is a probe or primer.

In embodiments, said nucleic acid comprises a detectable moiety and the level of ALAS1 mRNA is determined by detection of the amount of the detectable moiety.

In embodiments, said method further comprises obtaining the biological fluid sample from the subject. In embodiments, the biological fluid sample is separate from the tissue and contains exosomes. In embodiments of these methods, the efficacy of a porphyria treatment is assessed based on a comparison of the level of circulating extracellular ALAS1 mRNA in the subject relative to a reference value.

In embodiments, a decrease in the level of circulating extracellular ALAS1 mRNA in the subject in response to the porphyria treatment, relative to the reference value, indicates that the porphyria treatment is efficacious. In embodiments, the reference value is the level of circulating extracellular ALAS1 mRNA in the subject prior to the porphyria treatment.

All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety.

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 depicts the heme biosynthetic pathway.

FIG. 2A and FIG. 2B show a table summarizing certain porphyrias associated with genetic errors in heme metabolism.

FIG. 3A and FIG. 3B depict a human ALAS1 mRNA sequence transcript (Ref. Seq. NM_000688.4 (GI:40316942, record dated November 19, 2011), SEQ ID NO: 1).

FIG. 4A and FIG. 4B depict a human ALAS1 mRNA sequence transcript (Ref. Seq. NM_000688.5 (GI: 362999011, record dated April 1, 2012), SEQ ID NO: 382).

FIG. 5 shows the dose-response of the siRNA AD-53558 in suppressing mouse ALAS1 (mALAS1) mRNA relative to a PBS control. Results for a luciferase (LUC) AD-1955 control are also shown.

FIG. 6 shows the dose-response of the siRNA AD-53558 in suppressing ALAS1 mRNA in rats relative to a PBS control. Results for a luciferase (LUC) AD-1955 control are also shown.

FIG. 7 shows the durability of suppression of mouse ALAS1 (mALAS1) mRNA by the siRNA AD-53558 relative to a PBS control.

FIG. 8 shows means ± standard deviations of plasma ALA levels (in μM) at baseline, and after phenobarbital treatment in the experimental (ALAS1 siRNA) and control (LUC siRNA) groups.

FIG. 9 shows the plasma ALA levels (in μM) of individual animals at baseline, and after phenobarbital treatment in animals that received ALAS1 siRNA and control (LUC siRNA) treatment.

FIG. 10 shows means ± standard deviations of plasma PBG levels (in μM) at baseline, and after phenobarbital treatment in animals that received ALAS1 siRNA and control (LUC siRNA) treatment.

FIG. 11 shows the plasma PBG levels (in μM) of individual animals at baseline, and after phenobarbital treatment in animals that received ALAS1 siRNA and control (LUC siRNA) treatment.

FIG. 12 shows the relative mALAS1mRNA level in liver at baseline, and after phenobarbital treatment in select representative experimental (ALAS1 siRNA) and control (PBS) animals.

FIG. 13 shows the effects of three GalNAc conjugated mALAS1 siRNAs on mALAS1 expression (relative to a PBS control) in mouse liver tissue.

FIG. 14 shows plasma ALA and PBG levels over time after phenobarbital administration and treatment with ALAS1 siRNA or control LUC siRNA.

FIG. 15 shows the effects of a GalNAc conjugated ALAS1 siRNA on plasma ALA and plasma PBG levels in the mouse AIP phenobarbital induction model.

FIG. 16 shows dose-dependent effects of an ALAS1 siRNA on plasma ALA and PBG levels in the mouse AIP phenobarbital induction model. For the animals that received ALAS1 siRNA, the dose of siRNA administered (0.05 mg/kg, 0.1 mg/kg, 0.5 mg/kg, or 1.0 mg/kg) is shown on the horizontal axis.

FIG. 17, top panel shows the experimental design used to study suppression of ALA and PBG with an ALAS1 siRNA. The bottom panel shows the plasma ALA and PBG levels at baseline, in the control (Luc) condition, and following treatment with an ALAS1 siRNA at week 0, week 2, and week 4.

FIG. 18 shows the experimental design used to compare the effects of treatment with ALAS1 siRNA or hemin in an animal model of AIP (top) and the results for plasma ALA (μmol/L) levels (middle) and plasma PBG (μmol/L) levels (bottom).

FIG. 19 shows relative mRNA levels (ALAS1/GAPDH) in animals treated with 30 mg/kg, 10 mg/kg, or 3 mg/kg of AD-58632 compared with animals treated with PBS control.

FIG. 20 shows the experimental design used to investigate the dose response effect of the AD-58632 ALAS1 GalNAc conjugate in a rat AIP model.

FIG. 21 shows relative levels of liver PBGD mRNA (top graph) and relative levels of liver ALAS1 mRNA (bottom graph) in a rat model of AIP. Groups of animals were subjected to one of four treatments: (1) phenobarbital (PB) treatment only, (2) phenobarbital and porphobilinogen deaminase (PBGD) siRNA treatment, (3) phenobarbital, PBGD siRNA, and 30 mg/kg of ALAS1 siRNA, (4) phenobarbital, PBGD siRNA, and 10 mg/kg of ALAS1 siRNA.

FIG. 22 shows urinary PBG (top panel) and ALA (bottom panel) levels relative to creatinine levels in a rat model of AIP. Groups of animals were subjected to one of four treatments: (1) phenobarbital (PB) treatment only, (2) phenobarbital and porphobilinogen deaminase (PBGD) siRNA treatment, (3) phenobarbital, PBGD siRNA, and 30 mg/kg of ALAS1 siRNA, (4) phenobarbital, PBGD siRNA, and 10 mg/kg of ALAS1 siRNA.

FIG. 23 shows the suppression of ALAS-1 mRNA by AD-58632, compared with PBS control, in groups of rats that received five daily doses of siRNA at 6 mg/kg, 2 mg/kg, or 1 mg/kg versus single bolus doses of siRNA at 30 mg/kg, 10 mg/kg, or 5 mg/kg.

FIG. 24 shows the suppression of ALAS-1 mRNA by AD-58632, compared with PBS control, in groups of rats that received four weekly doses of siRNA at 10 mg/kg, 5 mg/kg, or 2.5 mg/kg.

FIG. 25 shows the suppression of ALAS-1 mRNA by AD-58632 and by five 19/19mer duplexes.

FIG. 26 shows the results of an evaluation of the effect of strand length and overhangs on the best two 19mers.

FIG. 27 is a graph that shows the levels of ALAS1 mRNA in liver (left bars) and in serum (right bars) for each treatment group in the NHP study described in Example 34.

FIG. 28 shows the suppression of ALAS-1 mRNA, compared with PBS control, in groups of rats that received 3 mg/kg or 10 mg/kg of AD-58632 or AD-60489.

FIG. 29 shows the experimental design used to investigate the effectiveness of ALAS1 siRNAs AD-58632 and AD-60489 in suppressing liver mRNA in non-human primates.

FIG. 30 shows the dose-dependent suppression of liver mRNA in non-human primates following treatment with 1.25 mg/kg, 2.5 mg/kg, or 5 mg/kg of AD-58632 or AD-60489.

FIG. 31 shows a comparison of the mRNA suppression results obtained from liver biopsies and from the cERD assay in a non-human primate study.

FIG. 32 shows the time course of suppression of mRNA as assessed using the cERD assay in a non-human primate study. The horizontal axis shows the time according to the study day.

FIG. 33 shows the suppression of ALAS1 mRNA in rats that received PBS or a single dose of 5 mg/kg of one of the indicated siRNA duplexes.

FIG. 34 shows the liver concentrations of the siRNA in rats that received a single dose of 5 mg/kg of the indicated siRNA.

FIG. 35 (top) shows the experimental design used to investigate the therapeutic efficacy of AD-60925 and AD-60926. FIG. 35(bottom) shows the relative levels of rat ALAS1/GAPDH mRNA in rats treated with (1) AF11-PBGD, (2) AF11-PBGD and PB, (3) AF-11PBGD, PB, and 3 mg/kg AD-60925, or (4) AF11-PBGD, PB, and AD-60926.

FIG. 36 shows the relative levels of urine PBG (top) and urine ALA (bottom) in rats treated with (1) AF11-PBGD, (2) AF11-PBGD and PB, (3) AF-11PBGD, PB, and 3 mg/kg AD-60925, or (4) AF11-PBGD, PB, and AD-60926.

FIG. 37 shows the relative levels of urine PBG (top) and urine ALA (bottom) over time in rats treated with (1) AF11-PBGD, (2) AF11-PBGD and PB, (3) AF-11PBGD, PB, and 3 mg/kg AD-60925, or (4) AF11-PBGD, PB, and AD-60926. The arrows indicate the timepoints when PB was administered.

FIG. 38 shows the relative levels of rat ALAS1 (rALAS1) mRNA in rats that received 4 doses of PBS or 2.5 mg/kg of one of the indicated siRNAs.

FIG. 39 shows the relative levels of rat ALAS1 (rALAS1) mRNA in rats that received a single dose of PBS or 2.5 mg/kg of one of the indicated siRNAs.

FIG. 40 (top) shows the relative levels of rat ALAS1 (rALAS1) mRNA in rats that received a single dose of PBS or 3 mg/kg of one of the indicated siRNAs. FIG. 40 (bottom) shows the concentration of siRNA in liver.

FIG. 41 (top) shows the suppression of rat ALAS1 (rALAS1) mRNA by AD-60489, AD-60519, and AD-60901. FIG. 41 (bottom) shows the concentration of siRNA in liver.

FIG. 42 shows the relative levels of rat ALAS1 (rALAS1) mRNA in rats that were treated with a single dose of PBS or 2.5 mg/kg of one of the indicated siRNAs.

FIG. 43 shows the relative levels of rat ALAS1 (rALAS1) mRNA in rats that were treated with PBS or one of the indicated siRNAs at a dose of 2.5 mg/kg twice per week for 2 weeks.

FIG. 44 (top) shows a schematic of the experimental design used to investigate the therapeutic efficacy of multiple biweekly doses of AD-60519. FIG. 44 (bottom) shows graphs depicting the suppression of urine PBG and urine ALA in rats that were treated with (i) PBGD siRNA and six doses of PBS, (ii) PBGD siRNA, PB, and six doses of PBS, (iii) PBGD siRNA, PB, and six doses of 2.5 mg/kg AD-60519, or (iv) PBGD siRNA, PB, and six doses of 5 mg/kg AD-60519.

FIG. 45 shows graphs depicting the suppression of serum PBG (upper graph) and serum ALA (lower graph) in a mouse AIP model that were treated with (i) PBGD siRNA and six doses of PBS (Baseline), (ii) PBGD siRNA, PB, and six doses of PBS (Saline), (iii) PBGD siRNA, PB, and six doses of 2.5 mg/kg AD-60519, or (iv) PBGD siRNA, PB, and six doses of 5 mg/kg AD-60519.

FIG. 46 (top) shows a schematic of the experimental design used to investigate the therapeutic efficacy of multiple weekly doses of AD-60519. FIG. 46 (bottom) shows a graph depicting the relative levels of rat ALAS1 mRNA (rALAS1/GAPDH) in rats that were treated with (i) PBGD siRNA and four doses of PBS, (ii) PBGD siRNA, PB, and four doses of PBS, (iii)

PBGD siRNA, PB, and four doses of 3 mg/kg AD-60519, (iv) PBGD siRNA, PB, and four doses of 1 mg/kg AD-60519, or (v) PBGD siRNA, PB, and four doses of 0.3 mg/kg AD-60519.

FIG. 47 shows graphs depicting the levels of urine PBG (upper graph) and urine ALA (lower graph) in rats that were treated with (i) PBGD siRNA and four doses of PBS, (ii) PBGD siRNA, PB, and four doses of PBS, (iii) PBGD siRNA, PB, and four doses of 3 mg/kg AD-60519, (iv) PBGD siRNA, PB, and four doses of 1 mg/kg AD-60519, or (v) PBGD siRNA, PB, and four doses of 0.3 mg/kg AD-60519.

FIG. 48 is a schematic that shows the design of a non-human primate study in which effects of ALAS1 siRNA GalNAc conjugates in suppressing liver ALAS1 mRNA and circulating ALAS1 mRNA were investigated

FIG. 49 is a graph that shows suppression of liver mRNA in non-human primates (NHPs) following treatment with ALAS1 siRNA GalNAc conjugates.

FIG. 50 is a graph that shows the normalized serum levels of ALAS1 mRNA in non-human primates (NHPs) at various times during the course of a study in which effects of treatment with ALAS1 siRNA GalNAc conjugates was investigated. The days on the horizontal axis correspond to the days in the schematic in FIG. 48.

FIG. 51 shows the normalized ALAS1 mRNA levels (shown as a fraction of the pre-dose level) as assessed in a rat single dose study that used urine cERD to monitor ALAS1 suppression.

FIG. 52 is a schematic that shows the design of a non-human primate study in which multidose and single dose effects of AD-60519 in suppressing liver ALAS1 mRNA and circulating ALAS1 mRNA were investigated.

FIG. 53 is a bar graph that shows the average relative liver ALAS1 mRNA levels (% of PBS control) at study day 24 (multidose groups) or at study day 4 (single dose groups). .

FIG. 54 is a graph that shows normalized serum ALAS1 mRNA levels (shown as a fraction of the pre-dose level) as assessed using cERD for the multidose groups (top graph, showing results up to day 24) and single dose groups (bottom graph, showing results up to day 22).

FIG. 55 is a graph that shows the liver mRNA, serum mRNA, and urine mRNA levels at study day 4 (in the single dose groups) or at study day 24 (in the multidose groups). Data for individual animals and the averages for each group are shown.

FIG. 56 is a graph that shows normalized serum ALAS1 mRNA levels (shown as a fraction of the pre-dose level) after 8 weeks as assessed using cERD for the multidose groups. Each graphical data point represents the remaining ALAS1 mRNA for the group average of 3 animal samples ± the standard deviation of the group.

FIG. 57 is a schematic of the structure of ALN-60519 (also referred to herein as AD-60519). FIG. 57 discloses SEQ ID NOS 5238-5239, respectively, in order of appearance.

FIG. 58 shows ALAS1 mRNA levels as assessed in matching serum or urine samples obtained from either AIP patients or normal healthy volunteers (NHV). ALAS1 mRNA levels in serum or urine were measured using the cERD method. In AIP patients A and B, a second set of serum and urine samples were collected to access ALAS1 mRNA variability over time.

Detailed Description of the Invention

iRNA directs the sequence-specific degradation of mRNA through a process known as RNA interference (RNAi). Described herein are iRNAs and methods of using them for inhibiting the expression of an ALAS1 gene in a cell or a mammal where the iRNA targets an ALAS1 gene. Also provided are compositions and methods for disorders related to ALAS1 expression, such as porphyrias (*e.g.*, ALA deyhdratase deficiency porphyria (ADP or Doss porphyria), acute intermittent porphyria, congenital erythropoietic porphyria, prophyria cutanea tarda, hereditary coproporphyria (coproporphyria), variegate porphyria, erythropoietic protoporphyria (EPP), X-linked sideroblastic anemia (XLSA), and and transient erythroporphyria of infancy).

Porphyrias are inherited or acquired disorders that can be caused by decreased or enhanced activity of specific enzymes in the heme biosynthetic pathway, also referred to herein as the porphyrin pathway (See FIG. 1). Porphyrins are the main precursors of heme. Porphyrins and porphyrin precursors include δ-aminolevulinic acid (ALA), porphopilinogen (PBG), hydroxymethylbilane (HMB), uroporphyrinogen I or III, coproporphyrinogen I or III, protoporphrinogen IX, and protoporphyrin IX. Heme is an essential part of hemoglobin,

myoglobin, catalases, peroxidases, and cytochromes, the latter including the respiratory and P450 liver cytochromes. Heme is synthesized in most or all human cells. About 85% of heme is made in erythroid cells, primarily for hemoglobin. Most of the remaining heme is made in the liver, 80% of which is used for the synthesis of cytochromes. Deficiency of specific enzymes in the porphyrin pathway leads to insufficient heme production and also to an accumulation of porphyrin precursors and/or porphyrins, which can be toxic to cell or organ function in high concentrations.

Porphyrias may manifest with neurological complications ("acute"), skin problems ("cutaneous") or both. Porphyrias may be classified by the primary site of the overproduction and accumulation of porphyrins or their precursors. In hepatic porphyrias, porphyrins and porphyrin precursors are overproduced predominantly in the liver, whereas in erythropoietic porphyrias, porphyrins are overproduced in the erythroid cells in the bone. The acute or hepatic porphyrias lead to dysfunction of the nervous system and neurologic manifestations that can affect both the central and peripheral nervous system, resulting in symptoms such as, for example, pain (e.g., abdominal pain and/or chronic neuropathic pain), vomiting, neuropathy (e.g., acute neuropathy, progressive neuropathy), muscle weakness, seizures, mental disturbances (e.g., hallucinations, depression anxiety, paranoia), cardiac arrhythmias, tachycardia, constipation, and diarrhea. The cutaneous or erythropoietic porphyrias primarily affect the skin, causing symptoms such as photosensitivity that can be painful, blisters, necrosis, itching, swelling, and increased hair growth on areas such as the forehead. Subsequent infection of skin lesions can lead to bone and tissue loss, as well as scarring, disfigurement, and loss of digits (e.g., fingers, toes). Most porphyrias are caused by mutations that encode enzymes in the heme biosynthetic pathway. A summary of porphyrias associated with genetic errors in heme metabolism is provided in FIG. 2.

Not all porphyrias are genetic. For example, patients with liver disease may develop porphyria as a result of liver dysfunction, and a transient form of erythroporphria (transient erythroporphyria of infancy) has been described in infancy (see Crawford, R.I. et al, *J Am Acad Dermatol.* 1995 Aug; 33(2 Pt 2):333-6.) Patients with PCT can acquire the deficient activity of uroporphyrinogen decarboxylase (URO-D), due to the formation of a ORO-D enzyme with lower than normal enzymatic activity (see Phillips et al. *Blood*, 98:3179-3185, 2001.)

Acute intermittent porphyria (AIP) (also be referred to as porphobilinogen (PBG) deaminase deficiency, or hydroxymethylbilane synthase (HMBS) deficiency), is the most common type of acute hepatic porphyria. Other types of acute hepatic porphyrias include hereditary coproporphyria (HCP), variegate porphyria (VP), and ALA deyhdratase deficiency porphyria (ADP). Acute hepatic porphyrias are described, e.g., in Balwani, M and Desnick, R.J., *Blood*, 120:4496-4504, 2012.

AIP is typically an autosomal dominant disease that is characterized by a deficiency of the enzyme porphobilinogen deaminase (PBG deaminase); this enzyme is also known as hydroxymethylbilane synthase (HMB synthase or HMBS). PBG deaminase is the third enzyme of the heme biosynthetic pathway (see FIG. 1) and catalyzes the head to tail condensation of four porphobilinogen molecules into the linear tetrapyrrole, hydroxymethylbilane (HMB). Alternatively spliced transcript variants encoding different isoforms of PBG deaminase have been described. Mutations in the PBG deaminase gene are associated with AIP. Such mutations may lead to decreased amounts of PBG deaminase and/or decreased activity of PBG deaminase (affected individuals typically have a ~50% reduction in PBG deaminase activity).

There are at least two different models of the pathophysiology of AIP and other acute hepatic porphyrias (see, *e.g.*, Lin CS-Y et al., Clinical Neurophysiology, 2011; 122:2336-44). According to one model, the decreased heme production resulting from PBG deaminase deficiency causes energy failure and axonal degeneration. According to the other, currently more favored model, the buildup of porphyrin precursors (e.g., ALA and PBG) results in neurotoxicity.

AIP has been found to have a prevalence as high as 1 in 10,000 in certain populations (*e.g.*, in Northern Sweden; see Floderus Y, et al. Clin Genet. 2002;62:288–97). The prevalence in the general population in United States and Europe, excluding the U.K., is estimated to be about 1 in 10,000 to 1 in 20,000. Clinical disease manifests itself in only approximately 10-15% of individuals who carry mutations that are known to be associated with AIP. However, the penetrance is as high as 40% in individuals with certain mutations (*e.g.*, the W198X mutation). AIP is typically latent prior to puberty. Symptoms are more common in females than in males. The prevalence of the disease is probably underestimated due to its incomplete penetrance and long periods of latency. In the United States, it is estimated that there are about 2000 patients

who have suffered at least one attack. It is estimated that there are about 150 active recurrent cases in France, Sweden, the U.K., and Poland; these patients are predominantly young women, with a median age of 30. See, e.g., Elder et al, *J Inherit Metab Dis.*, published online Nov 1, 2012.

AIP affects, for example, the visceral, peripheral, autonomic, and central nervous systems. Symptoms of AIP are variable and include gastrointestinal symptoms (*e.g.*, severe and poorly localized abdominal pain, nausea/vomiting, constipation, diarrhea, ileus), urinary symptoms (dysuria, urinary retention/incontinence, or dark urine, e.g., dark red urine), neurologic symptoms (*e.g.*, sensory neuropathy, motor neuropathy (e.g., affecting the cranial nerves and/or leading to weakness in the arms or legs), seizures, neuropathic pain (e.g., pain associated with progressive neuropathy, e.g., chronic neuropathic pain), neuropsychiatric symptoms (*e.g.*, mental confusion, anxiety, agitation, hallucination, hysteria, delirium, apathy, depression, phobias, psychosis, insomnia, somnolence, coma), autonomic nervous system involvement (resulting *e.g.*, in cardiovascular sysmptoms such as tachycardia, hypertension, and/or arrhythmias, as well as other symptoms, such as, *e.g.*, increased circulating catecholamine levels, sweating, restlessness, and/or tremor), dehydration, and electrolyte abnormalities. The most common symptoms are abdominal pain and tachycardia. Neurological manifestations include motor and autonomic neuropathy and seizures. Patients frequently have chronic neuropathic pain and develop a progressive neuropathy. Patients with recurring attacks often have a prodrome. Permanent paralysis may occur after a severe attack. Recovery from severe attacks that are not promptly treated may take weeks or months. An acute attack may be fatal, for example, due to paralysis of respiratory muscles or cardiovascular failure from electrolyte imbalance. (See, *e.g.*, Thunell S. Hydroxymethylbilane Synthase Deficiency. 2005 Sep 27 [Updated 2011 Sep 1]. In: Pagon RA, Bird TD, Dolan CR, et al., editors. GeneReviews™ [Internet]. Seattle (WA): University of Washington, Seattle; 1993- (hereinafter Thunell (1993)), which is hereby incorporated by reference in its entirety.) Prior to the availability of Hemin treatments, up to 20% of patients with AIP died from the disease.

In individuals who carry genes for AIP, the risk of hepatocellular cancer is increased. In those with recurrent attacks, the risk of hepatocellular cancer is particularly grave: after the age of 50, the risk is nearly 100-fold greater than in the general population.

Attacks of acute porphyria may be precipitated by endogenous or exogenous factors. The mechanisms by which such factors induce attacks may include, for example, increased demand for hepatic P450 enzymes and/or induction of ALAS1 activity in the liver. Increased demand for hepatic P450 enzymes results in decreased hepatic free heme, thereby inducing the synthesis of hepatic ALAS1.

Precipitating factors include fasting (or other forms of reduced or inadequate caloric intake, due to crash diets, long-distance athletics, etc.), metabolic stresses (*e.g.*, infections, surgery, international air travel, and psychological stress), endogenous hormones (e.g., progesterone), cigarette smoking, lipid-soluble foreign chemicals (including, *e.g.*, chemicals present in tobacco smoke, certain prescription drugs, organic solvents, biocides, components in alcoholic beverages), endocrine factors (e.g., reproductive hormones (women may experience exacerbations during the premenstrual period), synthetic estrogens, progesterones, ovulation stimulants, and hormone replacement therapy). See, for example, Thunell (1993).

Over 1000 drugs are contraindicated in the acute hepatic porphyrias (e.g., AIP, HCP, ADP, and VP) including, for example, alcohol, barbiturates, Carbamazepine, Carisoprodol, Clonazepam (high doses), Danazol, Diclofenac and possibly other NSAIDS, Ergots, estrogens, Ethyclorvynol, Glutethimide, Griseofulvin, Mephenytoin, Meprobamate (also mebutamate and tybutamate), Methyprylon, Metodopramide, Phenytoin, Primidone, progesterone and synthetic progestins, Pyrazinamide, Pyrazolones (aminopyrine and antipyrine), Rifampin, Succinimides (ethosuximide and methsuximide), sulfonamide antibiotics, and Valproic acid.

Objective signs of AIP include discoloration of the urine during an acute attack (the urine may appear red or red-brown), and increased concentrations of PBG and ALA in urine during an acute attack. Molecular genetic testing identifies mutations in the PBG deaminase (also known as HMBS) gene in more than 98% of affected individuals. Thunell (1993).

Diagnosis of porphria can involve assessment of family history, assessment of porphyrin precursor levels in urine, blood, or stool, and/or assessment of enzyme activity and DNA mutation analysis. The differential diagnosis of porphyrias may involve determining the type of porphyria by measuring individual levels of porphyrins or porphyrin precursors (e.g., ALA, PBG) in the urine, feces, and/or plasma (e.g., by chromatography and fluorometry) during an attack. The diagnosis of AIP can be confirmed by establishing that erythrocyte PBG deaminase

activity is at 50% or less of the normal level. DNA testing for mutations may be carried out in patients and at-risk family members. The diagnosis of AIP is typically confirmed by DNA testing to identify a specific caustative gene mutation (e.g., an HMBS mutation).

Current management of acute attacks of AIP involves hospitalization, monitoring of symptoms, and removal of unsafe drugs. Treatment of acute attacks typically requires hospitalization to control and treat acute sysmptoms, including, *e.g.*, abdominal pain, seizures, dehydration/hyponatremia, nausea/vomiting, tachycardia/hypertension, urinary retention/ileus. For example, abdominal pain may be treated, e.g., with narcotic analgesics, seizures may be treated with seizure precautions and possibly medications (although many anti-seizure medications are contraindicated), nausea/vomiting may be treated, e.g., with phenothiazines, and tachycardia/hypertension may be treated, e.g., with beta blockers. Treatment may include withdrawal of unsafe medications, monitoring of respiratory function, as well as muscle strength and neurological status. Mild attacks (e.g., those with no paresis or hyponatremia) may be treated with at least 300 g intravenous 10% glucose per day, although increasingly hemin is provided immediately. Severe attacks are typically treated as soon as possible with intravenous hemin (3-4 mg/kg daily for 4-14 days) and with IV glucose while waiting for the IV hemin to take effect. Typically, attacks are treated with IV hemin for 4 days and with IV glucose while waiting for administration of the IV hemin. Within 3-4 days following the start of hemin administration, there is usually clinical improvement accompanying by lowering of ALA and PBG levels.

Hemin (Panhematin® or hemin for injection, previously known as hematin) is the only heme product approved for use in the United States and was the first drug approved under the Orphan Drug Act. Panhematin® is hemin derived from processed red blood cells (PRBCs), and is Protoporphyrin IX containing a ferric iron ion (Heme B) with a chloride ligand. Heme acts to limit the hepatic and/or marrow synthesis of porphyrin. The exact mechanism by which hemin produces symptomatic improvement in patients with acute episodes of the hepatic porphyrias has not been elucidated; however, its action is likely due to the (feedback) inhibition of δ-aminolevulinic acid (ALA) synthase, the enzyme which limits the rate of the porphyrin/heme biosynthetic pathway. See Panhematin® product label, Lundbeck, Inc., October 2010.

Inhibition of ALA synthase should result in reduced production of ALA and PBG as well as porphyrins and porphyrin intermediates.

Drawbacks of heme products (e.g., hemin) include delayed impact on clinical symptoms and failure to prevent the recurrence of attacks. Adverse reactions associated with heme (e.g., hemin) administration may include phlebitis (e.g., thrombophlebitis), difficulty with venous access, anticoagulation (or coagulopathies), thrombocytopenia, renal shut down, or iron overload, which is particularly likely in patients requiring multiple courses of hemin treatment for recurrent attacks. To prevent phlebitis, an indwelling venous catheter is needed for access in patients with recurrent attacks. Renal damage can occur with high doses. Uncommonly reported side effects include fever, aching, malaise, hemolysis, anaphalaxis, and circulatory collapse. See Anderson, K.E., Approaches to Treatment and Prevention of Human Porphyrias, in *The Porphyrin Handbook: Medical Aspects of Porphyrins*, Edited by Karl M. Kadish, Kevin M. Smith, Roger Guilard (2003) (hereinafter Anderson).

Heme is difficult to prepare in a stable form for intravenous administration. It is insoluble at neutral pH but can be prepared as heme hydroxide at pH 8 or higher. Anderson. Panhematin is a lyophilized hemin preparation. When lyophilized hemin is solubilized for intravenous administration, degradation products form rapidly; these degradation products are responsible for a transient anticoagulant effect and for phlebitis at the site of infusion. Anderson. Heme albumin and heme arginate (Normosang, the European version of hemin) are more stable and may potentially cause less thrombophlebitis. However, heme arginate is not approved for use in the United States. Panhemin may be stabilized by solubilizing it for infusion in 30% human albumin rather than in sterile water; however, albumin adds intravascular volume-expanding effects and increases the cost of treatment as well as risk of pathogens since it is isolated from human blood. See, e.g., Anderson *supra*.

The successful treatment of an acute attack does not prevent or delay recurrence. There is a question of whether hemin itself can trigger recurring attacks due to induction of heme oxygenase. Nonetheless, in some areas (especially France), young women with multiply recurrent attacks are being treated with weekly hemin with the goal of achieving prophylaxis.

Limited experience with liver transplantation suggests that if successful, it is an effective treatment for AIP. There have been approximately 12 transplants in Europe in human patients, with curative or varying effects. Liver transplantation can restore normal excretion of ALA and PBG and prevent acute attacks. See, *e.g.*, Dar, F.S. et al. *Hepatobiliary Pancreat. Dis. Int.,* 9(1):93-96 (2010). Furthermore, if the liver of a patient with AIP is transplanted into another patient ("domino transplant"), the patient receiving the transplant may develop AIP.

Among the long-term clinical effects of acute porphyrias is chronic neuropathic pain that may result from a progressive neuropathy due to neurotoxic effects, e.g., of elevated porphyrin precursors (e.g., ALA and/or PBG). The neurotoxic effects can be associated with toxic heme intermediate production, for example, altered GABA signaling and/or production of iron-mediated oxidation and reactive oxygen species (ROS). Patients may suffer from neuropathic pain prior to or during an acute attack. Older patients may experience increased neuropathic pain with age for which various narcotic drugs are typically prescribed. Electromyogram abnormalities and decreased conduction times have been documented in patients with acute hepatic porphyrias. Of note, untreated, uninduced mice with AIP (PBG deaminase deficiency) develop a progressive motor neuropathy that has been shown to cause progressive quadriceps nerve axon degeneration and loss presumably due to constitutively elevated porphyrin precursor (ALA & PBG) levels, porphyrins and/or heme deficiency (Lindberg et al., J. Clin. Invest., 103(8): 1127–1134, 1999). In patients with acute porphyria (e.g., ADP, AIP, HCP, or VP), levels of porphyrin precursors (ALA & PBG) are often elevated in asymptomatic patients and in symptomatic patients between attacks. Thus, reduction of the porphyrin precursors and resumption of normal heme biosynthesis by reducing the level of ALAS1 expression and/or activity is expected to prevent and/or minimize development of chronic and progressive neuropathy. Treatment, e.g., chronic treatment (e.g., periodic treatment with iRNA as described herein, e.g., treatment according to a dosing regimen as described herein, e.g., weekly or biweekly treatment) can continuously reduce the ALAS1 expression in acute porphyria patients who have elevated levels of porphyrin precursors, porphyrins, porphyrin products or their metabolites. Such treatment may be provided as needed to prevent or reduce the frequency or severity of an individual patient's symptoms (e.g., pain and/or neuropathy) and/or to reduce a level of a porphyrin precursor, porphyrin, porphyrin product or metabolite.

The need exists for identifying novel therapeutics that can be used for the treatment of porphyrias. As discussed above, existing treatments such as heme products (e.g., hemin) have numerous drawbacks. For example, the impact of hemin on clinical symptoms is delayed, it is expensive, and it may have side effects (e.g., thrombophlebitis, anticoagulation, thrombocytopenia, iron overload, renal shutdown). Novel therapeutics such as those described herein can address these drawbacks and the unmet needs of patients acting faster, not inducing phlebitis, providing the convenience of subcutaneous administration, successfully preventing recurrent attacks, preventing or ameliorating pain (e.g., chronic neuropathic pain) and/or progressive neuropathy, and/or not causing certain adverse effects associated with hemin (e.g., iron overload, increased risk of hepatocellular cancer).

Patients with AIA include those who suffer from recurrent attacks and those who suffer from acute, sporadic attacks. In the pateints who suffer from recurrent attacks, about 5-10% have recurrent intermittent attacks (2-3 attacks per year) or recurrent attacks (>4 attacks per year). These patients are most likely to consider liver transplant or to receive prophylactic heme (e.g., hemin) infusions. The recurrent attack patients are likely to have poor quality of life due to long hospital stays, opiate addiction, and/or venous network toxicity. Chronic heme administration can induce heme oxygenase (HO-1). Thus, it can be difficult to wean patients off heme and some require more frequent treatment. Some clinicials are therefore restricting heme use to the most serious attacks. Accordingly, there is an unmet need for convenient, effective prophylaxis and treatments with better tolerability.

For patients who suffer from acute attacks, clinical guidelines suggest administration of heme as early as possible. However, given the challenges of diagnosis and lack of immediate drug availability, administration may be delayed. The slow onset of the effects of heme (e.g., hemin) and its poor tolerability slow the time to improvement. Persistence of severe abdominal pain, even after administration of heme, can require that patients receive opiates for multiple days.

Delayed administration of heme or continued exposure to precipitating factors can lead to more serious complications, including motor neuropathy and accompanying symptoms (e.g., weakness, paresis). Respiratory failure and paralysis can occur in severe cases. Recovery from neurological symptoms can take much longer to resolve. Accordingly, in the context of acute attacks, treatments that have a faster onset of action and better tolerability are needed.

Pharmacological validation of ALAS1 as a target for mRNA silencing is supported by at least the following findings: ALAS1 mRNA is strongly upregulated during an attack; panhematin down modulates ALAS-1; and addition of heme to liver cells in culture leads to reduced ALAS-1 mRNA. Several findings also indicate that suppression of ALAS1 mRNA can be achieved by targeting the liver. For example, liver transplant has been shown to be curative; and liver derived metabolites drive attacks (see e.g., Dar *et al. Hepatobiliary Pancreat Dis Int.* 9:93-6 (2010); Dowman *et al. Ann Intern Med* 154:571-2 (2011); and Wu *et al. Genes Dev* 23:2201-2209 (2009). Thus, reducing expression of ALAS1, e.g., in the liver, using iRNA compositions can be used to treat a porphyria. In certain embodiments, iRNA compositions can be used for prophylaxis and acute treatment of porphyrias. For example, iRNA compositions can be used prophylactically in a recurrent attack setting to induce long-term or chronic suppression of ALAS1 expression (leading to long-term or chronic suppression of ALA/PBG), and thus blunting the recurrent ALAS1 upregulation that drives the attacks. Such prophylactic treatment can reduce the number and the severity of the attacks. During an acute attack setting, administration of an iRNA composition can treat an acute attack, e.g., by reducing the levels of ALA/PBG.

The present disclosure provides methods and iRNA compositions for modulating the expression of an ALAS1 gene. In certain embodiments, expression of ALAS1 is reduced or inhibited using an ALAS1-specific iRNA, thereby leading to a decreased expression of an ALAS1 gene. Reduced expression of an ALAS1 gene may reduce the level of one or more porphyrin precursors, porphyrins, or porphyrin products or metabolites. Decreased expression of an ALAS1 gene, as well as related decreases in the level of one or more porphyrin precursors and/or porphyrins, can be useful in treating disorders related to ALAS1 expression, *e.g.*, porphyrias.

The iRNAs of the compositions featured 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 an ALAS1 gene (also referred to herein as an "ALAS1-specific iRNA"). The use of such an iRNA enables the targeted degradation of mRNAs of genes that are implicated in pathologies associated with ALAS1 expression in mammals, *e.g.*, porphyrias such

as ALA dehydratase deficiency porphyria (also known as Doss porphyria or plumboporphyria) or acute intermittent porphyria. Very low dosages of ALAS1-specific iRNAs can specifically and efficiently mediate RNAi, resulting in significant inhibition of expression of an ALAS1 gene. iRNAs targeting ALAS1 can specifically and efficiently mediate RNAi, resulting in significant inhibition of expression of an ALAS1 gene, *e.g.*, in cell based assays. Thus, methods and compositions including these iRNAs are useful for treating pathological processes related to ALAS1 expression, such as porphyrias (*e.g.*, X-linked sideroblastic anemia (XLSA), ALA deyhdratase deficiency porphyria (Doss porphyria), acute intermittent porphyria (AIP), congenital erythropoietic porphyria, prophyria cutanea tarda, hereditary coproporphyria (coproporphyria), variegate porphyria, erythropoietic protoporphyria (EPP), and transient erythroporphyria of infancy).

The following description discloses how to make and use compositions containing iRNAs to inhibit the expression of an ALAS1 gene, as well as compositions and methods for treating diseases and disorders caused by or modulated by the expression of this gene. Embodiments of the pharmaceutical compositions featured in the invention 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 an ALAS1 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 an ALAS1 gene.

Accordingly, in some aspects, pharmaceutical compositions containing an ALAS1 iRNA and a pharmaceutically acceptable carrier, methods of using the compositions to inhibit expression of an ALAS1 gene, and methods of using the pharmaceutical compositions to treat disorders related to ALAS1 expression 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 may be replaced in the nucleotide sequences of dsRNA featured in the invention 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 featured in the invention.

As used herein, "ALAS1" (also known as ALAS-1; δ-aminolevulinate synthase 1; δ-ALA synthase 1; 5'-aminolevulinic acid synthase 1; ALAS-H; ALASH; ALAS-N; ALAS3; EC2.3.1.37; 5-aminolevulinate synthase, nonspecific, mitochondrial; ALAS; MIG4; OTTHUMP00000212619; OTTHUMP00000212620; OTTHUMP00000212621; OTTHUMP00000212622; migration-inducing protein 4; EC 2.3.1) refers to a nuclear-encoded mitochondrial enzyme that is the first and typically rate-limiting enzyme in the mammalian heme biosynthetic pathway. ALAS1 catalyzes the condensation of glycine with succinyl-CoA to form δ-aminolevulinic acid (ALA). The human ALAS1 gene is expressed ubiquitously, is found on chromosome 3p21.1 and typically encodes a sequence of 640 amino acids. In contrast, the ALAS-2 gene, which encodes an isozyme, is expressed only in erythrocytes, is found on chromoxome Xp11.21, and typicallyencodes a sequence of 550 amino acids. As used herein an "ALAS1 protein" means any protein variant of ALAS1 from any species (*e.g.*, human, mouse, non-human primate), as well as any mutants and fragments thereof that retain an ALAS1 activity. Similarly, an "ALAS1 transcript" refers to any transcript variant of ALAS1, from any

species (*e.g.*, human, mouse, non-human primate). A sequence of a human ALAS1 mRNA transcript can be found at NM_000688.4 (FIG. 3A and FIG. 3B; SEQ ID NO:1). Another human ALAS1 mRNA transcript, can be found at NM_000688.5 (FIG. 4A and FIG. 4B; SEQ ID NO:382). The level of the mature encoded ALAS1 protein is regulated by heme: high levels of heme down-regulate the mature enzyme in mitochondria while low heme levels up-regulate. Multiple alternatively spliced variants, encoding the same protein, have been identified.

As used herein, the term "iRNA," "RNAi", "iRNA agent," or "RNAi agent" refers to an agent that contains RNA as that term is defined herein, and which mediates the targeted cleavage of an RNA transcript, e.g., via an RNA-induced silencing complex (RISC) pathway. In one embodiment, an iRNA as described herein effects inhibition of ALAS1 expression. Inhibition of ALAS1 expression may be assessed based on a reduction in the level of ALAS1 mRNA or a reduction in the level of the ALAS1 protein. As used herein, "target sequence" refers to a contiguous portion of the nucleotide sequence of an mRNA molecule formed during the transcription of an ALAS1 gene, including 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 therebetween. 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 may 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 may 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, 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, may 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 includes, but are not limited to, G:U Wobble or Hoogstein base pairing.

The terms "complementary," "fully complementary" and "substantially complementary" herein may 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 (mRNA) refers to a polynucleotide that is substantially complementary to a contiguous portion of the mRNA of interest (*e.g.*, an mRNA encoding an ALAS1 protein). For example, a polynucleotide is complementary to at least a part of an ALAS1 mRNA if the sequence is substantially complementary to a non-interrupted portion of an mRNA encoding ALAS1. As another example, a polynucleotide is complementary to at least a part of an ALAS1 mRNA if the sequence is substantially complementary to a non-interrupted portion of an mRNA encoding ALAS1.

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, e.g., 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.

In another embodiment, the iRNA agent may be a "single-stranded siRNA" that is introduced into a cell or organism to inhibit a target mRNA. Single-stranded RNAi agents bind to the RISC endonuclease Argonaute 2, which then cleaves the target mRNA. The single-stranded siRNAs are generally 15-30 nucleotides and are chemically modified. The design and testing of single-stranded siRNAs are described in U.S. Patent No. 8,101,348 and in Lima *et al.*, (2012) *Cell* 150: 883-894, the entire contents of each of which are hereby incorporated herein by reference. Any of the antisense nucleotide sequences described herein (e.g., sequences provided in Tables 2, 3, 6, 7, 8, 9, 14, 15, 18 and 20 or in Tables 21-40) may be used as a single-stranded siRNA as described herein or as chemically modified by the methods described in Lima *et al.*, (2012) *Cell* 150:883-894.

In another aspect, the RNA agent is a "single-stranded antisense RNA molecule". An single-stranded antisense RNA molecule is complementary to a sequence within the target mRNA. Single-stranded antisense RNA molecules can inhibit translation in a stoichiometric manner by base pairing to the mRNA and physically obstructing the translation machinery, see Dias, N. *et al.*, (2002) *Mol Cancer Ther* 1:347-355. Alternatively, the single-stranded antisense molecules inhibit a target mRNA by hydridizing to the target and cleaving the target through an

RNaseH cleavage event. The single-stranded antisense RNA molecule may be about 10 to about 30 nucleotides in length and have a sequence that is complementary to a target sequence. For example, the single-stranded antisense RNA molecule may comprise a sequence that is at least about 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, or more contiguous nucleotides from any one of the antisense nucleotide sequences described herein, e.g., sequences provided in any one of Tables 2, 3, 6, 7, 8, 9, 14, 15, 18 and 20 or in Tables 21-40.

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, in the ribose 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 nucleostide, 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, an acyclic 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, e.g., 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. In certain embodiments, the RNA molecule comprises a percentage of deoxyribonucleoses of at least 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95% or higher (but not 100%) deoxyribonucleosides, *e.g.*, in one or both strands. In other embodiments, the term "iRNA" does not encompass a double stranded DNA molecule (*e.g.*, a naturally-occurring double stranded DNA molecule or a 100% deoxynucleoside-containing DNA molecule). 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 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, 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. These applications are incorporated herein by reference in their entirety.

"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 may 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 known in the art.

As used herein, the term "modulate the expression of," refers to at an least partial "inhibition" or partial "activation" of an ALAS1 gene expression in a cell treated with an iRNA composition as described herein compared to the expression of ALAS1 in a control cell. A control cell includes an untreated cell, or a cell treated with a non-targeting control iRNA.

The terms "activate," "enhance," "up-regulate the expression of," "increase the expression of," and the like, in so far as they refer to an ALAS1 gene, herein refer to the at least partial activation of the expression of an ALAS1 gene, as manifested by an increase in the amount of ALAS1 mRNA, which may be isolated from or detected in a first cell or group of cells in which an ALAS1 gene is transcribed and which has or have been treated such that the expression of an ALAS1 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 an ALAS1 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, an ALAS1 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 an ALAS1 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 ALAS1 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 expression of," "down-regulate expression of," "suppress expression of," and the like, in so far as they refer to an ALAS1 gene, herein refer to the at least partial suppression of the expression of an ALAS1 gene, as assessed, *e.g.*, based on on ALAS1 mRNA expression, ALAS1 protein expression, or another parameter functionally linked to ALAS1 gene expression (e.g., ALA or PBG concentrations in plasma or urine). For example, inhibition of ALAS1 expression may be manifested by a reduction of the amount of ALAS1 mRNA which may be isolated from or detected in a first cell or group of cells in which an ALAS1 gene is transcribed and which has or have been treated such that the expression of an ALAS1 gene is inhibited, as compared to a control. The control may be a second cell or group of cells substantially identical to the first cell or group of cells, except that the second cell or group of cells have not been so treated (control cells). The degree of inhibition is usually expressed as a percentage of a control level, *e.g.*,

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

Alternatively, the degree of inhibition may be given in terms of a reduction of a parameter that is functionally linked to ALAS1 gene expression, *e.g.*, the amount of protein encoded by an ALAS1 gene, or the level of one or more porphyrins. The reduction of a parameter functionally linked to ALAS1 gene expression may similarly be expressed as a percentage of a control level. In principle, ALAS1 gene silencing may be determined in any cell expressing ALAS1, either constitutively or by genomic engineering, and by any appropriate assay. However, when a reference is needed in order to determine whether a given iRNA inhibits the expression of the ALAS1 gene by a certain degree and therefore is encompassed by the instant invention, the assays provided in the Examples below shall serve as such reference.

For example, in certain instances, expression of an ALAS1 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, an ALAS1 gene is suppressed by at least about 60%, 65%, 70%, 75%, or 80% by administration of an iRNA featured in the invention. In some embodiments, an ALAS1 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 ALAS1 expression, the terms "treat," "treating," "treatment," and the like, refer to relief from or alleviation of pathological processes related to ALAS1 expression (*e.g.*, pathological processes involving porphyrins or defects in the porphyrin pathway, such as, for example, porphyrias). In the context of the present invention insofar as it relates to any of the other conditions recited herein below (other than pathological processes related to ALAS1expression), the terms "treat," "treatment," and the like mean to prevent, relieve or alleviate at least one symptom associated with such condition, or to slow or reverse the progression or anticipated progression of such condition. For example, the methods featured herein, when employed to treat porphyria, may serve to reduce or prevent one or more symptoms associated with porphyria (e.g., pain), to reduce the severity or frequency of attacks associated with porphyria, to reduce the likelihood that an attack of one or more symptoms associated with porphyria will occur upon exposure to a precipitating condition, to shorten an attack associated with porphyria, and/or to reduce the risk of developing conditions associated with porphyria (*e.g.*, hepatocellular cancer or neuropathy (e.g., progressive neuropathy),). Thus, unless the context clearly indicates otherwise, the terms "treat," "treatment," and the like are intended to encompass prophylaxis, e.g., prevention of disorders and/or symptoms of disorders related to ALAS1 expression.

By "lower" in the context of a disease marker or symptom is meant a statistically or clinically 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 typically 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 related to ALAS1expression. The specific

amount that is therapeutically effective can be readily determined by an ordinary medical practitioner, and may vary depending on factors known in the art, such as, for example, the type of pathological process, the patient's history and age, the stage of pathological process, and the administration of other agents.

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, in a method of treating a disorder related to ALAS1 expression (e.g., in a method of treating a porphyria), an effective amount includes an amount effective to reduce one or more symptoms associated with a porphyria, an amount effective to reduce the frequency of attacks, an amount effective to reduce the likelihood that an attack of one or more symptoms associated with porphyria will occur upon exposure to a precipitating factor, or an amount effective to reduce the risk of developing conditions associated with porphyria (*e.g.*, neuropathy (e.g., progressive neuropathy), hepatocellular cancer). 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 ALAS1 can reduce ALAS1 protein levels by any measurable amount, e.g., by at least 10%, 20%, 30%, 40% or 50%.

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.

The term "about" when referring to a number or a numerical range means that the number or numerical range referred to is an approximation within experimental variability (or within statistical experimental error), and thus the number or numerical range may vary from, for example, between 1% and 15% of the stated number or numerical range.

II. iRNA Agents

Described herein are iRNA agents that inhibit the expression of an ALAS1gene. In one embodiment, the iRNA agent includes double-stranded ribonucleic acid (dsRNA) molecules for inhibiting the expression of an ALAS1 gene in a cell or in a subject (*e.g.*, in a mammal, *e.g.*, in a human having a porphyria), where 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 an ALAS1gene, and where 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 ALAS1 gene, inhibits the expression of the ALAS1 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 an ALAS1 gene in a cell or mammal. Expression of an ALAS1 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 ALAS1 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 sufficiently 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, derived from the sequence of an mRNA formed during the expression of an ALAS1 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, *e.g.*, 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, then, 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 ALAS1 expression is not generated in the target cell by cleavage of a larger dsRNA.

A dsRNA as described herein may 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, an ALAS1 gene is a human ALAS1 gene. In another embodiment the ALAS1 gene is a mouse or a rat ALAS1 gene.

In specific embodiments, the first sequence is a sense strand of a dsRNA that includes a sense sequence disclosed herein, e.g., in Tables 21-40, and the second sequence is an antisense strand of a dsRNA that includes an antisense sequence disclosed herein, e.g., in Tables 21-40.

In specific embodiments, the first sequence is a sense strand of a dsRNA that includes a sense sequence from Table 2 or Table 3, and the second sequence is an antisense strand of a dsRNA that includes an antisense sequence from Table 2 or Table 3. In embodiments, the first sequence is a sense strand of a dsRNA that includes a sense sequence from Table 2, 3, 6, 7, 8, 9, 14, or 15, and the second sequence is an antisense strand of a dsRNA that includes an antisense sequence from Table 2, 3, 6, 7, 8, 9, 14, or 15. In embodiments, the first sequence is a sense strand of a dsRNA that includes a sense sequence from Table 2, 3, 6, 7, 8, 9, 14, 15, 18 or 20, and the second sequence is an antisense strand of a dsRNA that includes an antisense sequence from Table 2, 3, 6, 7, 8, 9, 14, 15, 18 or 20.

In one aspect, a dsRNA can include at least sense and antisense nucleotide sequences, whereby the sense strand is selected from the sense sequences provided herein, e.g., in Tables 21-40, and the corresponding antisense strand of the sense strand is selected from the antisense sequences provided herein, e.g., in Tables 21-40.

In one aspect, a dsRNA can include at least sense and antisense nucleotide sequences, whereby the sense strand is selected from the groups of sequences provided in Tables 2 and 3, and the corresponding antisense strand of the sense strand is selected from Tables 2 and 3. In a further aspect, a dsRNA can include at least sense and antisense nucleotide sequences, whereby the sense strand is selected from the groups of sequences provided in Tables 2, 3, 6, 7, 8, 9, 14, and 15, and the corresponding antisense strand of the sense strand is selected from Tables 2, 3, 6, 7, 8, 9, 14, and 15. In a further aspect, a dsRNA can include at least sense and antisense nucleotide sequences, whereby the sense strand is selected from the groups of sequences provided in Tables 2, 3, 6, 7, 8, 9, 14, 15, 18 and 20, and the corresponding antisense strand of the sense strand is selected from Tables 2, 3, 6, 7, 8, 9, 14, 15, 18 and 20.

In embodiments, the iRNA is AD-60501, AD-60519, AD-60901, AD-60495, AD-60900, AD-60935, AD-60879, AD-61190, AD-61191, AD-60865, AD-60861, AD-60876, AD-61193, AD-60519, AD-60519, AD-60901, AD-60405, AD-60887, AD-60923, AD-60434, AD-60892, AD-60419, AD-60924, AD-60445, AD-60925, AD-60926, AD-60820, AD-60843, AD-60819,

AD-61140, AD-61141, AD-61142, AD-60835, AD-60839, AD-61143, AD-61144, AD-61145, AD-61146, AD-60892, or AD-60419 (*e.g.*, including the nucleotide sequence and/or one or more (*e.g.*, all) of the modifications of the aforesaid dsRNAs). In embodiments, the iRNA comprises an antisense strand that comprises, or consists of, an antisense sequence (including one or more (*e.g.*, all the modifications)) selected from the antisense sequence of AD-60501, AD-60519, AD-60901, AD-60495, AD-60900, AD-60935, AD-60879, AD-61190, AD-61191, AD-60865, AD-60861, AD-60876, AD-61193, AD-60519, AD-60519, AD-60901, AD-60405, AD-60887, AD-60923, AD-60434, AD-60892, AD-60419, AD-60924, AD-60445, AD-60925, AD-60926, AD-60820, AD-60843, AD-60819, AD-61140, AD-61141, AD-61142, AD-60835, AD-60839, AD-61143, AD-61144, AD-61145, AD-61146, AD-60892, or AD-60419. In embodiments, the iRNA comprises a sense strand that comprises, or consists of, a sense sequence (and/or one or more (*e.g.*, all) of the modifications)) selected from AD-60501, AD-60519, AD-60901, AD-60495, AD-60900, AD-60935, AD-60879, AD-61190, AD-61191, AD-60865, AD-60861, AD-60876, AD-61193, AD-60519, AD-60519, AD-60901, AD-60405, AD-60887, AD-60923, AD-60434, AD-60892, AD-60419, AD-60924, AD-60445, AD-60925, AD-60926, AD-60820, AD-60843, AD-60819, AD-61140, AD-61141, AD-61142, AD-60835, AD-60839, AD-61143, AD-61144, AD-61145, AD-61146, AD-60892, or AD-60419.

In embodiments, the iRNA comprises (i) an antisense strand that comprises, or consists of, the sequence of UAAGAUGAGACACUCUUUCUGGU or UAAGAUGAGACACUCTUUCUGGU and/or (ii) a sense strand that comprises, or consists of, the sequence of CAGAAAGAGUGUCUCAUCUUA. In embodiments, one or more nucleotides of the antisense strand and/or sense strand are modified as described herein.

In embodiments, the iRNA comprises (i) an antisense strand that comprises, or consists of, the antisense sequence of AD-60489 and/or (ii) a sense strand that comprises, or consists of, the sense sequence of AD-60489 (and/or one or more (*e.g.*, all) of the modifications of the sense strand and/or antisense strand of AD-60489).

In embodiments, the iRNA comprises (i) an antisense strand that comprises, or consists of, the antisense sequence of AD-60519 and/or (ii) a sense strand that comprises, or consists of, the sense sequence of AD-60519 (and/or one or more (*e.g.*, all) of the modifications of the sense strand and/or antisense strand of AD-60489).

In embodiments, the iRNA comprises (i) an antisense strand that comprises, or consists of, the antisense sequence of AD-61193 and/or (ii) a sense strand that comprises, or consists of, the sense sequence of AD-61193 (and/or one or more (*e.g.*, all) of the modifications of the sense strand and/or antisense strand of AD-60489).

In embodiments, the iRNA comprises (i) an antisense strand that comprises, or consists of, the antisense sequence of AD-60819 and/or (ii) a sense sequence that comprises, or consists of, the sense sequence of AD-60819 (and/or one or more (*e.g.*, all) of the modifications of the sense strand and/or antisense strand of AD-60489).

In embodiments, the iRNA for inhibiting expression of ALAS1 is provided, wherein the dsRNA comprises (i) an antisense strand that comprises, or consists of, the antisense sequence of AD-60489, AD-60519, AD-61193, or AD-60819 (or a corresponding unmodified antisense sequence) and/or (ii) a sense strand that comprises, or consists of, the sense sequence of AD-60489, AD-60519, AD-61193, or AD-60819 (or a corresponding unmodified antisense sequence). In embodiments, the iRNA comprises (i) an antisense strand that consists of the antisense sequence of AD-60489, AD-60519, AD-61193, or AD-60819 and/or (ii) a sense strand that consists of the sense sequence of AD-60489, AD-60519, AD-61193, or AD-60819, except that the antisense strand and/or sense strand of the dsRNA differs by 1, 2, or 3 nucleotides from the corresponding antisense and/or sense sequence of AD-60489, AD-60519, AD-61193, or AD-60819.

The sequences and modifications of AD-60489, AD-60519, AD-61193, and AD-60819 are shown in Table 44 disclosed herein.

In one embodiment, the iRNA is ALN-60519. ALN-60519 is a chemically synthesized double stranded oligonucleotide covalently linked to a ligand containing three N-acetylgalactosamine (GalNAc) residues (depicted in FIG. 57). In one embodiment, all nucleotides of ALN-60519 are 2'-OMe or 2'-F modified and are connected through 3'-5' phosphodiester linkages, thus forming the sugar-phosphate backbone of the oligonucleotide. The sense strand and the antisense strand of ALN-60519 contain 21 and 23 nucleotides, respectively. The 3'-end of the sense strand of ALN-60519 is conjugated to the triantennary GalNAc moiety (referred to as L96) through a phosphodiester linkage. The antisense strand contains four phosphorothioate linkages - two at the 3' end and two at the 5' end. The sense strand of ALN-

60519 contains two phosphorothioate linkages at the 5' end. The 21 nucleotides of the sense strand of ALN-60519 hybridize with the complementary 21 nucleotides of the antisense strand, thus forming 21 nucleotide base pairs and a two-base overhang at the 3'-end of the antisense strand. The two single strands, the sense strand and the antisense strand, of ALN-60519 can be synthesized by conventional solid phase oligonucleotide synthesis, employing standard phosphoramidite chemistry with the 5'-hydroxyl group protected as dimethoxytriphenylmethyl (DMT) ether. Each strand can be assembled from the 3' to the 5' terminus by sequential addition of protected nucleoside phosphoramidites.

In these aspects, 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 by the expression of an ALAS1 gene gene. As such, a dsRNA will include two oligonucleotides, where one oligonucleotide is described herein as the sense strand, and the second oligonucleotide is described as the corresponding antisense strand. 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 the tables herein, dsRNAs described herein can include at least one strand of a length of minimally 21 nucleotides. It can be reasonably expected that shorter duplexes having one of the sequences of disclosed herein 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 disclosed herein, and differing in their ability to inhibit the expression of an ALAS1gene 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 the tables herein, identify a site in an ALAS1 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 herein, e.g., in Tables 2, 3, 6, 7, 8, 9, 14, 15, 18, 20, and in Tables 21-40, coupled to additional nucleotide sequences taken from the region contiguous to the selected sequence in an ALAS1gene.

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 the tables herein, 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 the tables herein, further optimization can be achieved by systematically either adding or removing nucleotides to generate longer or shorter sequences and testing those and 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 an ALAS1 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 an ALAS1 gene. Consideration of the efficacy of iRNAs with mismatches in inhibiting expression of an ALAS1 gene is important, especially if the particular region of complementarity in an ALAS1 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. 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 having an acyclic sugar) 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 this invention 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 US 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.

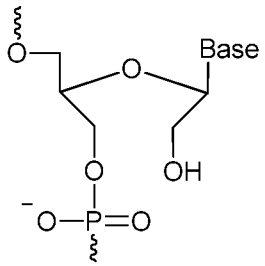
Some embodiments featured in the invention include RNAs with phosphorothioate backbones and oligonucleosides with heteroatom backbones, and in particular --CH_2--NH--CH_2--, --CH_2--N(CH_3)--O--CH_2--[known as a methylene (methylimino) or MMI backbone], --CH_2--O--N(CH_3)--CH_2--, --CH_2--N(CH_3)--N(CH_3)--CH_2-- 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 may 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.

In other embodiments, an iRNA agent comprises one or more (*e.g.*, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more) acyclic nucleotides (or nucleosides). In certain embodiments, the sense strand or the antisense strand, or both sense strand and antisense strand, include less than five acyclic

nucleotides per strand (*e.g.*, four, three, two or one acyclic nucleotides per strand). The one or more acyclic nucleotides can be found, for example, in the double-stranded region, of the sense or antisense strand, or both strands; at the 5'-end, the 3'-end, both of the 5' and 3'-ends of the sense or antisense strand, or both strands, of the iRNA agent. In one embodiment, one or more acyclic nucleotides are present at positions 1 to 8 of the sense or antisense strand, or both. In one embodiment, one or more acyclic nucleotides are found in the antisense strand at positions 4 to 10 (*e.g.*, positions 6-8) from the 5'-end of the antisense strand. In another embodiment, the one or more acyclic nucleotides are found at one or both 3'-terminal overhangs of the iRNA agent.

The term "acyclic nucleotide" or "acyclic nucleoside" as used herein refers to any nucleotide or nucleoside having an acyclic sugar, *e.g.*, an acyclic ribose. An exemplary acyclic nucleotide or nucleoside can include a nucleobase, *e.g.*, a naturally-occurring or a modified nucleobase (*e.g.*, a nucleobase as described herein). In certain embodiments, a bond between any of the ribose carbons (C1, C2, C3, C4, or C5), is independently or in combination absent from the nucleotide. In one embodiment, the bond between C2-C3 carbons of the ribose ring is absent, *e.g.*, an acyclic 2'-3'-seco-nucleotide monomer. In other embodiments, the bond between C1-C2, C3-C4, or C4-C5 is absent (*e.g.*, a 1'-2', 3'-4' or 4'-5'-seco nucleotide monomer). Exemplary acyclic nucleotides are disclosed in US 8,314,227, incorporated herein by reference in its entirely. For example, an acyclic nucleotide can include any of monomers D-J in Figures 1-2 of US 8,314,227. In one embodiment, the acyclic nucleotide includes the following monomer:



wherein Base is a nucleobase, *e.g.*, a naturally-occurring or a modified nucleobase (*e.g.*, a nucleobase as described herein).

In certain embodiments, the acyclic nucleotide can be modified or derivatized, e.g., by coupling the acyclic nucleotide to another moiety, *e.g.*, a ligand (*e.g.*, a GalNAc, a cholesterol ligand), an alkyl, a polyamine, a sugar, a polypeptide, among others.

In other embodiments, the iRNA agent includes one or more acyclic nucleotides and one or more LNAs (*e.g.*, an LNA as described herein). For example, one or more acyclic nucleotides and/or one or more LNAs can be present in the sense strand, the antisense strand, or both. The number of acyclic nucleotides in one strand can be the same or different from the number of LNAs in the opposing strand. In certain embodiments, the sense strand and/or the antisense strand comprises less than five LNAs (*e.g.*, four, three, two or one LNAs) located in the double-stranded region or a 3'-overhang. In other embodiments, one or two LNAs are located in the double stranded region or the 3'-overhang of the sense strand. Alternatively, or in combination, the sense strand and/or antisense strand comprises less than five acyclic nucleotides (*e.g.*, four, three, two or one acyclic nucleotides) in the double-stranded region or a 3'-overhang. In one embodiment, the sense strand of the iRNA agent comprises one or two LNAs in the 3'-overhang of the sense strand, and one or two acyclic nucleotides in the double-standed region of the antisense strand (*e.g.*, at positions 4 to 10 (*e.g.*, positions 6-8) from the 5'-end of the antisense strand) of the iRNA agent.

In other embodiments, inclusion of one or more acyclic nucleotides (alone or in addition to one or more LNAs) in the iRNA agent results in one or more (or all) of: (i) a reduction in an off-target effect; (ii) a reduction in passenger strand participation in RNAi; (iii) an increase in specificity of the guide strand for its target mRNA; (iv) a reduction in a microRNA off-target effect; (v) an increase in stability; or (vi) an increase in resistance to degradation, of the iRNA molecule.

Other modifications include 2'-methoxy (2'-OCH_3), 2'-aminopropoxy (2'-$OCH_2CH_2CH_2NH_2$) and 2'-fluoro (2'-F). Similar modifications may 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 may 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 (*e.g.*, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more) locked nucleic acids (LNA), (also referred to herein as "locked nucleotides"). In one embodiment, a locked nucleic acid is a nucleotide having a modified ribose moiety in which the ribose moiety comprises an extra bridge connecting, *e.g.*, 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, increase thermal stability, 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; 7,399,845; and 8,314,227, each of which is herein incorporated by reference in its entirety. Exemplary LNAs include but are not limited to, a 2', 4'-C methylene bicyclo nucleotide (see for example Wengel *et al.,* International PCT Publication No. WO 00/66604 and WO 99/14226).

In other embodiments, the iRNA agents include one or more (*e.g.*, about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more) G-clamp nucleotides. A G-clamp nucleotide is a modified cytosine analog wherein the modifications confer the ability to hydrogen bond both Watson-Crick and Hoogsteen faces of a complementary guanine within a duplex, see for example Lin and Matteucci, 1998, *J. Am. Chem. Soc.,* 120, 8531-8532. A single G-clamp analog substitution within an oligonucleotide can result in substantially enhanced helical thermal stability and mismatch discrimination when hybridized to complementary oligonucleotides. The inclusion of such

nucleotides in the iRNA molecules can result in enhanced affinity and specificity to nucleic acid targets, complementary sequences, or template strands.

Potentially stabilizing modifications to the ends of RNA molecules can include N-(acetylaminocaproyl)-4-hydroxyprolinol (Hyp-C6-NHAc), N-(caproyl-4-hydroxyprolinol (Hyp-C6), N-(acetyl-4-hydroxyprolinol (Hyp-NHAc), thymidine-2'-O-deoxythymidine (ether), N-(aminocaproyl)-4-hydroxyprolinol (Hyp-C6-amino), 2-docosanoyl-uridine-3"- phosphate, inverted base dT(idT) and others. Disclosure of this modification can be found in PCT Publication No. WO 2011/005861.

iRNA Motifs

In one embodiment, the sense strand sequence may be represented by formula (I):

$5'\ n_p\text{-}N_a\text{-}(X\ X\ X)_i\text{-}N_b\text{-}Y\ Y\ Y\text{-}N_b\text{-}(Z\ Z\ Z)_j\text{-}N_a\text{-}n_q\ 3'$ (I)

wherein:

i and j are each independently 0 or 1;

p and q are each independently 0-6;

each N_a independently represents an oligonucleotide sequence comprising 0-25 modified nucleotides, each sequence comprising at least two differently modified nucleotides;

each N_b independently represents an oligonucleotide sequence comprising 0-10 modified nucleotides;

each n_p and n_q independently represent an overhang nucleotide;

wherein Nb and Y do not have the same modification; and

XXX, YYY and ZZZ each independently represent one motif of three identical modifications on three consecutive nucleotides. Preferably YYY is all 2'-F modified nucleotides.

In one embodiment, the N_a and/or N_b comprise modifications of alternating pattern.

In one embodiment, the YYY motif occurs at or near the cleavage site of the sense strand. For example, when the RNAi agent has a duplex region of 17-23 nucleotides in length, the YYY motif can occur at or the vicinity of the cleavage site (*e.g.*: can occur at positions 6, 7, 8; 7, 8, 9; 8, 9, 10; 9, 10, 11; 10, 11,12 or 11, 12, 13) of - the sense strand, the count starting from the 1st nucleotide, from the 5'-end; or optionally, the count starting at the 1st paired nucleotide within the duplex region, from the 5'- end.

In one embodiment, i is 1 and j is 0, or i is 0 and j is 1, or both i and j are 1. The sense strand can therefore be represented by the following formulas:

5' n_p-N_a-YYY-N_b-ZZZ-N_a-n_q 3' (Ib);

5' n_p-N_a-XXX-N_b-YYY-N_a-n_q 3' (Ic); or

5' n_p-N_a-XXX-N_b-YYY-N_b-ZZZ-N_a-n_q 3' (Id).

When the sense strand is represented by formula (Ib), N_b represents an oligonucleotide sequence comprising 0-10, 0-7, 0-5, 0-4, 0-2 or 0 modified nucleotides. Each N_a independently can represent an oligonucleotide sequence comprising 2-20, 2-15, or 2-10 modified nucleotides.

When the sense strand is represented as formula (Ic), N_b represents an oligonucleotide sequence comprising 0-10, 0-7, 0-5, 0-4, 0-2 or 0 modified nucleotides. Each N_a can independently represent an oligonucleotide sequence comprising 2-20, 2-15, or 2-10 modified nucleotides.

When the sense strand is represented as formula (Id), each N_b independently represents an oligonucleotide sequence comprising 0-10, 0-7, 0-5, 0-4, 0-2 or 0 modified nucleotides. Preferably, N_b is 0, 1, 2, 3, 4, 5 or 6. Each N_a can independently represent an oligonucleotide sequence comprising 2-20, 2-15, or 2-10 modified nucleotides.

Each of X, Y and Z may be the same or different from each other.

In other embodiments, i is 0 and j is 0, and the sense strand may be represented by the formula:

5' n_p-N_a-YYY- N_a-n_q 3' (Ia).

When the sense strand is represented by formula (Ia), each N_a independently can represent an oligonucleotide sequence comprising 2-20, 2-15, or 2-10 modified nucleotides.

In one embodiment, the antisense strand sequence of the RNAi may be represented by formula (II):

5' $n_{q'}$-N_a'-(Z'Z'Z')$_k$-N_b'-Y'Y'Y'-N_b'-(X'X'X')$_l$-N'_a-n_p' 3' (II)

wherein:

k and l are each independently 0 or 1;

p' and q' are each independently 0-6;

each N_a' independently represents an oligonucleotide sequence comprising 0-25 modified nucleotides, each sequence comprising at least two differently modified nucleotides;

each N_b' independently represents an oligonucleotide sequence comprising 0-10 modified nucleotides;

each n_p' and n_q' independently represent an overhang nucleotide;

wherein N_b' and Y' do not have the same modification;

and

X'X'X', Y'Y'Y' and Z'Z'Z' each independently represent one motif of three identical modifications on three consecutive nucleotides.

In one embodiment, the N_a' and/or N_b' comprise modifications of alternating pattern.

The Y'Y'Y' motif occurs at or near the cleavage site of the antisense strand. For example, when the RNAi agent has a duplex region of 17-23nucleotidein length, the Y'Y'Y' motif can occur at positions 9, 10, 11;10, 11, 12; 11, 12, 13; 12, 13, 14 ; or 13, 14, 15 of the antisense strand, with the count starting from the 1st nucleotide, from the 5'-end; or optionally, the count starting at the 1st paired nucleotide within the duplex region, from the 5'- end. Preferably, the Y'Y'Y' motif occurs at positions 11, 12, 13.

In one embodiment, Y'Y'Y' motif is all 2'-OMe modified nucleotides.

In one embodiment, k is 1 and l is 0, or k is 0 and l is 1, or both k and l are 1.

The antisense strand can therefore be represented by the following formulas:

5' $n_{q'}$-N_a'-Z'Z'Z'-N_b'-Y'Y'Y'-N_a'-$n_{p'}$ 3' (IIb);

5' $n_{q'}$-N_a'-Y'Y'Y'-N_b'-X'X'X'-$n_{p'}$ 3' (IIc); or

5' $n_{q'}$-N_a'- Z'Z'Z'-N_b'-Y'Y'Y'-N_b'- X'X'X'-N_a'-$n_{p'}$ 3' (IId).

When the antisense strand is represented by formula (IIb), N_b' represents an oligonucleotide sequence comprising 0-10, 0-7, 0-5, 0-4, 0-2 or 0 modified nucleotides. Each N_a' independently represents an oligonucleotide sequence comprising 2-20, 2-15, or 2-10 modified nucleotides.

When the antisense strand is represented as formula (IIc), N_b' represents an oligonucleotide sequence comprising 0-10, 0-7, 0-5, 0-4, 0-2 or 0 modified nucleotides. Each N_a' independently represents an oligonucleotide sequence comprising 2-20, 2-15, or 2-10 modified nucleotides.

When the antisense strand is represented as formula (IId), each N_b' independently represents an oligonucleotide sequence comprising 0-10, 0-7, 0-5, 0-4, 0-2 or 0 modified nucleotides. Each N_a' independently represents an oligonucleotide sequence comprising 2-20, 2-15, or 2-10 modified nucleotides. Preferably, N_b is 0, 1, 2, 3, 4, 5 or 6.

In other embodiments, k is 0 and l is 0 and the antisense strand may be represented by the formula:

5' $n_{p'}$-$N_{a'}$-Y'Y'Y'- $N_{a'}$-$n_{q'}$ 3' (Ia).

When the antisense strand is represented as formula (IIa), each N_a' independently represents an oligonucleotide sequence comprising 2-20, 2-15, or 2-10 modified nucleotides.

Each of X', Y' and Z' may be the same or different from each other.

Each nucleotide of the sense strand and antisense strand may be independently modified with LNA, HNA, CeNA, 2'-methoxyethyl, 2'-O-methyl, 2'-O-allyl, 2'-C- allyl, 2'-hydroxyl, or 2'-fluoro. For example, each nucleotide of the sense strand and antisense strand is independently modified with 2'-O-methyl or 2'-fluoro. Each X, Y, Z, X', Y' and Z', in particular, may represent a 2'-O-methyl modification or a 2'-fluoro modification.

In one embodiment, the sense strand of the RNAi agent may contain YYY motif occurring at 9, 10 and 11 positions of the strand when the duplex region is 21 nt, the count starting from the 1st nucleotide from the 5'-end, or optionally, the count starting at the 1st paired nucleotide within the duplex region, from the 5'- end; and Y represents 2'-F modification. The

sense strand may additionally contain XXX motif or ZZZ motifs as wing modifications at the opposite end of the duplex region; and XXX and ZZZ each independently represents a 2'-OMe modification or 2'-F modification.

In one embodiment the antisense strand may contain Y′Y′Y′ motif occurring at positions 11, 12, 13 of the strand, the count starting from the 1st nucleotide from the 5'-end, or optionally, the count starting at the 1st paired nucleotide within the duplex region, from the 5'- end; and Y′ represents 2'-O-methyl modification. The antisense strand may additionally contain X′X′X′ motif or Z′Z′Z′ motifs as wing modifications at the opposite end of the duplex region; and X′X′X′ and Z′Z′Z′ each independently represents a 2'-OMe modification or 2'-F modification.

The sense strand represented by any one of the above formulas (Ia), (Ib), (Ic), and (Id) forms a duplex with a antisense strand being represented by any one of formulas (IIa), (IIb), (IIc), and (IId), respectively.

Accordingly, the RNAi agents for use in the methods of the invention may comprise a sense strand and an antisense strand, each strand having 14 to 30 nucleotides, the RNAi duplex represented by formula (III):

sense: 5' n_p -N_a-(X X X)$_i$ -N_b- Y Y Y -N_b -(Z Z Z)$_j$-N_a-n_q 3'

antisense: 3' n_p'-N_a'-(X'X'X')$_k$-N_b'-Y'Y'Y'-N_b'-(Z'Z'Z')$_l$-N_a'-n_q' 5'

(III)

wherein:

i, j, k, and l are each independently 0 or 1;

p, p′, q, and q′ are each independently 0-6;

each N_a and N_a' independently represents an oligonucleotide sequence comprising 0-25 modified nucleotides, each sequence comprising at least two differently modified nucleotides;

each N_b and N_b' independently represents an oligonucleotide sequence comprising 0-10 modified nucleotides;

wherein

each n_p', n_p, n_q', and n_q, each of which may or may not be present, independently represents an overhang nucleotide; and

XXX, YYY, ZZZ, X′X′X′, Y′Y′Y′, and Z′Z′Z′ each independently represent one motif of three identical modifications on three consecutive nucleotides.

In one embodiment, i is 0 and j is 0; or i is 1 and j is 0; or i is 0 and j is 1; or both i and j are 0; or both i and j are 1. In another embodiment, k is 0 and l is 0; or k is 1 and l is 0; k is 0 and l is 1; or both k and l are 0; or both k and l are 1.

Exemplary combinations of the sense strand and antisense strand forming a RNAi duplex include the formulas below:

5' n_p - N_a -Y Y Y -N_a-n_q 3'

3' n_p'-N_a'-Y′Y′Y′ -N_a'n_q' 5'

(IIIa)

5' n_p -N_a -Y Y Y -N_b -Z Z Z -N_a-n_q 3'

3' n_p'-N_a'-Y′Y′Y′-N_b'-Z′Z′Z′-N_a'n_q' 5'

(IIIb)

5' n_p-N_a- X X X -N_b -Y Y Y - N_a-n_q 3'

3' n_p'-N_a'-X′X′X′-N_b'-Y′Y′Y′-N_a'-n_q' 5'

(IIIc)

5' n_p -N_a -X X X -N_b-Y Y Y -N_b- Z Z Z -N_a-n_q 3'

3' n_p'-N_a'-X′X′X′-N_b'-Y′Y′Y′-N_b'-Z′Z′Z′-N_a-n_q' 5'

(IIId)

When the RNAi agent is represented by formula (IIIa), each N_a independently represents an oligonucleotide sequence comprising 2-20, 2-15, or 2-10 modified nucleotides.

When the RNAi agent is represented by formula (IIIb), each N_b independently represents an oligonucleotide sequence comprising 1-10, 1-7, 1-5 or 1-4 modified nucleotides. Each N_a independently represents an oligonucleotide sequence comprising 2-20, 2-15, or 2-10 modified nucleotides.

When the RNAi agent is represented as formula (IIIc), each N_b, N_b' independently represents an oligonucleotide sequence comprising 0-10, 0-7, 0-5, 0-4, 0-2 or 0modified nucleotides. Each N_a independently represents an oligonucleotide sequence comprising 2-20, 2-15, or 2-10 modified nucleotides.

When the RNAi agent is represented as formula (IIId), each N_b, N_b' independently represents an oligonucleotide sequence comprising 0-10, 0-7, 0-5, 0-4, 0-2 or 0modified nucleotides. Each N_a, N_a' independently represents an oligonucleotide sequence comprising 2-20, 2-15, or 2-10 modified nucleotides. Each of N_a, N_a', N_b and N_b' independently comprises modifications of alternating pattern.

Each of X, Y and Z in formulas (III), (IIIa), (IIIb), (IIIc), and (IIId) may be the same or different from each other.

When the RNAi agent is represented by formula (III), (IIIa), (IIIb), (IIIc), and (IIId), at least one of the Y nucleotides may form a base pair with one of the Y′ nucleotides. Alternatively, at least two of the Y nucleotides form base pairs with the corresponding Y′ nucleotides; or all three of the Y nucleotides all form base pairs with the corresponding Y′ nucleotides.

When the RNAi agent is represented by formula (IIIb) or (IIId), at least one of the Z nucleotides may form a base pair with one of the Z′ nucleotides. Alternatively, at least two of the Z nucleotides form base pairs with the corresponding Z′ nucleotides; or all three of the Z nucleotides all form base pairs with the corresponding Z′ nucleotides.

When the RNAi agent is represented as formula (IIIc) or (IIId), at least one of the X nucleotides may form a base pair with one of the X′ nucleotides. Alternatively, at least two of the X nucleotides form base pairs with the corresponding X′ nucleotides; or all three of the X nucleotides all form base pairs with the corresponding X′ nucleotides.

In one embodiment, the modification on the Y nucleotide is different than the modification on the Y' nucleotide, the modification on the Z nucleotide is different than the modification on the Z' nucleotide, and/or the modification on the X nucleotide is different than the modification on the X' nucleotide.

In one embodiment, when the RNAi agent is represented by formula (IIId), the N_a modifications are 2′-O-methyl or 2′-fluoro modifications. In another embodiment, when the RNAi agent is represented by formula (IIId), the N_a modifications are 2′-O-methyl or 2′-fluoro modifications and $n_p' > 0$ and at least one n_p' is linked to a neighboring nucleotide a via phosphorothioate linkage. In yet another embodiment, when the RNAi agent is represented by

formula (IIId), the N_a modifications are 2′-O-methyl or 2′-fluoro modifications , n_p' >0 and at least one n_p' is linked to a neighboring nucleotide via phosphorothioate linkage, and the sense strand is conjugated to one or more GalNAc derivatives attached through a bivalent or trivalent branched linker. In another embodiment, when the RNAi agent is represented by formula (IIId), the N_a modifications are 2′-O-methyl or 2′-fluoro modifications , n_p' >0 and at least one n_p' is linked to a neighboring nucleotide via phosphorothioate linkage, the sense strand comprises at least one phosphorothioate linkage, and the sense strand is conjugated to one or more GalNAc derivatives attached through a bivalent or trivalent branched linker.

In one embodiment, when the RNAi agent is represented by formula (IIIa), the N_a modifications are 2′-O-methyl or 2′-fluoro modifications , n_p' >0 and at least one n_p' is linked to a neighboring nucleotide via phosphorothioate linkage, the sense strand comprises at least one phosphorothioate linkage, and the sense strand is conjugated to one or more GalNAc derivatives attached through a bivalent or trivalent branched linker.

In one embodiment, the RNAi agent is a multimer containing at least two duplexes represented by formula (III), (IIIa), (IIIb), (IIIc), and (IIId), wherein the duplexes are connected by a linker. The linker can be cleavable or non-cleavable. Optionally, the multimer further comprises a ligand. Each of the duplexes can target the same gene or two different genes; or each of the duplexes can target same gene at two different target sites.

In one embodiment, the RNAi agent is a multimer containing three, four, five, six or more duplexes represented by formula (III), (IIIa), (IIIb), (IIIc), and (IIId), wherein the duplexes are connected by a linker. The linker can be cleavable or non-cleavable. Optionally, the multimer further comprises a ligand. Each of the duplexes can target the same gene or two different genes; or each of the duplexes can target same gene at two different target sites.

In one embodiment, two RNAi agents represented by formula (III), (IIIa), (IIIb), (IIIc), and (IIId) are linked to each other at the 5' end, and one or both of the 3' ends and are optionally conjugated to to a ligand. Each of the agents can target the same gene or two different genes; or each of the agents can target same gene at two different target sites.

iRNA Conjugates

The iRNA agents disclosed herein can be in the form of conjugates. The conjugate may be attached at any suitable location in the iRNA molecule, e.g., at the 3' end or the 5' end of the sense or the antisense strand. The conjugates are optionally attached via a linker.

In some embodiments, an iRNA agent described herein is chemically linked to one or more ligands, moieties or conjugates, which may confer functionality, e.g., by affecting (e.g., enhancing) the activity, cellular distribution 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 some embodiments, a ligand provides an enhanced affinity for a selected target, *e.g*, molecule, cell or cell type, 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. Typical 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-glycolied) 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, biotin, or an RGD peptide or RGD peptide mimetic.

In some embodiments, the ligand is a GalNAc ligand that comprises one or more N-acetylgalactosamine (GalNAc) derivatives. Additional description of GalNAc ligands is provided in the section titled Carbohydrate Conjugates.

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 pharmacokinetic modulator (PK 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 backbone 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.

Ligand-conjugated oligonucleotides of the invention may be synthesized by the use of an oligonucleotide that bears a pendant reactive functionality, such as that derived from the attachment of a linking molecule onto the oligonucleotide (described below). This reactive

oligonucleotide may be reacted directly with commercially-available ligands, ligands that are synthesized bearing any of a variety of protecting groups, or ligands that have a linking moiety attached thereto.

The oligonucleotides used in the conjugates of the present invention may be conveniently and routinely made through the well-known technique of solid-phase synthesis. Equipment for such synthesis is sold by several vendors including, for example, Applied Biosystems (Foster City, Calif.). Any other means for such synthesis known in the art may additionally or alternatively be employed. It is also known to use similar techniques to prepare other oligonucleotides, such as the phosphorothioates and alkylated derivatives.

In the ligand-conjugated oligonucleotides and ligand-molecule bearing sequence-specific linked nucleosides of the present invention, the oligonucleotides and oligonucleosides may be assembled on a suitable DNA synthesizer utilizing standard nucleotide or nucleoside precursors, or nucleotide or nucleoside conjugate precursors that already bear the linking moiety, ligand-nucleotide or nucleoside-conjugate precursors that already bear the ligand molecule, or non-nucleoside ligand-bearing building blocks.

When using nucleotide-conjugate precursors that already bear a linking moiety, the synthesis of the sequence-specific linked nucleosides is typically completed, and the ligand molecule is then reacted with the linking moiety to form the ligand-conjugated oligonucleotide. In some embodiments, the oligonucleotides or linked nucleosides of the present invention are synthesized by an automated synthesizer using phosphoramidites derived from ligand-nucleoside conjugates in addition to the standard phosphoramidites and non-standard phosphoramidites that are commercially available and routinely used in oligonucleotide synthesis.

Lipid Conjugates

In one embodiment, the ligand is a lipid or lipid-based molecule. Such a lipid or lipid-based molecule can typically bind a serum protein, such as 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 (*e.g.*, inhibit) 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 one embodiment, the lipid based ligand binds HSA. For example, the ligand can bind HSA with a sufficient affinity such that distribution of the conjugate to a non-kidney tissue is enhanced. However, the affinity is typically not so strong that the HSA-ligand binding cannot be reversed.

In another embodiment, the lipid based ligand binds HSA weakly or not at all, such that distribution of the conjugate to the kidney is enhanced. 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).

Cell Permeation Agents

In another aspect, the ligand is a cell-permeation agent, such as a helical cell-permeation agent. In one embodiment, 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 typically an α-helical agent, and can have a lipophilic and a lipophobic phase.

The ligand can be a peptide or peptidomimetic. 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:3367). An RFGF analogue (*e.g.*, amino acid sequence AALLPVLLAAP (SEQ ID NO:3368)) 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:3369)) and the *Drosophila Antennapedia* protein (RQIKIWFQNRRMKWKK (SEQ ID NO: 3370)) 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). Typically, the peptide or peptidomimetic tethered to a dsRNA agent via an incorporated monomer unit 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 for use in the compositions and methods of the invention may be linear or cyclic, and may be modified, *e.g.,* glycosylated or methylated, to facilitate targeting to a specific tissue(s). RGD-containing peptides and peptidiomimemtics may include D-amino acids,

as well as synthetic RGD mimics. In addition to RGD, one can use other moieties that target the integrin ligand. Preferred conjugates of this ligand target PECAM-1 or VEGF.

An RGD peptide moiety can be used to target a particular cell type, e.g., 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). Typically, 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).

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

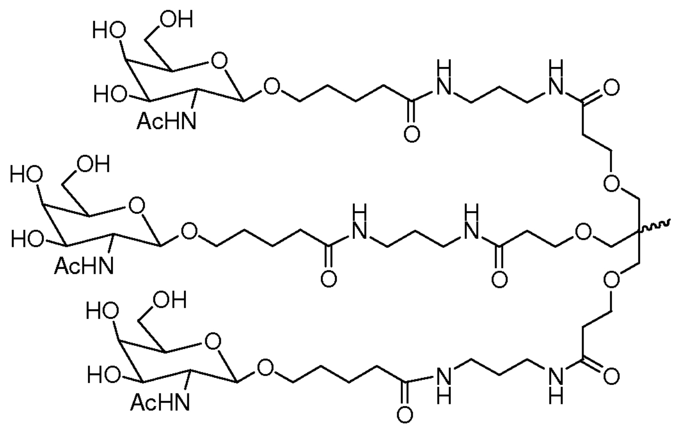
In some embodiments of the compositions and methods of the invention, an iRNA oligonucleotide further comprises a carbohydrate. The carbohydrate conjugated iRNA are advantageous for the *in vivo* delivery of nucleic acids, as well as compositions suitable for *in vivo* therapeutic use, as described herein. 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 can 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 can 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, 5, 6, 7, 8, or 9 monosaccharide units), and polysaccharides such as starches, glycogen, cellulose and polysaccharide gums. Specific monosaccharides include C5 and above (*e.g.,* C5, C6, C7, or C8) sugars; di- and trisaccharides include sugars having two or three monosaccharide units (*e.g.,* C5, C6, C7, or C8).

In one embodiment, a carbohydrate conjugate comprises a monosaccharide. In one embodiment, the monosaccharide is an N-acetylgalactosamine (GalNAc). GalNAc conjugates are described, for example, in U.S. Patent No. 8,106,022, the entire content of which is hereby incorporated herein by reference. In some embodiments, the GalNAc conjugate serves as a ligand that targets the iRNA to particular cells. In some embodiments, the GalNAc conjugate targets the iRNA to liver cells, e.g., by serving as a ligand for the asialoglycoprotein receptor of liver cells (e.g., hepatocytes).

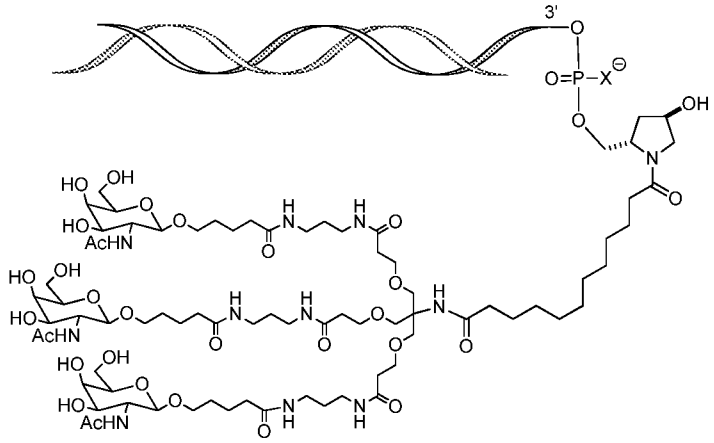
In some embodiments, the carbohydrate conjugate comprises one or more GalNAc derivatives. The GalNAc derivatives may be attached via a linker, e.g., a bivalent or trivalent branched linker. In some embodiments the GalNAc conjugate is conjugated to the 3' end of the sense strand. In some embodiments, the GalNAc conjugate is conjugated to the iRNA agent (e.g., to the 3' end of the sense strand) via a linker, e.g., a linker as described herein.

In some embodiments, the GalNAc conjugate is

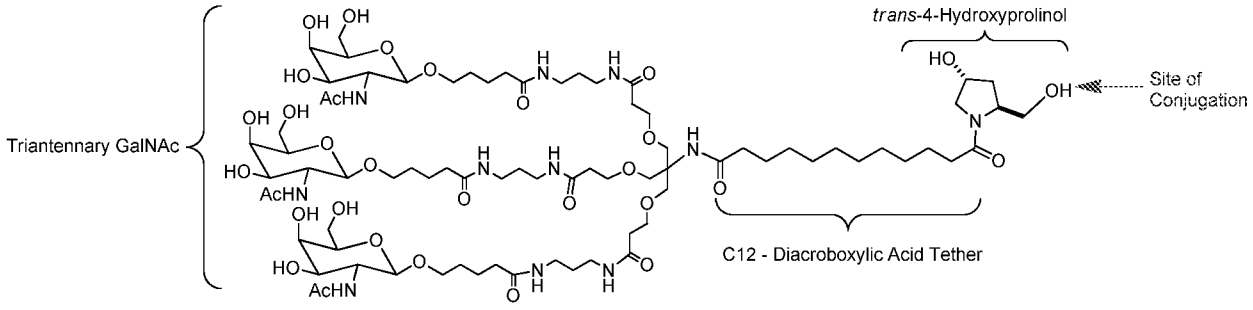


Formula II.

In some embodiments, the RNAi agent is attached to the carbohydrate conjugate via a linker as shown in the following schematic, wherein X is O or S

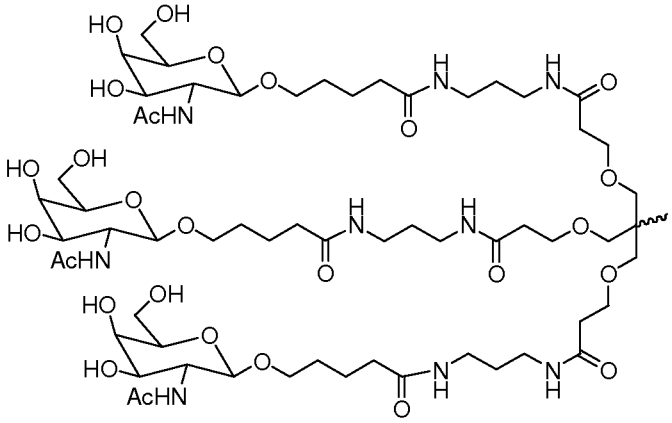


In some embodiments, the RNAi agent is conjugated to L96 as defined in Table 1 and shown below

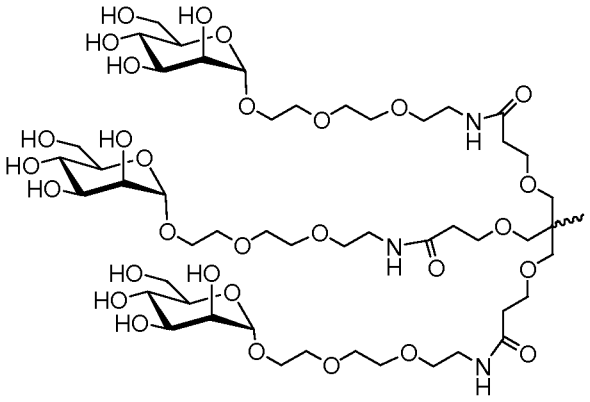


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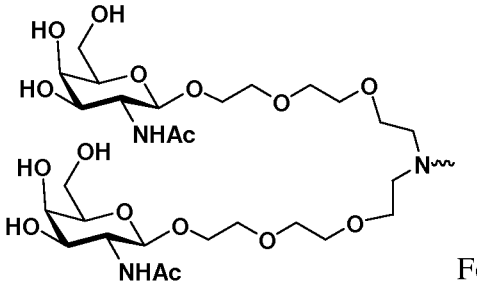
In some embodiments, a carbohydrate conjugate for use in the compositions and methods of the invention is selected from the group consisting of:



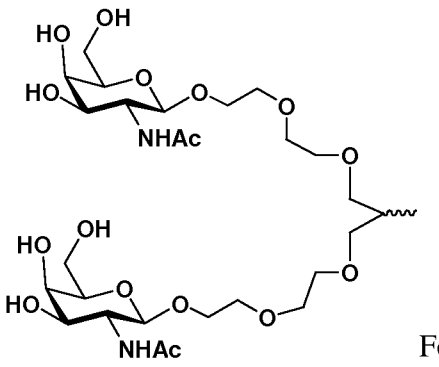
Formula II,



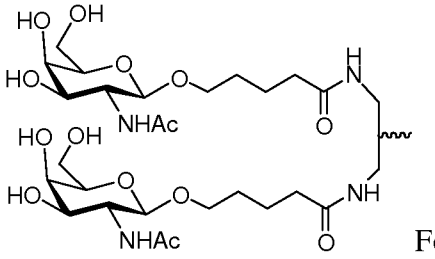
Formula III,



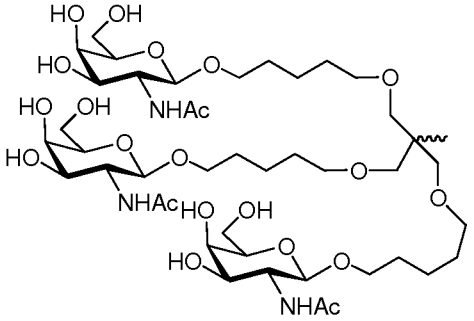
Formula IV,



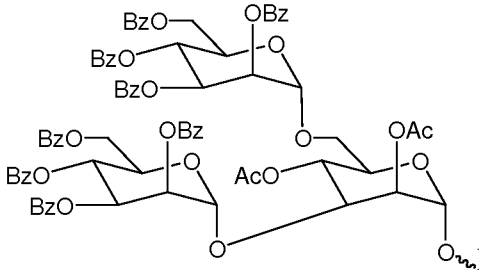
Formula V,



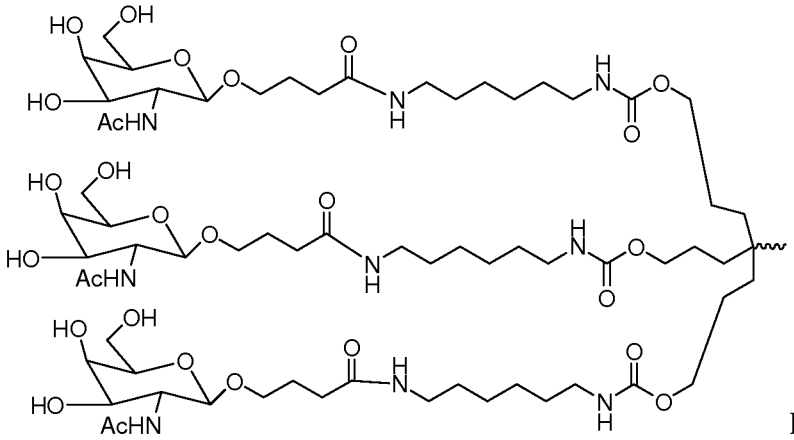
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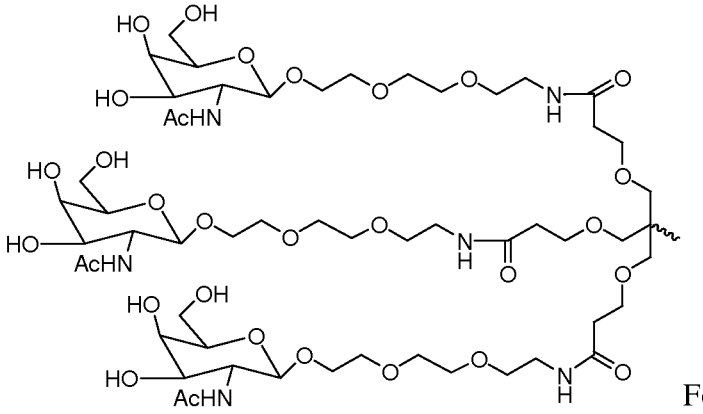
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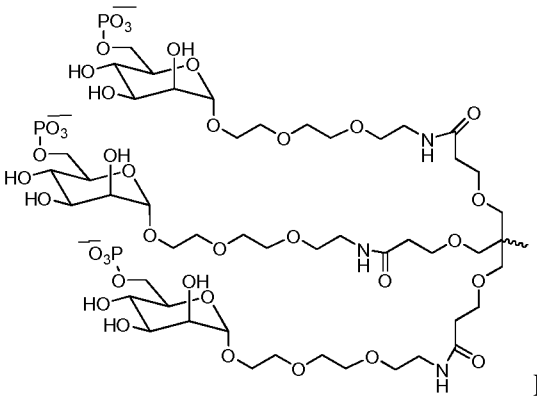
Formula VIII,



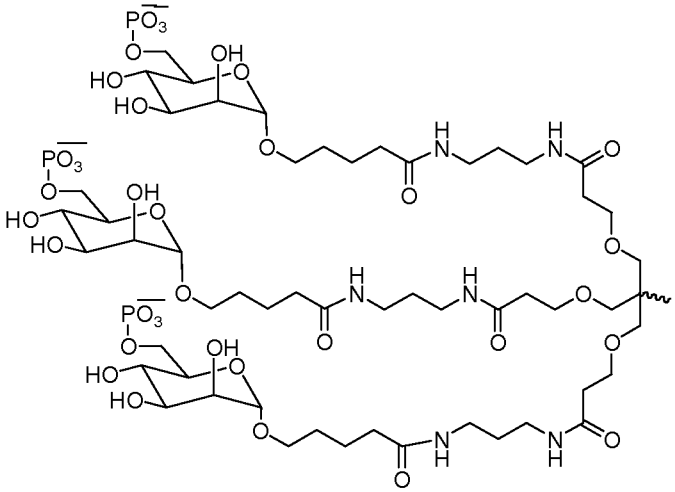
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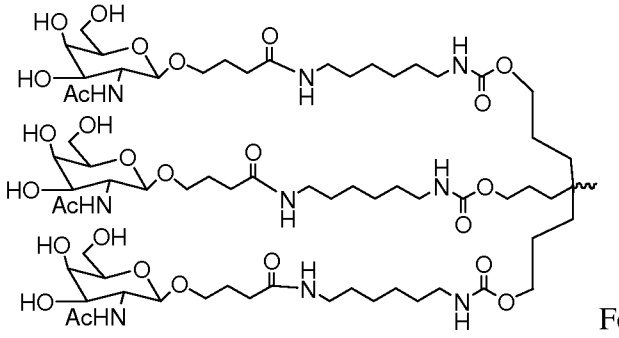
Formula X,



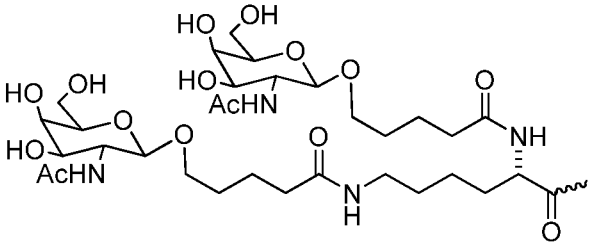
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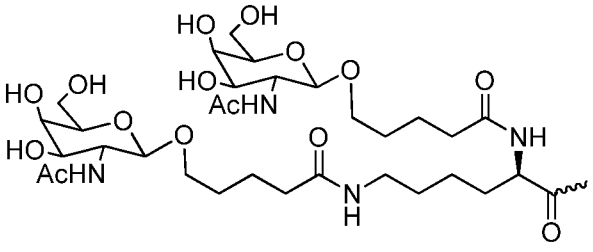
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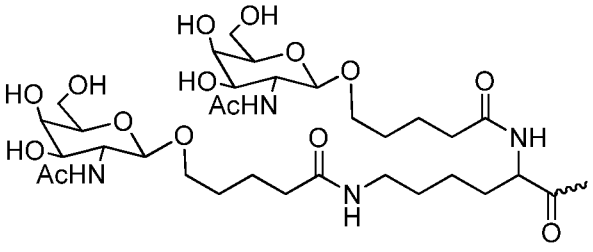
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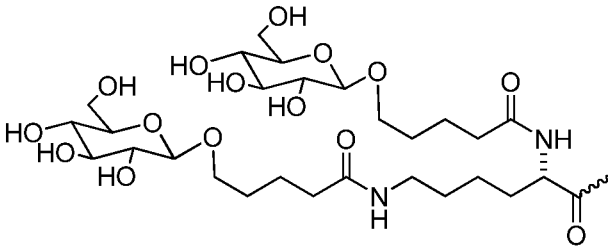
Formula XIV,



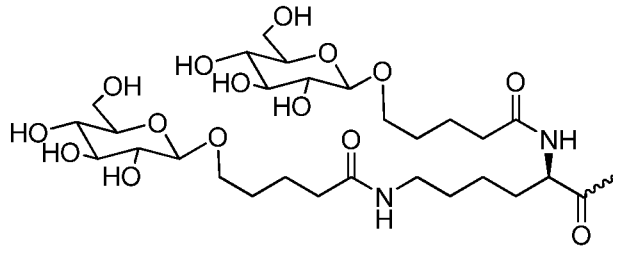
Formula XV,



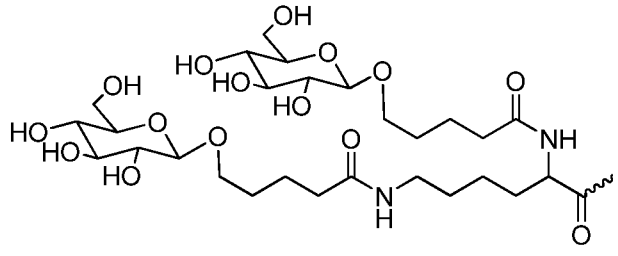
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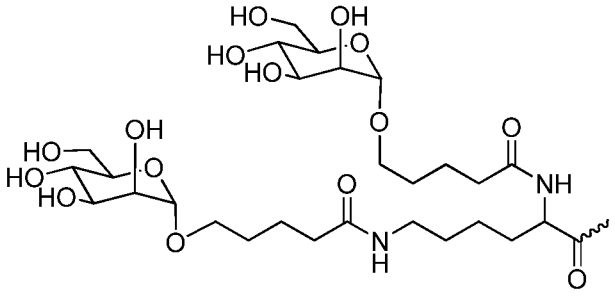
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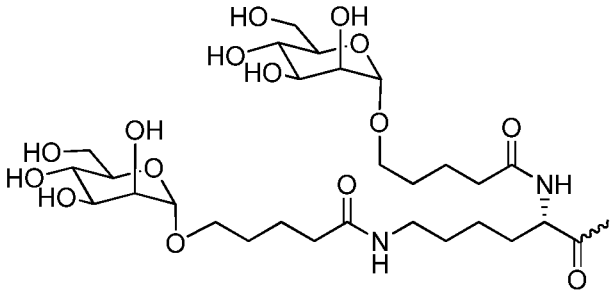
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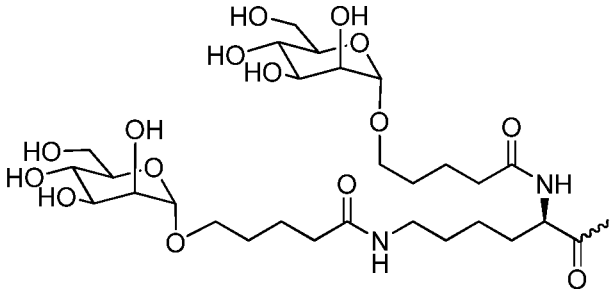
Formula XIX,



Formula XX,

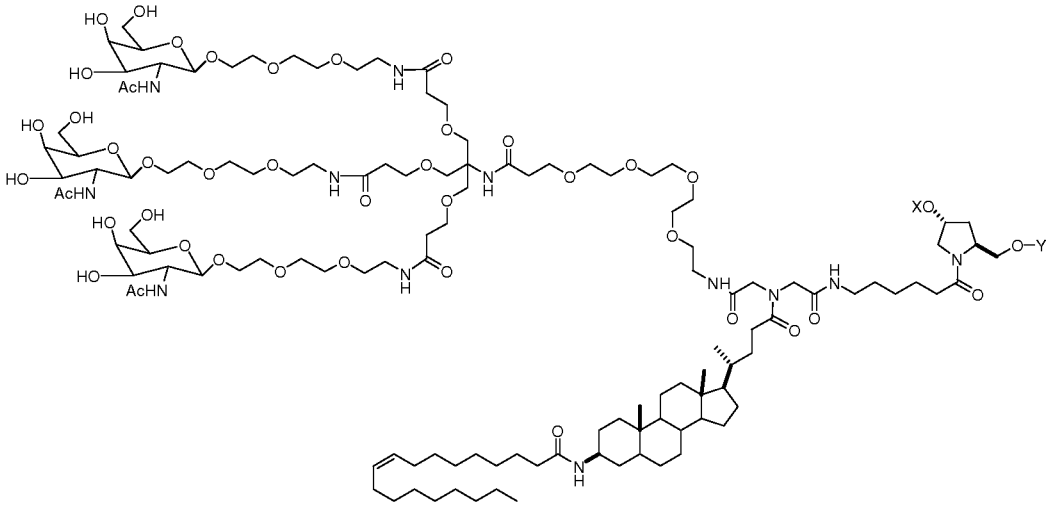


Formula XXI,



Formula XXII.

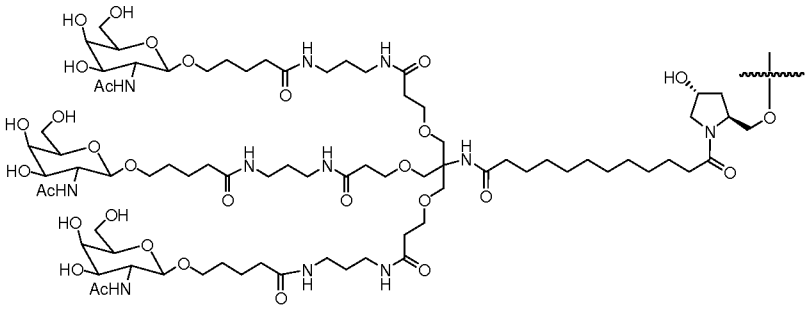
Another representative carbohydrate conjugate for use in the embodiments described herein includes, but is not limited to,



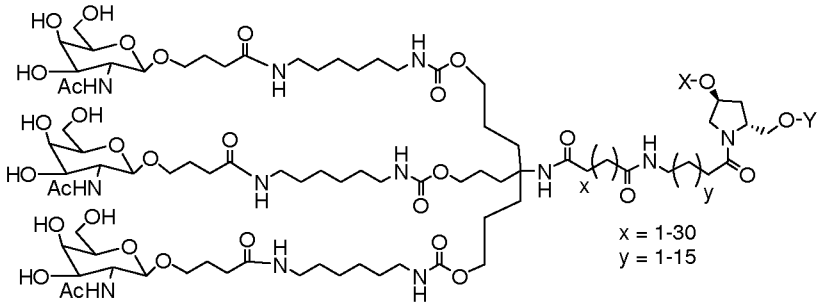
(Formula XXIII), when one of X or Y is an oligonucleotide, the other is a hydrogen.

In some embodiments, the carbohydrate conjugate further comprises one or more additional ligands as described above, such as, but not limited to, a PK modulator and/or a cell permeation peptide.

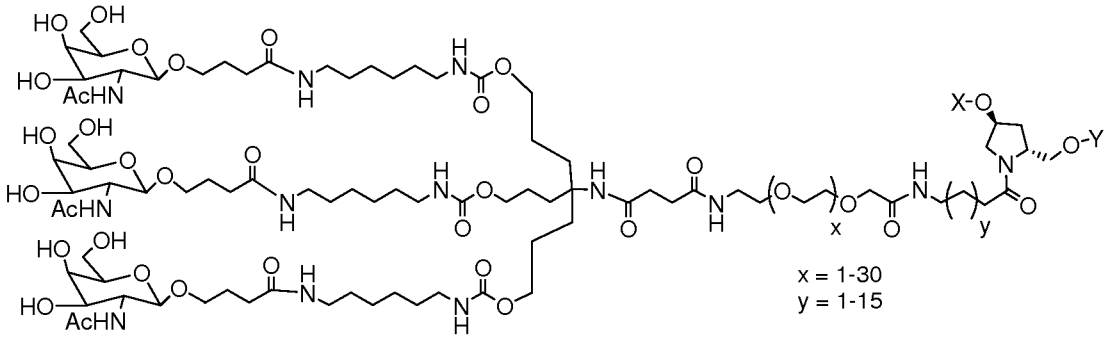
In one embodiment, an iRNA of the invention is conjugated to a carbohydrate through a linker. Non-limiting examples of iRNA carbohydrate conjugates with linkers of the compositions and methods of the invention include, but are not limited to,



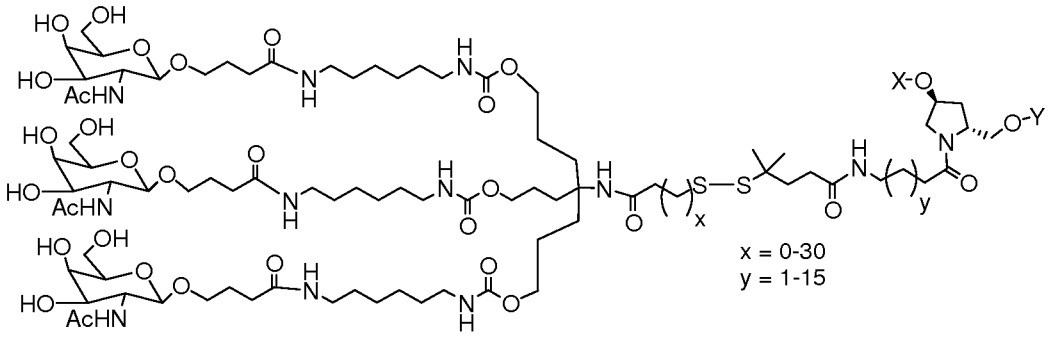
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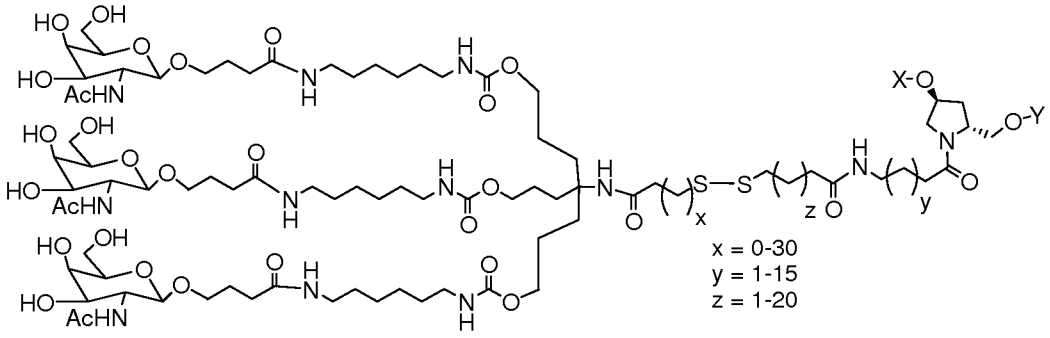
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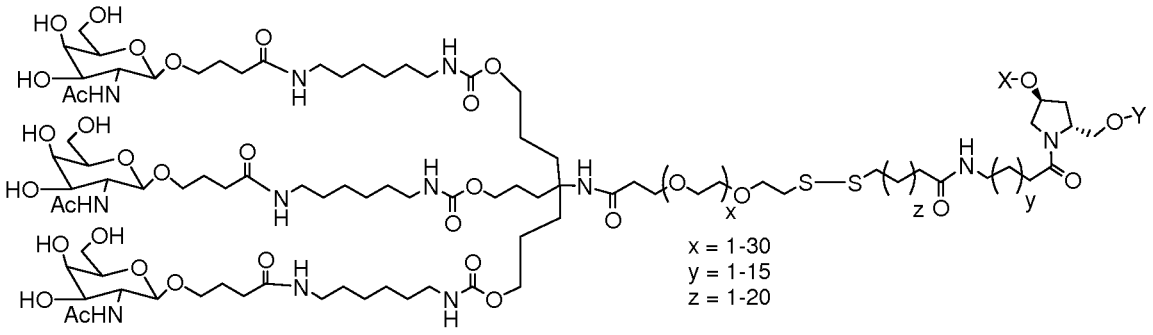
(Formula XXVI),



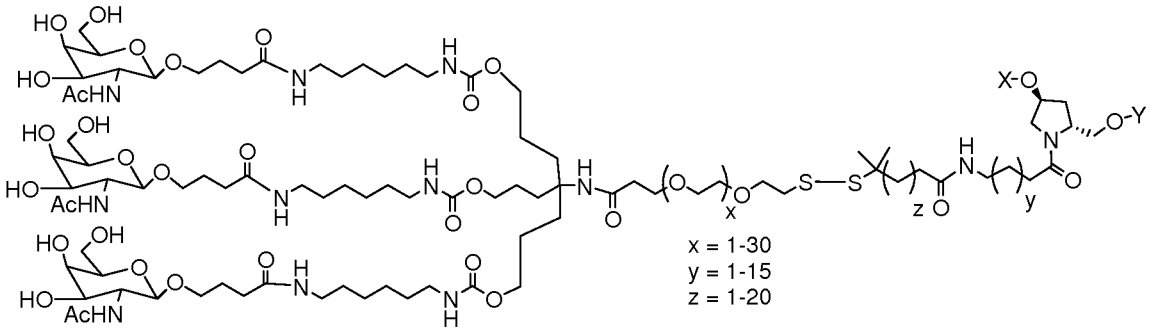
(Formula XXVII),



(Formula XXVIII),



(Formula XXIX), and



(Formula XXX), when one of X or Y is an oligonucleotide, the other is a hydrogen.

Linkers

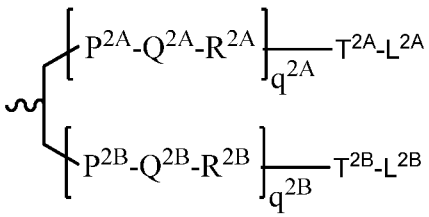
In some embodiments, the conjugate or ligand described herein can be attached to an iRNA oligonucleotide with various linkers that can be cleavable or non-cleavable.

The term "linker" or “linking group” means an organic moiety that connects two parts of a compound, *e.g.,* covalently attaches two parts of a compound. Linkers typically comprise a direct bond or an atom such as oxygen or sulfur, a unit such as NR8, 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(R8), C(O), substituted or unsubstituted aryl, substituted or unsubstituted heteroaryl, substituted or unsubstituted heterocyclic; where R8 is hydrogen, acyl, aliphatic or substituted aliphatic. In one embodiment, the linker is between about 1-24 atoms, 2-24, 3-24, 4-24, 5-24, 6-24, 6-18, 7-18, 8-18 atoms, 7-17, 8-17, 6-16, 7-16, or 8-16 atoms.

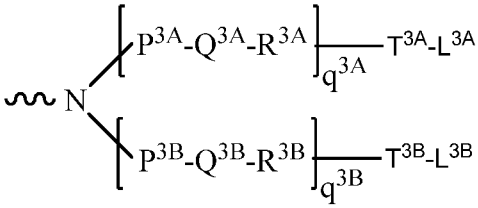
In one embodiment, a dsRNA of the invention is conjugated to a bivalent or trivalent branched linker selected from the group of structures shown in any of formula (XXXI) – (XXXIV):

Formula XXXI

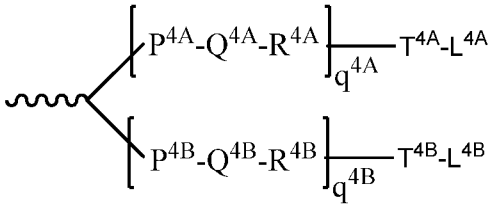


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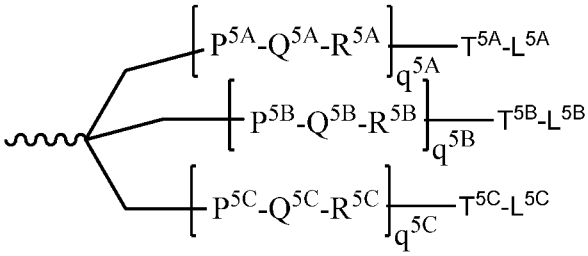
Formula XXXII



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Formula XXXIII

Formula XXXIV

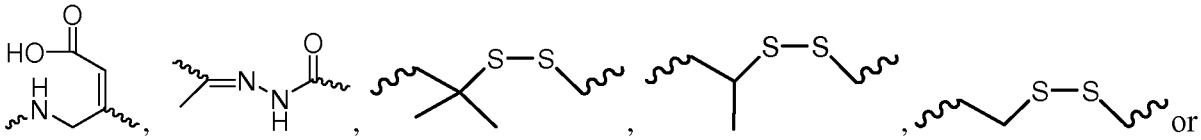
wherein:

q2A, q2B, q3A, q3B, q4A, q4B, q5A, q5B and q5C represent independently for each occurrence 0-20 and wherein the repeating unit can be the same or different;

P^{2A}, P^{2B}, P^{3A}, P^{3B}, P^{4A}, P^{4B}, P^{5A}, P^{5B}, P^{5C}, T^{2A}, T^{2B}, T^{3A}, T^{3B}, T^{4A}, T^{4B}, T^{4A}, T^{5B}, T^{5C} are each independently for each occurrence absent, CO, NH, O, S, OC(O), NHC(O), CH_2, CH_2NH or CH_2O;

Q^{2A}, Q^{2B}, Q^{3A}, Q^{3B}, Q^{4A}, Q^{4B}, Q^{5A}, Q^{5B}, Q^{5C} are independently for each occurrence absent, alkylene, substituted alkylene wherin one or more methylenes can be interrupted or terminated by one or more of O, S, S(O), SO_2, $N(R^N)$, C(R')=C(R''), C≡C or C(O);

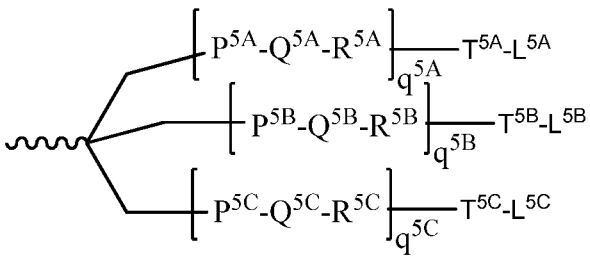
R^{2A}, R^{2B}, R^{3A}, R^{3B}, R^{4A}, R^{4B}, R^{5A}, R^{5B}, R^{5C} are each independently for each occurrence absent, NH, O, S, CH_2, C(O)O, C(O)NH, $NHCH(R^a)C(O)$, $-C(O)-CH(R^a)-NH-$, CO, CH=N-O,



heterocyclyl;

L^{2A}, L^{2B}, L^{3A}, L^{3B}, L^{4A}, L^{4B}, L^{5A}, L^{5B} and L^{5C} represent the ligand; *i.e.* each independently for each occurrence a monosaccharide (such as GalNAc), disaccharide, trisaccharide, tetrasaccharide, oligosaccharide, or polysaccharide; andR^a is H or amino acid side chain.Trivalent conjugating GalNAc derivatives are particularly useful for use with RNAi agents for inhibiting the expression of a target gene, such as those of formula (XXXV):

Formula XXXV



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wherein L^{5A}, L^{5B} and L^{5C} represent a monosaccharide, such as GalNAc derivative.

Examples of suitable bivalent and trivalent branched linker groups conjugating GalNAc derivatives include, but are not limited to, the structures recited above as formulas II, VII, XI, X, and XIII.

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 about 10 times, 20, times, 30 times, 40 times, 50 times, 60 times, 70 times, 80 times, 90 times or more, or at least about 100 times faster in a 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 a 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, a liver-targeting ligand can be linked to a cationic lipid 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 can 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 about 2, 4, 10, 20, 30, 40, 50, 60, 70, 80, 90, or about 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

In one embodiment, a cleavable linking group is a redox cleavable linking group that is 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 one, candidate compounds are cleaved by at most about 10% in the blood. In other embodiments, useful candidate compounds are degraded at least about 2, 4, 10, 20, 30, 40, 50, 60, 70, 80, 90, or about 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

In another embodiment, a cleavable linker comprises a phosphate-based cleavable linking group. A phosphate-based cleavable linking group is 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

In another embodiment, a cleavable linker comprises an acid cleavable linking group. An *a*cid cleavable linking group is a linking group that is 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, 5.75, 5.5, 5.25, 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 cleavable linking groups

In another embodiment, a cleavable linker comprises an ester-based cleavable linking group. An ester-based cleavable linking group is 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 cleavable linking groups

In yet another embodiment, a cleavable linker comprises a peptide-based cleavable linking group. A peptide-based cleavable linking group is 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 – NHCHRAC(O)NHCHRBC(O)-, where RA and RB are the R groups of the two adjacent amino acids. These candidates can be evaluated using methods analogous to those described above.

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; 8,106,022, the entire contents of 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 may 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 the present invention, are iRNA compounds, *e.g.*, dsRNAs, that 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 RNAs bearing an aminolinker 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. These alternatives are discussed further below.

Direct delivery

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 other groups, e.g., a lipid or carbohydrate group as described herein. Such conjugates can be used to target iRNA to particular cells, e.g., liver cells, e.g., hepatocytes. For example, GalNAc conjugates or lipid (e.g., LNP) formulations can be used to target iRNA to particular cells, e.g., liver cells, e.g., hepatocytes.

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, liposomes, 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 ALAS1 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, International 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, a dsRNA is expressed as an inverted repeat joined by a linker polynucleotide sequence such that the dsRNA has a stem and loop structure.

An iRNA expression vector is typically a DNA plasmid or viral vector. An expression vector compatible with eukaryotic cells, *e.g.*, 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 contain 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.

An iRNA expression plasmid can be transfected into a target cell as a complex with a cationic lipid carrier (*e.g.,* Oligofectamine) or a non-cationic lipid-based carrier (*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 typical 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, the invention provides pharmaceutical compositions containing an iRNA, as described herein, and a pharmaceutically acceptable carrier. The pharmaceutical composition containing the iRNA is useful for treating a disease or disorder related to the expression or activity of an ALAS1 gene (e.g., a disorder involving the porphyrin pathway). Such pharmaceutical compositions are formulated based on the mode of delivery. For example, compositions can be formulated for systemic administration via parenteral delivery, *e.g.*, by intravenous (IV) delivery. In some embodiments, a composition provided herein (e.g., an LNP formulation) is formulated for intravenous delivery. In some embodiments, a composition

provided herein (e.g., a composition comprising a GalNAc conjugate) is formulated for subcutaneous delivery.

The pharmaceutical compositions featured herein are administered in a dosage sufficient to inhibit expression of an ALAS1 gene. 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 the iRNA may be 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 can 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 ALAS1 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 related to ALAS1 expression (*e.g.*,

pathological processes involving porphyrins or defects in the porphyrin pathway, such as, for example, porphyrias). Such models can be used for *in vivo* testing of iRNA, as well as for determining a therapeutically effective dose and/or an effective dosing regimen.

A suitable mouse model is, for example, a mouse containing a transgene expressing human ALAS1. Mice that have knock-in mutations (e.g., mutations that are associated with acute hepatic porphyrias in humans) can be used to determine the therapeutically effective dosage and/or duration of administration of ALAS1 siRNA .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 a tissue that produces erythrocytes. For example, the iRNA can be delivered to bone marrow, liver (*e.g.*, hepatocyes of liver), lymph glands, spleen, lungs (*e.g.*, pleura of lungs) or spine. In one embodiment, the iRNA is delivered to bone marrow.

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 DNA 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 DNA 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 cyclosporin-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.*) describe 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, an ALAS1 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 may 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), l,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), l,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

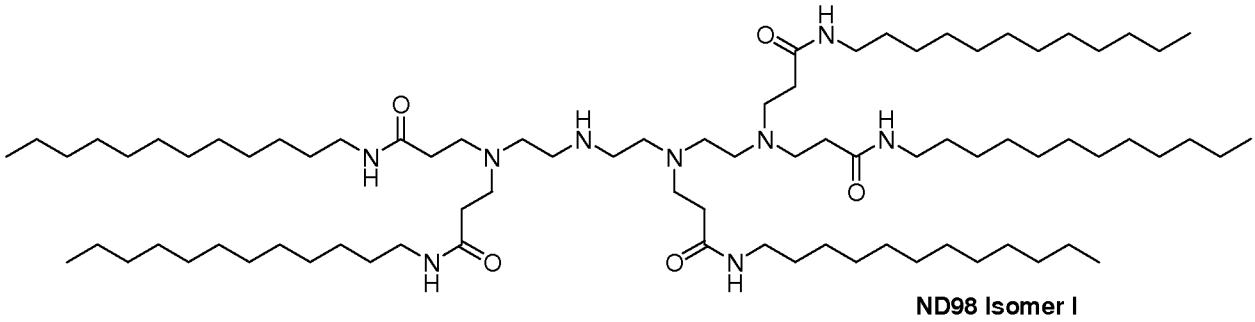
PEG-dialkyloxypropyl (DAA), a PEG-phospholipid, a PEG-ceramide (Cer), or a mixture thereof. The PEG-DAA conjugate may be, for example, a PEG-dilauryloxypropyl (Ci_2), a PEG-dimyristyloxypropyl (Ci_4), a PEG-dipalmityloxypropyl (Ci_6), or a PEG- distearyloxypropyl ($C]_8$). The conjugated lipid that prevents 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.

In some embodiments, the iRNA is formulated in a lipid nanoparticle (LNP).

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), Cholesterol (Sigma-Aldrich), and PEG-Ceramide C16 (Avanti Polar Lipids) can be used to prepare lipid-dsRNA nanoparticles (*e.g.,* 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 1

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 provided in the following table.

Table 10: Exemplary lipid formulations

	Cationic Lipid	**cationic lipid/non-cationic lipid/cholesterol/PEG-lipid conjugate Lipid:siRNA ratio**
SNALP	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
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
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 (l,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, 2009 and International Application No. PCT/US10/33777, filed May 5, 2010, which are hereby incorporated by reference.

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 may 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 may 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.

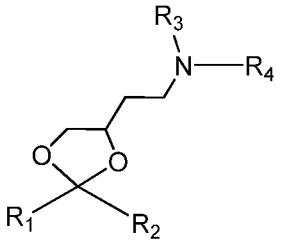
The terms "optionally substituted alkyl", "optionally substituted alkenyl", "optionally substituted alkynyl", "optionally substituted acyl", and "optionally substituted heterocycle" means that, when substituted, at least one hydrogen atom is replaced with a substituent. In the case of an oxo substituent (=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 may 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

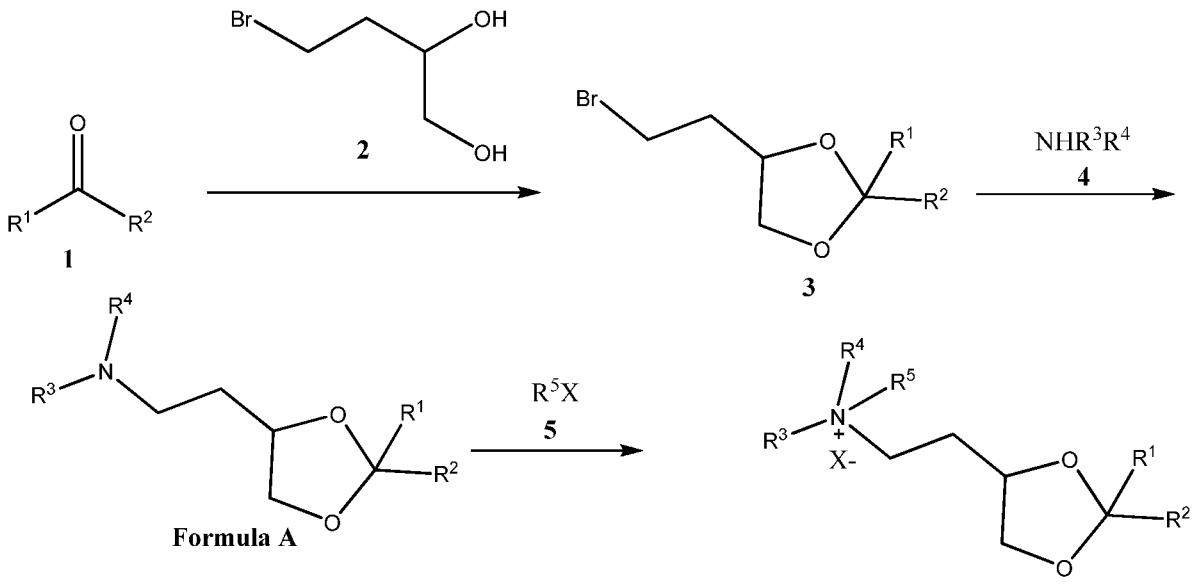
In one embodiments, nucleic acid-lipid particles featured in the invention are formulated using a cationic lipid of formula A:



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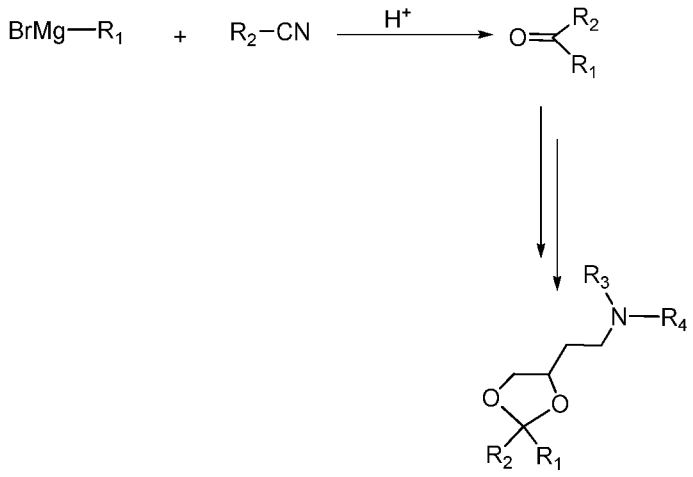
where R1 and R2 are independently alkyl, alkenyl or alkynyl, each can be optionally substituted, and R3 and R4 are independently lower alkyl or R3 and R4 can be taken together to form an optionally substituted heterocyclic ring. In some embodiments, the cationic lipid is XTC (2,2-Dilinoleyl-4-dimethylaminoethyl-[1,3]-dioxolane). 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



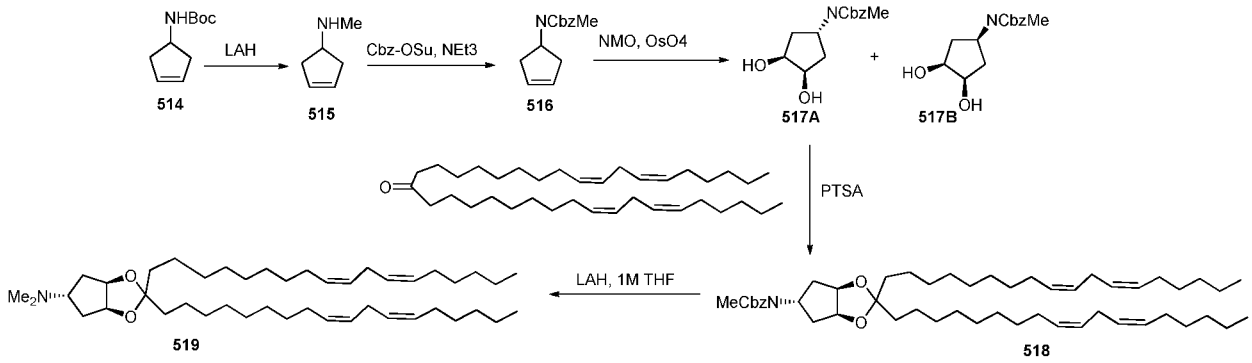
Alternatively, the ketone 1 starting material can be prepared according to Scheme 2. Grignard reagent 6 and cyanide 7 can be purchased or prepared according to methods known to those of ordinary skill in the art. Reaction of 6 and 7 yields ketone 1. Conversion of ketone 1 to the corresponding lipids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

The pharmaceutical formulations featured in 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 featured in 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 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 may 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 include, 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 may 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 biologic agents which function by a non-RNAi mechanism. Examples of such biologic agents include agents that interfere with an interaction of ALAS1 and at least one ALAS1 binding partner.

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 typical.

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 in the invention lies generally within a range of circulating concentrations that include the ED50 with little or no toxicity. The dosage may 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 may 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 featured in the invention can be administered in combination with other known agents effective in treatment of diseases or disorders related to ALAS1 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 related to expression of an ALAS1 gene

The invention relates in particular to the use of an iRNA targeting ALAS1 to inhibit ALAS1 expression and/or to treat a disease, disorder, or pathological process that is related to ALAS1 expression.

As used herein, "a disorder related to ALAS1 expression," a "disease related to ALAS1 expression, a "pathological process related to ALAS1 expression," or the like includes any condition, disorder, or disease in which ALAS1 expression is altered (e.g., elevated), the level of one or more porphyrins is altered (e.g., elevated), the level or activity of one or more enzymes in the heme biosynthetic pathway (porphyrin pathway) is altered, or other mechisms that lead to pathological changes in the heme biosynthetic pathway. For example, an iRNA targeting an ALAS1 gene, or a combination thereof, may be used for treatment of conditions in which levels of a porphyrin or a porphyrin precursor (e.g., ALA or PBG) are elevated (*e.g.*, certain porphyrias), or conditions in which there are defects in the enzymes of the heme biosynthetic pathway (*e.g.*, certain porphyrias). Disorders related to ALAS1 expression include, for example, X-linked sideroblastic anemia (XLSA), ALA deyhdratase deficiency porphyria (Doss porphyria), acute intermittent porphyria (AIP), congenital erythropoietic porphyria, prophyria cutanea tarda, hereditary coproporphyria (coproporphyria), variegate porphyria, erythropoietic protoporphyria (EPP), and transient erythroporphyria of infancy.

As used herein, a "subject" to be treated according to the methods described herein, includes a human or non-human animal, *e.g.*, a mammal. The mammal may be, for example, a rodent (*e.g.*, a rat or mouse) or a primate (*e.g.*, a monkey). In some embodiments, the subject is a human.

In some embodiments, the subject is suffering from a disorder related to ALAS1 expression (*e.g.*, has been diagnosed with a porphyria or has suffered from one or more symptoms of porphyria and is a carrier of a mutation associated with porphyria) or is at risk of developing a disorder related to ALAS1 expression (*e.g.*, a subject with a family history of porphyria, or a subject who is a carrier of a genetic mutation associated with porphyria).

Classifications of porphyrias, including acute hepatic porphyrias, are described, e.g., in Balwani, M. & Desnick, R.J., *Blood*, 120(23), published online as Blood First Edition paper, July 12, 102; DOI 10.1182/blood-2012-05-423186. As described in Balwain & Desnick, acute intermittent porphyria (AIP) hereditary coproporphyria (HCP), variegate porphyria (VP) are autosomal dominant porphyrias and ALA deyhdratase deficiency porphyria (ADP) is autosomal recessive. In rare cases, AIP, HCP, and VP occur as homozygous dominant forms. In addition, there is a rare homozygous recessive form of porphyria cutanea tarda (PCT), which is the single hepatic cutaneous porphyria, and is also known as hepatoerythropoietic porphyria. The clinical and laboratory features of these porphyrias are described in Table 11 below.

Table 11: Human hepatic porphyrias: clinical and laboratory features

Porphyria	Deficient enzyme	Inheritance	Principal symptoms, NV or CP	Enzyme activity, % of normal	Increased porphyrin precursors and/or porphyrins*		
					Erythrocytes	Urine	Stool
Acute hepatic porphyrias							
ADP	ALA-dehydratase	AR	NV	~5	Zn-protoporphyrin	ALA, coproporphyrin III	_
AIP	HMB-synthase	AD	NV	~50	_	ALA, PBG, uroporphyrin	_
HCP	COPRO-oxidase	AD	NV and CP	~50	_	ALA, PBG, coproporphyrin III	coproporphyrin III
VP	PROTO-oxidase	AD	NV and CP	~50	_	ALA, PBG coproporphyrin III	coproporphyrin III, protoporphyrin
Hepatic cutaneous porphyrias							
PCT	URO-decarboxylase	Sporadic or AD	CP	<20	_	uroporphyrin, 7-carboxylate porphyrin	uroporphyrin, 7-carboxylate porphyrin

AR indicates autosomal recessive; AD, autosomal dominant; NV, neurovisceral; CP, cutaneous photosensitivity; and -, not applicable.

*Increases that may be important for diagnosis.

In some embodiments, the subject has or is at risk for developing a porphyria, e.g., a hepatic porphyria, e.g., AIP, HCP, VP, ADP, or hepatoerythropoietic porphyria.

In some embodiments, the porphyria is an acute hepatic porphyria, e.g., an acute hepatic porphyria iselected from acute intermittent porphyria (AIP), hereditary coproporphyria (HCP), variegate porphyria (VP), and ALA deyhdratase deficiency porphyria (ADP).

In some embodiments, the porphyria is a dual porphyria, e.g., at least two porphyrias. In some embodiments, the dual porphyria comprises two or more porphyrias selected from acute intermittent porphyria (AIP) hereditary coproporphyria (HCP), variegate porphyria (VP), and ALA deyhdratase deficiency porphyria (ADP).

In some embodiments, the porphyria is a homozygous dominant hepatic porphyria (e.g., homozygous dominant AIP, HCP, or VP) or hepatoerythropoietic porphyria,In some embodiments, the porphyria is AIP, HCP, VP, or hepatoerythropoietic porphyria, or a combination thereof (e.g., a dual porphyria). In embodiments, the AIP, HCP, or VP is either heterozygous dominant or homozygous dominant.

In embodiments, the subject has or is at risk for developing a porphyria, e.g., ADP, and shows an elevated level (e.g., an elevated urine level) of ALA and/or coproporphyrin III. In embodiments, the subject has or is at risk for developing a porphyria, e.g., ADP, and shows an elevated level of erythrocyte Zn-protoporphyrin.

In embodiments, the subject has or is at risk for developing a porphyria, e.g., AIP, and shows an elevated level (e.g., an elevated urine level) of ALA, PBG, and/or uroporphyrin.

In embodiments, the subject has or is at risk for developing a porphyria, e.g., HCP, and shows an elevated level (e.g., an elevated urine level) of ALA, PBG, and/or coproporphyrin III. In embodiments, the subject has or is at risk for developing a porphyria, e.g., HCP, and shows an elevated level (e.g., an elevated stool level) of coproporphyrin III.

In embodiments, the subject has or is at risk for developing a porphyria, e.g., VP, and shows an elevated level (e.g., an elevated urine level) of ALA, PBG, and/or coproporphyrin III.

In embodiments, the subject has or is at risk for developing a porphyria, e.g., HCP, and shows an elevated level (e.g., an elevated stool level) of coproporphyrin III and/or protoporphyrin.

In embodiments, the subject has or is at risk for developing a porphyria, e.g., PCT, (e.g.,hepatoerythropoietic porphyria) and shows an elevated level (e.g., an elevated urine level) of uroporphyrin and/or 7-carboxylate porphyrin. In embodiments, the subject has or is at risk for

developing a porphyria, e.g., PCT, (e.g.,hepatoerythropoietic porphyria) and shows an elevated level (e.g., an elevated stool level) of uroporphyrin and/or 7-carboxylate porphyrin.

A mutation associated with porphyria includes any mutation in a gene encoding an enzyme in the heme biosynthetic pathway (porphyrin pathway) or a gene which alters the expression of a gene in the heme biosynthetic pathway . In many embodiments, the subject carries one or more mutations in an enzyme of the porphyrin pathway (*e.g.*, a mutation in ALA deydratase or PBG deaminase). In some embodiments, the subject is suffereing from an acute porphyria (e.g., AIP, ALA deydratase deficiency porphyria).

In some cases, patients with an acute hepatic porphyria (e.g., AIP), or patients who carry mutations associated with an acute hepatic porphyria (e.g., AIP) but who are asymptomatic, have elevated ALA and/or PBG levels compared with healthy individuals. See, e.g., Floderus, Y. et al, Clinical Chemistry, 52(4): 701-707, 2006; Sardh et al., Clinical Pharmacokinetics, 46(4): 335-349, 2007. In such cases, the level of ALA and/or PBG can be elevated even when the patient is not having, or has never had, an attack. In some such cases, the patient is otherwise completely asymptomatic. In some such cases, the patient suffers from pain, e.g., neuropathic pain, which can be chronic pain (e.g., chronic neuropathic pain). In some cases, the patient has a neuropathy. In some cases, the patient has a progressive neuropathy.

In some embodiments, the subject to be treated according to the methods described herein has an elevated level of a porphyrin or a porphyrin precursor, e.g., ALA and/or PBG. Levels of a porphyrin or a porphyrin precursor can be assessed using methods known in the art or methods described herein. For example, methods of assessing uring and plasma ALA and PBG levels, as well as urine and plasma porphyrin levels, are disclosed in Floderus, Y. et al, Clinical Chemistry, 52(4): 701-707, 2006; and Sardh et al., Clinical Pharmacokinetics, 46(4): 335-349, 2007, the entire contents of which are hereby incorporated in their entirety.

In some embodiments, the subject is an animal model of a porphyria, e.g., a mouse model of a porphyria (e.g., a mutant mouse as described in Lindberg et al. Nature Genetics, 12: 195-199, 1996). In some embodiments, the subject is a human, e.g., a human who has or is at risk for developing a porphyria, as described herein. In some embodiments, the subject is not having an acute attack of porphyria. In some embodiments, the subject has never had an attack. In some

embodiments, the patient suffers from chronic pain. In some embodiments, the patient has nerve damage. In embodiments, the subject has EMG changes and/or changes in nerve conduction velocity. In some embodiments, the subject is asymptomatic. In some embodiments, the subject is at risk for developing a porphyria (e.g., carries a gene mutation associated with a porphyria) and is asymptomatic. In some embodiments, the subject has previously had an acute attack but is asymptomatic at the time of treatment.

In some embodiments, the subject is at risk for developing a porphyria and is treated prophylactically to prevent the development of a porphyria. . In some embodiments the subject has an elevated level of a porphyrin or a porphyrin precursor, e.g., ALA and/or PBG. In some embodiments, the prophylactic treatment begins at puberty. In some embodiments the treatment lowers the level (e.g., the plasma level or the urine level) of a porphyrin or a porphyrin precursor, e.g., ALA and/or PBG. In some embodiments, the treatment prevents the development of an elevated level of a porphyrin or a porphyrin precursor, e.g., ALA and/or PBG. In some embodiments, the treatment prevents the development of, or decreases the frequency or severity of, a symptom associated with a porphyria, e.g., pain or nerve damage.

In some embodiments, the level of a porphyrin or a porphyrin precursor, e.g., ALA or PBG, is elevated, e.g., in a sample of plasma or urine from the subject. In some embodiments, the level of a porphyrin or a porphyrin precursor, e.g., ALA or PBG, in the subject is assessed based on the absolute level of the porphyrin or the porphyrin precursor, e.g., ALA or PBG in a sample from the subject. In some embodiments, the level of a porphyrin or a porphyrin precursor, e.g., ALA or PBG, in the subject is assessed based on the relative level of the porphyrin or porphyrin precursor, e.g., ALA or PBG, in a sample from the subject. In some embodiments, the relative level is relative to the level of another protein or compound, e.g., the level of creatinine, in a sample from the subject. In some embodiments, the sample is a urine sample. In some embodiments, the sample is a plasma sample. In some embodiments, the sample is a stool sample.

An elevated level of a porphyrin or a porphyrin precursor, e.g., ALA and/or PBG, can be established, e.g., by showing that the subject has a level of a porphyrin or a porphyrin precursor, e.g., ALA and/or PBG (e.g., a plasma or urine level of ALA and/or PBG) that is greater than, or greater than or equal to, a reference value. A physician with expertise in the treatment of

porphyrias would be able to determine whether the level of a porphyrin or a porphyrin precursor, (e.g., ALA and/or PBG) is elevated, e.g., for the purpose of diagnosing a porphyria or for determining whether a subject is at risk for developing a porphyria, e.g., a subject may be predisposed to an acute attack or to pathology associated with a porphyria, such as, e.g., chronic pain (e.g., neuropathic pain) and neuropathy (e.g., progressive neuropathy).

As used herein, a "reference value" refers to a value from the subject when the subject is not in a disease state, or a value from a normal or healthy subject, or a value from a reference sample or population, e.g., a group of normal or healthy subjects (e.g., a group of subjects that does not carry a mutation associated with a porphyria and/or a group of subjects that does not suffer from symptoms associated with a porphyria).

In some embodiments, the reference value is a pre-disease level in the same individual. In some embodiments, the reference value is a level in a reference sample or population. In some embodiments, the reference value is the mean or median value in a reference sample or population. In some embodiments, the reference value the value that is is two standard deviations above the mean in a reference sample or population. In some embodiments, the reference value is the value that is 2.5, 3, 3.5, 4, 4.5, or 5 standard deviations above the mean in a reference sample or population.

In some embodiments, wherein the subject has an elevated level of a porphyrin or a porphyrin precursor, e.g., ALA and/or PBG, the subject has a level of ALA and/or PBG that is at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, or 90% higher than a reference value. In some embodiments, the subject has a level of a porphyrin or a porphyrin precursor, e.g., ALA and/or PBG, that is at least 2, 3, 4, 5, 6, 7, 8, 9, or 10 fold higher than a reference value.

In some embodiments, the reference value is an upper reference limit. As used herein, an "upper reference limit" refers to a level that is the upper limit of the 95% confidence interval for a reference sample or population, e.g., a group of normal (e.g., wild type) or healthy individuals, e.g., individuals who do not carry a genetic mutation associated with a porphyria and/or individuals who do not suffer from a porphyria. Accordingly, a lower reference limit refers to a level that is the lower limit of the same 95% confidence interval.

In some embodiments wherein the subject has an elevated level, e.g., a plasma level or a urine level, of a porphyrin or a porphyrin precursor, e.g., ALA or PBG, the level is greater than

or equal to 2 times, 3 times, 4 times, or 5 times that of a reference value, e.g., an upper reference limit. In some embodiments, the subject has a urine level of a porphyrin or a porphyrin precursor, e.g., ALA or PBG, that is greater than 4 times that of an upper reference limit.

In some embodiments, the reference value is a value provided in Floderus, Y. et al, Clinical Chemistry, 52(4): 701-707, 2006 or Sardh et al., Clinical Pharmacokinetics, 46(4): 335-349, 2007. In some embodiments, the reference value is a value provided in Table 1 of Sardh et al.

In some embodiments, the subject is a human and has a urine level of PBG that is greater than or equal to 4.8 mmol/mol creatinine. In certain embodiments, the subject is a human and has a urine level of PBG that is greater than, or greater than or equal to, about 3, 4, 5, 6, 7, or 8 mmol/mol creatinine.

In embodiments, the reference value for plasma PBG is 0.12 μmol/L. In embodiments, the subject is a human and has a plasma PBG level that is greater than, or greater than or equal to, 0.10 μmol/L, 0.12 μmol/L, 0.24 μmol/L, 0.36 μmol/L, 0.48 μmol/L, or 0.60 μmol/L. In embodiments, the subject is a human and has a plasma level of PBG that is greater than, or greater than or equal to, 0.48 μmol/L.

In embodiments, the reference value for urine PBG is 1.2 mmol/mol creatinine. In embodiments, the subject is a human and has a urine PBG level that is greater than, or greater than or equal to, 1.0 mmol/mol creatinine, 1.2 mmol/mol creatinine, 2.4 mmol/mol creatinine, 3.6 mmol/mol creatinine, 4.8 mmol/mol creatinine, or 6.0 mmol/mol creatinine. In embodiments, the subject is a human and has a urine level of PBG that is greater than, or greater than or equal to, 4.8 mmol/mol creatinine.

In embodiments, the reference value for plasma ALA is 0.12 μmol/L. In embodiments, the subject is a human and has a plasma ALA level that is greater than, or greater than or equal to, 0.10 μmol/L, 0.12 μmol/L, 0.24 μmol/L, 0.36 μmol/L, 0.48 μmol/L, or 0.60 μmol/L. In embodiments, the subject is a human and has a plasma ALA level that is greater than, or greater than or equal to 0.48 μmol/L.

In embodiments, the reference value for urine ALA is 3.1 mmol/mol creatinine. In embodiments, the subject is a human and has a urine ALA level that is greater than, or greater

than or equal to, 2.5 mmol/mol creatinine, 3.1 mmol/mol creatinine, 6.2 mmol/mol creatinine, 9.3 mmol/mol creatinine, 12.4 mmol/mol creatinine, or 15.5 mmol/mol creatinine.

In embodiments, the reference value for plasma porphyrin is 10 nmol/L. In embodiments, the subject is a human and has a plasma porphyrin level that is greater than, or greater than or equal to, 10 nmol/L. In embodiments, the subject is a human and has a plasma porphyrin level that is greater than, or greater than or equal to, 8, 10, 15, 20, 25, 30, 35, 40, 45, or 50 nmol/L. the subject is a human and has a plasma porphyrin level that is greater than, or greater than or equal to 40 nmol/L.In embodiments, the reference value for urine porphyrin is 25 µmol/mol creatinine. In embodiments, the subject is a human and has a urine porphyrin level that is greater than, or greater than or equal to, 25 µmol/mol creatinine. In embodiments, the subject is a human and has a urine porphyrin level that is greater than, or equal to, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, or 80 µmol/mol creatinine.

In some embodiments, the subject has a level, e.g., a plasma level or a urine level, of a porphyrin or a porphyrin precursor, e.g., ALA or PBG, that is greater than that of 99% of individuals in a sample of healthy individuals.

In some embodiments, the subject has a level, e.g., a plasma level or a urine level, of ALA or PBG that is greater than two standard deviations above the mean level in a sample of healthy individuals.

In some embodiments, the subject has a urine level of ALA that is 1.6 or more times that of the mean level in a normal subject (e.g., a subject that does not carry a mutation associated with a porphyria). In some embodiments, the subject has a plasma level of ALA that is 2 or 3 times that of the mean level in a normal subject. In some embodiments, the subject has a urine level of PBG that is four or more times that of the mean level in a normal subject. In some embodiments, the subject has a plasma level of PBG that is four or more times that of the mean level in a normal subject.

In some embodiments, the method is effective to decrease the level of a porphyrin or a porphyrin precursor, e.g., ALA and/or PBG. In embodiments, the method is effective to produce a predetermined reduction in the elevated level of the porphyrin or porphyrin precursor, e.g., ALA or PBG. In some embodiments, the predetermined reduction is a decrease of at least 10%,

20%, 30%, 40%, or 50%. In some embodiments, the predetermined reduction is a reduction that is effective to prevent or ameliorate symptoms, e.g., pain or recurring attacks.

In some embodiments, the predetermined reduction is a reduction that is at least 1, 2, 3, or more standard deviations, wherein the standard deviation is determined based on the values from a reference sample, e.g., a reference sample as described herein.

In some embodiments, the predetermined reduction is a reduction that brings the level of the porphyrin or porphyrin precursor to a level that is less than, or to a level that is less than or equal to, a reference value (e.g., a reference value as described herein).

In some embodiments, the subject to be treated according to the methods described suffers from pain, e.g., chronic pain. In some embodiments, the subject has or is at risk for developing a porphyria, e.g. an acute hepatic porphyria, e.g., AIP. In embodiments, the method is effective to treat the pain, e.g., by reducing the severity of the pain or curing the pain. In embodiments, the method is effective to decrease or prevent nerve damage.

In some embodiments, the subject to be treated according to the methods described herein (a) has an elevated level of ALA and/or PBG and (b) suffers from pain, e.g., chronic pain. In embodiments, the method is effective to decrease an elevated level of ALA and/or PBG and/or to treat the pain, e.g., by reducing the severity of the pain or curing the pain.

In some embodiments, the subject is an animal that serves as a model for a disorder related to ALAS1 expression.

In some embodiments the subject is an animal that serves as a model for porphyria (*e.g.*, a genetically modified animal with one or more mutations. In some embodiments, the porphyria is AIP and the subject is an animal model of AIP. In one such embodiment, the subject is a genetically modified mouse that is deficient in porphobilinogen deaminase , such as, for example, the mouse described in Lindberg *et al.*, *Nature Genetics*, 12:195-199, 1996, or the homozygous R167Q mouse described in Yasuda, M., Yu, C. Zhang, J., Clavero, S., Edelmann, W., Gan, L., Phillips, J.D., & Desnick, R.J. Acute intermittent porphyria: A severely affected knock-in mouse that mimics the human homozygous dominant phenotype. (Abstract of Presentation on October 14, 2011 at the American Society of Human Genetics; Program No. 1308F; accessed online on April 4, 2012 at ichg2011.org/cgi-bin/showdetail.pl?absno=21167);

both of these references are hereby incorporated herein in their entirety. Several knock-in models for mutations causing homozygous dominant AIP in humans have been generated. The mutations employed include, *e.g.*, R167Q, R173Q, and R173W in PBG deaminase. Viable homozygotes included the R167Q/R176Q and R167Q/R173Q, both of which exhibit constitutively elevated ALA and PBG levels analogous to the phenotype in human homozygous dominant AIP; in some embodiments, such a viable homozygous AIP mouse model is the subject.

In one embodiment, a subject to be treated according to the methods described herein, (*e.g.*, a human subject or patient), is at risk of developing, or has been diagnosed, with a disorder related to ALAS1 expression, e.g. a porphyria. In some embodiments, the subject is a subject who has suffered one or more acute attacks of one or more porphyric symptoms. In other embodiments, the subject is a subject who has suffered chronically from one or more symptoms of porphyria (e.g., pain, e.g., neuropathic pain and or neuropathy, e.g., progressive neuropathy). In some embodiments, the subject carries a genetic alteration (e.g., a mutation) as described herein but is otherwise asymptomatic. In some embodiments, the subject has previously been treated with a heme product (e.g., hemin, heme arginate, or heme albumin), as described herein.

In some embodiments, a subject (*e.g.*, a subject with a porphyria, such as, *e.g.*, AIP) to be treated according to the methods described herein has recently experienced or is currently experiencing a prodrome. In some such embodiments, the subject is administered a combination treatment, e.g., an iRNA as described herein, and one or more additional treatments known to be effective against porphyria (*e.g.*, glucose and/or a heme product such as hemin, as described herein) or its associated symptoms.

In one embodiment, an iRNA as described herein is administered in combination with glucose or dextrose. For example, 10-20% dextrose in normal saline may be provided intravenously. Typically, when glucose is administered, at least 300 g of 10% glucose is administered intravenously daily. The iRNA (e.g., an iRNA in an LNP formulation) may also be administered intravenously, as part of the same infusion that is used to administer the glucose or dextrose, or as a separate infusion that is administered before, concurrently, or after the administration of the glucose or dextrose. In some embodiments, the iRNA is administered via a different route of administration (*e.g.*, subcutaneously). In yet another embodiment, the iRNA is

administered in combination with total parenteral nutrition. The iRNA may be administered before, concurrent with, or after the administration of total parenteral nutrition.

In one embodiment, the iRNA is administered in combination with a heme product (*e.g.*, hemin, heme arginate, or heme albumin). In a further embodiment, the iRNA is administered in combination with a heme product and glucose, a heme product and dextrose, or a heme product and total parenteral nutrition.

A "prodrome," as used herein, includes any symptom that the individual subject has previously experienced immediately prior to developing an acute attack. Typical symptoms of a prodrome include, e.g., abdominal pain, nausea, headaches, psychological symptoms (e.g., anxiety), restlessness and/or insomnia. In some embodiments, the subject experiences pain (e.g., abdominal pain and/or a headache) during the prodrome. In some embodiments, the subject experiences nausea during the prodrome. In some embodiments, the subject experiences psychological symptoms (e.g., anxiety) during the prodrome. In some embodiments, the subject becomes restless and/or suffers from insomnia during the prodrome.

An acute "attack" of porphyria involves the onset of one or more symptoms of porphyria, typically in a patient who carries a mutation associated with porphyria (*e.g.*, a mutation in a gene that encodes an enzyme in the porphyrin pathway).

In certain embodiments, administration of an ALAS1 iRNA results in a decrease in the level of one or more porphyrins or porphyrin precursors, as described herein (*e.g.,* ALA and/or PBG). The decrease may be measured relative to any appropriate control or reference value. For example, the decrease in the level of one or more porphyrins or porphyrin precursors may be established in an individual subject, *e.g.*, as a decrease of at least 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50% or more compared with the level prior to treatment (*e.g.*, immediately prior to treatment). A decrease in the level of a porphyrin precursor, a porphyrin, or or a porphyrin metabolite may be measured using any method known in the art. For example, the level of PBG and/or ALA in urine or plasma may be assessed, using the Watson-Schwartz test, ion exchange chromatography, or high-performance liquid chromatography – mass spectrometry. See, *e.g.*, Thunell (1993).

In some embodiments, administration of an ALAS1 siRNA is effective to reduce the level of ALA and/or PBG in the subject. The level of ALA or PBG in the subject can be

assessed, e.g., based on the absolute level of ALA or PBG, or based on the relative level of ALA or PBG (e.g., relative to the level of another protein or compound, e.g., the level of creatinine) in a sample from the subject. In some embodiments, the sample is a urine sample. In some embodiments, the sample is a plasma sample.

In certain embodiments, an iRNA that targets ALAS1 is administered in combination one or more additional treatments, *e.g.*, another treatment known to be effective in treating porphyria or symptoms of porphyria. For example, the other treatment may be glucose (*e.g.*, IV glucose) or a heme product (*e.g.*, hemin, heme arginate, or heme albumin). The additional treatment(s) may be administered before, after, or concurrent with the administration of iRNA.

The iRNA and an additional therapeutic agent can be administered in combination in the same composition, *e.g.*, intravenously, or the additional therapeutic agent can be administered as part of a separate composition or by another method described herein.

In some embodiments, administration of iRNA, or administration of iRNA in combination one or more additional treatments (*e.g.*, glucose, dextrose or the like), decreases the frequency of acute attacks (e.g., by preventing acute attacks so that they no longer occur, or by reducing the number of attacks that occur in a certain time period, e.g., fewer attacks occur per year). In some such embodiments, the iRNA is administered according to a regular dosing regimen, *e.g.*, daily, weekly, biweekly, or monthly.

In some embodiments, the iRNA is administered after an acute attack of porphyria. In some such embodiments, the iRNA is in a composition, e.g. a composition comprising a lipid formulation, e.g. an LNP formulation.

In some embodiments, the iRNA is administered during an acute attack of porphyria. In some such embodiments, the iRNA is in a composition, e.g. a composition comprising a lipid formulation (e.g., an LNP formulation) or a composition comprising a GalNAc conjugate.

In some embodiments, administration of an ALAS1 siRNA is effective to lessen the severity of the attack (e.g., by ameliorating one or more signs or symptoms associated with the attack). In some embodiments, administration of an ALAS1 siRNA is effective to shorten the duration of an attack. In some embodiments, administration of an ALAS1 siRNA is effective to stop an attack. In some embodiments, the iRNA is administered prophylactically to prevent an acute attack of porphyria. In some such embodiments, the iRNA is in the form of a GalNAc

conjugate, e.g., in a composition comprising a GalNAc conjugate. In some embodiments, the prophylactic administration is before, during, or after exposure to or occurrence of a precipitating factor. In some embodiments, the subject is at risk of developing porphyria.

In some embodiments, the siRNA is administered during a prodrome. In some embodiments, the prodrome is characterized by pain (e.g., headache and/or abdominal pain), nausea, psychological symptoms (e.g., anxiety), restlessness and/or insomnia.

In some embodiments, the siRNA is administered during a particular phase of the menstrual cycle, e.g., during the luteal phase.

In some embodiments, administration of an ALAS1 siRNA is effective to prevent attacks (e.g., recurrent attacks that are associated with a prodrome and/or with a precipitating factor, e.g., with a particular phase of the menstrual cycle, e.g., the luteal phase). In some embodiments, administration of an ALAS1 siRNA is effective to reduce the frequency of attacks. In embodiments, administration of an ALAS1 siRNA is effective to lessen the severity of the attack (e.g., by ameliorating one or more signs or symptoms associated with the attack). In some embodiments, administration of an ALAS1 siRNA is effective to shorten the duration of an attack. In some embodiments, administration of an ALAS1 siRNA is effective to stop an attack.

In some embodiments administration of an ALAS1 siRNA is effective to prevent or decrease the frequency or severity of pain, e.g., neuropathic pain.

In some embodiments administration of an ALAS1 siRNA is effective to prevent or decrease the frequency or severity of neuropathy

Effects of administration of an ALAS1 siRNA can be established, for example, by comparison with an appropriate control. For example, a decrease in the frequency of acute attacks, as well as a decrease in the level of one or more porphyrins or porphyrin precursors, may be established, for example, in a group of patients with AIP, as a decreased frequency compared with an appropriate control group. A control group (e.g., a group of similar individuals or the same group of individuals in a crossover design) may include, for example, an untreated population, a population that has been treated with a conventional treatment for porphyria (*e.g.*, a conventional treatment for AIP may include glucose, hemin, or both); a population that has been

treated with placebo, or a non-targeting iRNA, optionally in combination with one or more conventional treatments for porphyria (*e.g.*, glucose, *e.g.*, IV glucose), and the like.

A subject "at risk" of developing porphyria, as used herein, includes a subject with a family history of porphyria and/or a history of one or more recurring or chronic porphyric symptoms, and/or a subject who carries a genetic alteration (e.g., a mutation) in a gene encoding an enzyme of the heme biosynthetic pathway, and a subject who carries a genetic alteration, e.g., a mutation. known to be associated with porphyria.

In embodiments, the alteration, e.g., the mutation, makes an individual susceptible to an acute attack (e.g., upon exposure to a precipitating factor, e.g., a drug, dieting or other precipitating factor, e.g., a precipitating factor as disclosed herein). In embodiments, the alteration, e.g., the mutation, is associated with elevated levels of a porphyrin or a porphyrin precursor (e.g., ALA and/or PBG). In embodiments, the alteration, e.g., the mutation, is associated with chronic pain (e.g., chronic neuropathic pain) and/or neuropathy (e.g., progressive neuropathy). In embodiments, the , the alteration, e.g., the mutation, is associated with changes in EMG and/or nerve conduction velocities.

In embodiments, the alteration is a mutation in the ALAS1 gene. In embodiments, the alteration is a mutation in the ALAS1 gene promoter, or in regions upstream or downstream from the ALAS1 gene. In embodiments, the alteration is a mutation in transcription factors or other genes that interact with ALAS1. In embodiments, the alteration is an alteration, e.g., a mutation, in a gene that encodes an enzyme in the heme biosynthetic pathway.

In some embodiments, the subject has an genetic alteration as described herein (e.g., a genetic mutation known to be associated with a porphyria). In some such embodiments, the subject has an elevated level (e.g., urine or plasma level) of ALA and/or PBG. In some such embodiments, the subject does not have an elevated level of ALA and/or PBG. In embodiments, the subject has a genetic alteration as described herein and has other symptoms, e.g., chronic pain, EMG changes, changes in nerve conduction velocity, and/or other symptoms associated with a porphyria. In embodiments, the subject has a genetic alteration but does not suffer from acute attacks.

In embodiments, the subject has a mutation associated with AIP, HCP, VP, or ADP.

In some embodiments, the porphyria is AIP. In some such embodiments, the subject has an alteration, e.g., at least one mutation, in the PBG deaminase gene. Many PBG deaminase mutations are known in the art, for example, as reported in Hrdinka, M. *et al.* Physiological Research, 55 (Suppl 2):S119-136 (2006). In some embodiments, the subject is heterozygous for a PBG deaminase mutation. In other embodiments, the subject is homozygous for a PBG deaminase mutation. A homozygous subject may carry two identical mutations or two different mutations in the PBG deaminase gene.

In some embodiments, the porphyria is HCP. In some such embodiments, the subject has an alteration, e.g., at least one mutation, in the gene that encodes the enzyme coproporphyrinogen III oxidase.

In some embodiments, the porphyria is VP. In some such embodiments, the subject has an alteration, e.g., at least one mutation, in the gene that encodes protoporphrinogen oxidase.

In embodiments, the porphyria is ADP, e.g., autosomal recessive ADP. In some such embodiments, the subject has an alteration, e.g., at least one mutation, in the gene that encodes ALA deydratase.

Methods of treatment provided herein may serve to ameliorate one or more symptoms associated with porphyria, to reduce the frequency of attacks associated with porphyria, to reduce the likelihood that an attack of one or more symptoms associated with porphyria will occur upon exposure to a precipitating factor, or to reduce the risk of developing conditions associated with porphyria (*e.g.*, neuropathy (e.g., progressive neuropathy), hepatocellular cancer). Additionally, the methods provided herein may serve to decrease the level of one or more porphyrin precursors, porphyrins and/or related porphyrin products or metabolites. The level of a porphyrin precursor or a porhyrin may be measured in any biological sample, such as, *e.g.*, urine, blood, feces, cerebrospinal fluid, or a tissue sample. The sample may be present within a subject or may be obtained or extracted from the subject. In some embodiments, the porphyria is AIP, and the level of PBG and/or ALA is decreased. In some embodiments, the porphyrin product or metabolite is porphobilin, porphobilinogen, or uroporphyrin. A decrease in the level of a porphyrin product or metabolite may be measured using any method known in the art. For example, the level of PBG and/or ALA in urine or plasma may be assessed, using the

Watson-Schwartz test, ion exchange chromatography, or high-performance liquid chromatography – mass spectrometry. See, *e.g.*, Thunell (1993).

Methods described herein may also serve to reduce chronically elevated levels of porphyrin precursors (e.g., ALA and/or PBG) in subjects suffering from a porphyria (e.g., an acute hepatic porphyria, e.g., AIP) or at risk for developing a porphyria. Methods for assessing plasma and urine levels (e.g., chronically elevated levels) of porphyrin precursors include, e.g., HPLC-mass spectrometry and ion-exchange chromatography. The levels of porphyrin precursors may be expressed as the level relative to another protein or compound, e.g., creatinine. See, e.g., Floderus, Y. et al, Clinical Chemistry, 52(4): 701-707, 2006; Sardh et al., Clinical Pharmacokinetics, 46(4): 335-349, 2007

A "precipitating factor" as used herein, refers to an endogenous or exogenous factor that may induce an acute attack of one or more symptoms associated with porphyria. Precipitating factors include fasting (or other forms of reduced or inadequate caloric intake, due to crash diets, long-distance athletics, etc.), metabolic stresses (*e.g.*, infections, surgery, international air travel, and psychological stress), endogenous hormones (e.g., progesterone), cigarette smoking, lipid-soluble foreign chemicals (including, *e.g.*, chemicals present in tobacco smoke, certain prescription drugs, organic solvents, biocides, components in alcoholic beverages), endocrine factors (e.g., reproductive hormones (women may experience exacerbations during the premenstrual period), synthetic estrogens, progesterones, ovulation stimulants, and hormone replacement therapy). See, for example, Thunell (1993). Common precipitating factors include cytochrome P450 inducing drugs and phenobarbital.

Symptoms associated with porphyria may include abdominal pain or cramping, headaches, effects caused by nervous system abnormalities, and light sensitivity, causing rashes, blistering, and scarring of the skin (photodermatitis). In certain embodiments, the porphyria is AIP. Symptoms of AIP include gastrointestinal symptoms (*e.g.*, severe and poorly localized abdominal pain, nausea/vomiting, constipation, diarrhea, ileus), urinary symptoms (dysuria, urinary retention/incontinence, or dark urine), neurologic symptoms (*e.g.*, sensory neuropathy, motor neuropathy (e.g., affecting the cranial nerves and/or leading to weakness in the arms or legs), seizures, neuropathic pain, progressive neuropathy, headaches, neuropsychiatric symptoms (*e.g.*, mental confusion, anxiety, agitation, hallucination, hysteria, delirium, apathy, depression,

phobias, psychosis, insomnia, somnolence, coma), autonomic nervous system involvement (resulting *e.g.*, in cardiovascular sysmptoms such as tachycardia, hypertension, and/or arrhythmias, as well as other symptoms, such as, *e.g.*, increased circulating catecholamine levels, sweating, restlessness, and/or tremor), dehydration, and electrolyte abnormalities.

In some embodiments, an iRNA targeting ALAS1 is administered together with (*e.g.*, before, after, or concurrent with) another treatment that may serve to alleviate one or more of the above symptoms. For example, abdominal pain may be treated, *e.g.*, with narcotic analgesics, seizures may be treated, *e.g.*, with anti-seizure medications, nausea/vomiting may be treated, *e.g.*, with phenothiazines, and tachycardia/hypertension may be treated, *e.g.*, with beta blockers.

The term "decrease" (or "increase") is intended to refer to a measurable change, e.g., a statistically significant change. The change may be, for example, at least 5%, 10%, 20%, 30%, 40%, 50% or more change (*e.g.*, decrease (or increase) relative to a reference value, *e.g.*, a reference where no iRNA is provided).

The invention further relates to the use of an iRNA or a pharmaceutical composition thereof, *e.g.*, for treating a disorder related to ALAS1 expression, 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 the disorder. In one embodiment, the iRNA or pharmaceutical composition thereof can be administered in conjunction with a heme product (*e.g.*, hemin, heme arginate, or heme albumin, as described herein) and/or in conjunction with intravenous glucose infusions. In some embodiments, the iRNA or pharmaceutical composition thereof is used prophylactically, *e.g.*, to prevent or ameliorate symptoms of an anticipated attack of acute porphyria. The prophylactic use may be timed according to the exposure or anticipated exposure of the subject to a precipitating factor. As described herein, a precipitating factor may be any endogenous or exogenous factor known to precipitate an acute attack. For example, the premenstrual phase is an endogenous precipitating factor, and a cytochrome P450 inducing drug is an exogenous precipitating factor.

The effective amount for the treatment of a disorder related to ALAS1 expression (e.g., a porphyria such as AIP) depends on the type of disorder to be treated, the severity of the symptoms, the subject being treated, the sex, age and general condition of the subject, the mode

of administration and so forth. For any given case, an appropriate "effective amount" can be determined by one of ordinary skill in the art using routine experimentation. 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. In connection with the administration of an iRNA targeting ALAS1 or pharmaceutical composition thereof, "effective against" a disorder related to ALAS1 expression indicates that administration in a clinically appropriate manner results in a beneficial effect, e.g., for an individual patient or for at least a fraction of patients, e.g., a statistically significant fraction of patients. Beneficial effects include, e.g., prevention of or reduction of symptoms or other effects. For example, beneficial effects include, e.g., an improvement (e.g., decrease in the severity or frequency) of symptoms, a reduction in the severity or frequency of attacks, a reduced risk of developing associated disease (e.g., neuropathy (e.g., progressive neuropathy), hepatocellular cancer), an improved ability to tolerate a precipitating factor, an improvement in quality of life, a reduction in the expression of ALAS1, a reduction in a level (e.g., a plasma or urine level) of a porphyrin or a porphyrin precursor (e.g., ALA and/or PBG) or other effect generally recognized as positive by medical doctors familiar with treating the particular type of disorder.

A treatment or preventive effect is evident when there is an improvement, e.g., 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, *e.g.*, 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 (e.g., plasma or urinary ALA or PBG) or symptom is observed.

Patients can be administered a therapeutic amount of iRNA. The therapeutic amount can be, e.g., 0.05-50 mg/kg. For example, the therapeutic amount can be 0.05, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 1.5, 2.0, or 2.5, 3.0, 3.5, 4.0, 4.5, 5, 10, 15, 20, 25, 30, 35, 40, 45, or 50 mg/kg dsRNA.

In some embodiments, the iRNA is formulated as a lipid formulation, e.g., an LNP formulation as described herein. In some such embodiments, the therapeutic amount is 0.05-5 mg/kg, e.g., 0.05, 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, or 5.0 mg/kg dsRNA. In some embodiments, the lipid formulation, e.g., LNP formulation, is administered intravenously.

In some embodiments, the iRNA is 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.

In some embodiments, the iRNA is in the form of a GalNAc conjugate as described herein. In some such embodiments, the therapeutic amount is 0.5-50 mg, e.g., 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, 45, or 50 mg/kg dsRNA. In some embodiments, the GalNAc conjugate is administered subcutaneously.

In some embodiments, the administration is repeated, for example, on a regular basis, such as, daily, 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.

In some embodiments, the iRNA agent is administered in two or more doses. In some embodiments, the number or amount of subsequent doses is dependent on the achievement of a desired effect, *e.g.*, suppression of a ALAS gene, reduction of a level of a porphyrin or porphyrin precursor (e.g., ALA and/or PBG), or the achievement of a therapeutic or prophylactic effect, *e.g.*, reduction or prevention of one or more symptoms associated with porphyria (e.g., pain, e.g., neuropathic pain), and/or prevention of attacks or reduction in the frequency and/or severity of attacks associated with porphyria.

In some embodiments, the iRNA agent is administered according to a schedule. For example, the iRNA agent may be administered once per week, twice per week, three times per week, four times per week, or five times per week. In some embodiments, the schedule involves regularly spaced administrations, *e.g.*, hourly, every four hours, every six hours, every eight hours, every twelve hours, daily, every 2 days, every 3 days, every 4 days, every 5 days, weekly, biweekly, or monthly. In embodiments, the iRNA agent is administered weekly or biweekly to

achieve a desired effect, e.g., to decrease the level of ALA and/or PBG, to decrease pain, and/or to prevent acute attacks.

In embodiments, the schedule involves closely spaced administrations followed by a longer period of time during which the agent is not administered. For example, the schedule may involve an initial set of doses that are administered in a relatively short period of time (*e.g.*, about every 6 hours, about every 12 hours, about every 24 hours, about every 48 hours, or about every 72 hours) followed by a longer time period (*e.g.*, about 1 week, about 2 weeks, about 3 weeks, about 4 weeks, about 5 weeks, about 6 weeks, about 7 weeks, or about 8 weeks) during which the iRNA agent is not administered. In one embodiment, the iRNA agent is initially administered hourly and is later administered at a longer interval (*e.g.*, daily, weekly, biweekly, or monthly). In another embodiment, the iRNA agent is initially administered daily and is later administered at a longer interval (*e.g.*, weekly, biweekly, or monthly). In certain embodiments, the longer interval increases over time or is determined based on the achievement of a desired effect. In a specific embodiment, the iRNA agent is administered once daily during an acute attack, followed by weekly dosing starting on the eighth day of administration. In another specific embodiment, the iRNA agent is administered every other day during a first week followed by weekly dosing starting on the eighth day of administration.

In one embodiment, the iRNA agent is administered to prevent or reduce the severity or frequency of recurring attacks, e.g., cyclical attacks associated with a precipitating factor. In some embodiments, the precipitating factor is the menstrual cycle. In some embodiments, the iRNA is administered repeatedly, e.g., at regular intervals to prevent or reduce the severity or frequency of recurring attacks, e.g., cyclical attacks associated with a precipitating factor, e.g., the menstrual cycle, e.g., a particular phase of the menstrual cycle, e.g., the luteal phase. In some embodiments, the iRNA is administered during a particular phase of the menstrual cycle or based on hormone levels of the patient being treated (e.g., based on hormone levels that are associated with a particular phase of the menstrual cycle). In some embodiments, the iRNA is administered on one or more particular days of the menstrual cycle, e.g., on day 1, 2, 3, 4, 5, 6, 7, 8. 9. 10. 11. 12. 13, 14, 15, 16, 17, 18, 19, 20 , 21, 22, 23, 24, 25, 26, 27, or on day 28 (or later day for

subjects who have a longer menstrual cycle). In some embodiments, the iRNA is administered during the luteal phase, e.g., on one or more days between days 14-28 of the menstrual cycle (or later, in subjects who have a menstrual cycle longer than 28 days). In some embodiments, ovulation of the subject is assessed (e.g., using a blood or urine test that detects a hormone associated with ovulation, e.g., LH) and the iRNA is administered at a predetermined interval after ovulation. In some embodiments, the iRNA is administered immediately after ovulation. In some embodiments, the iRNA is administered 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, or 18 days after ovulation.Any of these schedules may optionally be repeated for one or more iterations. The number of iterations may depend on the achievement of a desired effect, *e.g.*, the suppression of a ALAS1 gene and/or the achievement of a therapeutic or prophylactic effect, *e.g.*, reduce or prevent one or more symptoms associated with porphyria, to reduce the frequency of attacks associated with porphyria.

In some embodiments, an initial dose of the iRNA agent is administered and the level of ALA or PBG is tested, e.g., 1-48 hours, e.g., 2, 4, 8, 12, or 24 hours following administration of the initial dose. In some embodiments, if the level of ALA and/or PBG has decreased (e.g., to achieve a predetermined reduction, e.g., a normalization), and/or if the symptoms associated with porphyria (e.g., pain) have improved (e.g., such that the patient is asymptomatic), no further dose is administered, whereas if the level of ALA and/or PBG has not decreased (e.g., has not achieved a predetermined reduction, e.g., has not normalized), a further dose of ALA or PBG is administered. In some embodiments, the further dose is administered 12, 24, 36, 48, 60, or 72 hours after the initial dose. In some embodiments, if the initial dose is not effective to decrease the level of ALA and/or PBG, the further dose is modified, e.g., increased to achieve a desired decrease (e.g., a predetermined reduction, e.g., a normalization) in ALA or PBG levels.

In some embodiments, the predetermined reduction is a decrease of at least 10%, 20%, 30%, 40%, or 50%. In some embodiments, the predetermined reduction is a reduction that is effective to prevent or ameliorate symptoms, e.g., pain, prodromal symptoms, or recurring attacks.

In some embodiments, the predetermined reduction is a reduction of at least 1, 2, 3, or more standard deviations, wherein the standard deviation is determined based on the values from a reference sample, e.g., a reference sample as described herein.

In some embodiments, the predetermined reduction is a reduction that brings the level of the porphyrin or porphyrin precursor to a level that is less than, or to a level that is less than or equal to, a reference value (e.g., a reference value as described herein).

As used herein, a "normalization" in ALA or PBG levels (or a "normal" or "normalized" level) refers to a level (e.g., a urine and/or plasma level) of either ALA, or PBG, or both, that is within the expected range for a healthy individual, an individual who is asymptomatic (e.g., an individual who does not experience pain and/or suffer from neuropathy), or an individual who does not have a mutation associated with a porphyria. For example, in some embodiments, a normalized level is within two standard deviations of the normal mean. In some embodiments, a normalized level is within normal reference limits, e.g., within the 95% confidence interval for an appropriate control sample, e.g., a sample of healthy individuals or individuals who do not carry a gene mutation associated with a porphyria. In some embodiments, the ALA and/or PBG level of the subject (e.g., the urine and/or plasma ALA and/or PBG level) is monitored at intervals, a further dose of the iRNA agent is administered when the level increases above the reference value

Administration of the iRNA may reduce ALAS1 mRNA or protein 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. Administration of the iRNA may reduce levels of products associated with ALAS1 gene expression, *e.g.*, levels of one or more porphyrins or porphyrin precursors (e.g., the level of ALA and/or PBG). Administration of the iRNA agent may also inhibit or prevent the upregulation of ALAS1 mRNA or protein levels during an acute attack of AIP.

Before administration of a full dose of the iRNA, patients can be administered a smaller dose, such as a 5% infusion dose, and monitored for adverse effects, such as an allergic reaction,

or for elevated lipid levels or blood pressure. In another example, the patient can be monitored for unwanted effects.

Methods for modulating expression of an ALAS1 gene

In yet another aspect, the invention provides a method for modulating (*e.g.*, inhibiting or activating) the expression of an ALAS1 gene, *e.g.*, in a cell or in a subject. In some embodiments, the cell is *ex vivo*, *in vitro*, or *in vivo*. In some embodiments, the cell is an erythroid cell or a hepatocyte. In some embodiments, the cell is in a subject (e.g., a mammal, such as, for example, a human). In some embodiments, the subject (*e.g.*, the human) is at risk, or is diagnosed with a disease related to ALAS1 expression, as described above.

In one embodiment, the method includes contacting the cell with an iRNA as described herein, in an amount effective to decrease the expression of an ALAS1 gene in the cell. "Contacting," as used herein, includes directly contacting a cell, as well as indirectly contacting a cell. For example, a cell within a subject (*e.g.*, an erythroid cell or a liver cell, such as a hepatocyte) may be contacted when a composition comprising an iRNA is administered (*e.g.*, intravenously or subcutaneously) to the subject.

The expression of an ALAS1 gene may be assessed based on the level of expression of an ALAS1 mRNA, an ALAS1 protein, or the level of a parameter functionally linked to the level of expression of an ALAS1 gene (*e.g.*, the level of a porphyrin or the incidence or severity of a symptom related to a porphyria). In some embodiments, the expression of ALAS1 is inhibited by at least 5%, at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, or at least 95%. In some embodiments, the iRNA has an IC_{50} in the range of 0.001-0.01 nM, 0.001-0.10 nM, 0.001-1.0 nM, 0.001-10 nM, 0.01-0.05 nM, 0.01-0.50 nM, 0.02-0.60 nM, 0.01-1.0 nM, 0.01-1.5 nM, 0.01-10 nM. The IC_{50} value may be normalized relative to an appropriate control value, *e.g.*, the IC_{50} of a non-targeting iRNA.

In some embodiments, the method includes introducing into the cell an iRNA as described herein and maintaining the cell for a time sufficient to obtain degradation of the

mRNA transcript of an ALAS1 gene, thereby inhibiting the expression of the ALAS1 gene in the cell.

In one embodiment, the method includes administering a composition described herein, *e.g.*, a composition comprising an iRNA that targets ALAS1, to the mammal such that expression of the target ALAS1 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. In some embodiments, the decrease in expression of ALAS1 is detectable within 1 hour, 2 hours, 4 hours, 8 hours, 12 hours, or 24 hours of the first administration.

In another embodiment, the method includes administering a composition as described herein to a mammal such that expression of the target ALAS1 gene is increased by *e.g.*, at least 10% compared to an untreated animal. In some embodiments, the activation of ALAS1 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 ALAS1 expression by stabilizing the ALAS1 mRNA transcript, interacting with a promoter in the genome, and/or inhibiting an inhibitor of ALAS1 expression.

The iRNAs useful for the methods and compositions featured in the invention specifically target RNAs (primary or processed) of an ALAS1 gene. Compositions and methods for inhibiting the expression of an ALAS1 gene 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 ALAS1 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. In some such embodiments, the compositions comprise a lipid formulated siRNA (*e.g.*, an LNP formulation, such as an LNP11 formulation) for intravenous infusion. In particular

embodiments, such compositions may be used to treat acute attacks of porphyria and/or for prophylaxis (*e.g.*, to decrease the severity or frequency of attacks).

In other embodiments, the compositions are administered subcutaneously. In some such embodiments, the compositions comprise an iRNA conjugated to a GalNAc ligand. In particular embodiments, such compositions may be used to treat acute attacks of porphyria or for prophylaxis (*e.g.*, to decrease the severity or frequency of attacks).

Methods for decreasing a level of a porphyrin or porphyrin precursor

In another aspect, the invention provides a method for decreasing a level of a porphyrin or a porphyrin precursor, *e.g.*, in a cell or in a subject.

In some embodiments, the cell is *ex vivo*, *in vitro*, or *in vivo*. In some embodiments, the cell is an erythroid cell or a hepatocyte. In some embodiments, the cell is a hepatocyte. In some embodiments, the cell is in a subject (*e.g.*, a mammal, such as, for example, a human).

In some embodiments, the subject (*e.g.*, the human) is at risk, or is diagnosed with a porphyria, as described herein. In some embodiments, the method is effective to treat a porphyria as described herein (*e.g.*, by ameliorating one or more symptoms associated with a porphyria, reducing the frequency of attacks associated with a porphyria, reducing the likelihood that an attack of one or more symptoms associated with porphyria will occur upon exposure to a precipitating factor, or reducing the risk of developing conditions associated with a porphyria (*e.g.*, neuropathy (e.g., progressive neuropathy), hepatocellular cancer). In one embodiment, the method includes contacting the cell with an RNAi, as described herein, in an amount sufficient to decrease the level of the porphyrin or porphyrin precursor (e.g., ALA or PBG) in the cell, or in another related cell or group of cells, or in the subject. "Contacting," as used herein, includes directly contacting a cell, as well as indirectly contacting a cell. For example, a cell within a subject (*e.g.*, an erythroid cell or a liver cell, such as a hepatocyte) may be contacted when a composition comprising an RNAi is administered (*e.g.*, intravenously or subcutaneously) to the subject. "Another related cell or group of cells," as used herein, includes any cell or group of cells in which the level of the porphyrin or porphyrin precursor decreases as a result of the contacting. For example, the cell may be part of a tissue present within a subject (*e.g.*, a liver cell present within a subject), and contacting the cell within the subject (*e.g.*, contacting one or

more liver cells present within a subject) with the RNAi may result in a decrease in the level of the porphyrin or porphyrin precursor in another related cell or group of cells (*e.g.*, nerve cells of the subject), or in a tissue or fluid of the subject (*e.g.*, in the urine, blood, plasma, or cerebrospinal fluid of the subject).

In some embodiments, the porphyrin or porphyrin precursor is selected from the group consisting of δ-aminolevulinic acid (ALA), porphopilinogen (PBG), hydroxymethylbilane (HMB), uroporphyrinogen III, coproporphyrinogen III, protoporphrinogen IX, and protoporphyrin IX In some embodiments the porphyrin precursor is ALA. In some embodiments, the porphyrin precursor is PBG. In some embodiments, the method decreases the level of ALA and PBG. The level of a porphyrin or a porphyrin precursor may be measured as described herein and as known in the art.

Assays and Methods for Monitoring RNAi activity

In another aspect, the invention provides assays and methods for monitoring ALAS1 mRNA levels. RNAi activity in the liver can be monitored by detecting mRNA levels or 5'RACE product in tissue, or by detecting the level of circulating secreted protein.

Alternatively, or in combination, circulating extracellular levels of ALAS1 mRNA can be detected, e.g., by cERD assays (Circulating Extracellular RNA Detection). In some embodiments, the ALAS1 mRNA level can be detected in a bodily fluid sample, e.g., a serum or urine sample. In some embodiments, exosomes are shed into bodily fluids from different cells types, which contain mRNA and miRNA derived from a tissue of origin. Such exosomes can be used to monitor the level of RNAi in circulation. In one embodiment, a sample, e.g., a serum or urine sample from a subject treated with an iRNA described herein can be purified with low speed spin, followed by a spin at about 160,000g for about 2 hours to form a pellet. RNA can be extracted and analyzed to measure the levels of ALAS1 mRNA. Exemplary methods and assays are disclosed in PCT/US2012/043584, published as WO 2012/177906, the contents of which are incorporated by reference.

Accordingly, an assay, or method, is provided for detecting the level of circulating extracellular ALAS1 mRNA in a subject. The assay, or method includes providing RNA (*e.g.*, extracellular RNA) from a biological fluid sample (e.g., urine, blood or plasma sample) from the

subject, said biological fluid sample comprising the ALAS1 mRNA; and detecting the level of circulating extracellular ALAS1 mRNA in the sample.

In one embedment, the assay or method includes the step of obtaining an ALAS1 cDNA from the ALAS1 mRNA; and contacting the ALAS1 cDNA with a nucleic acid complementary (e.g., probe and/or primer) to the ALAS1 cDNA or a portion thereof, thereby producing a reaction mix; and detecting (e.g., measuring) the level of ALAS1 cDNA in the reaction mix, wherein the ALAS1 cDNA level is indicative of the ALAS1 mRNA level, thereby assaying the level of circulating extracellular ALAS1 mRNA in the subject.

In one embodiment, the assay or method includes acquiring a biological fluid sample from a subject, where the biological sample is separate from the tissue, and where the biological sample contains exosomes. The assay or method can further include detecting the levels of an RNA in the biological sample, where the RNA is expressed from the gene in the tissue of the subject, where the exosomes are not purified from the biological sample prior to detecting levels of RNA in the biological sample.

In embodiments, said biological fluid sample is a blood sample. In embodiments, said biological fluid sample is a serum sample. In another embodiment, the biological fluid sample is a urine sample.

In embodiments, the the method comprises PCR, qPCR or 5'-RACE.

In embodiments, said nucleic acid is a probe or primer.

In embodiments, said nucleic acid comprises a detectable moiety and the level of ALAS1 mRNA is determined by detection of the amount of the detectable moiety.

In embodiments, said method further comprises obtaining the biological fluid sample from the subject.

In embodiments of these methods, the efficacy of a porphyria treatment is assessed based on a comparison of the level of circulating extracellular ALAS1 mRNA in the subject relative to a reference value.

In embodiments, a decrease in the level of circulating extracellular ALAS1 mRNA in the subject in response to the porphyria treatment, relative to the reference value, indicates that the porphyria treatment is efficacious. In embodiments, the reference value is the level of circulating extracellular ALAS1 mRNA in the subject prior to the porphyria treatment.

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. siRNA 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.

All 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 oligonucleotidess 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.

siRNA preparation

For the general preparation of siRNA, 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.

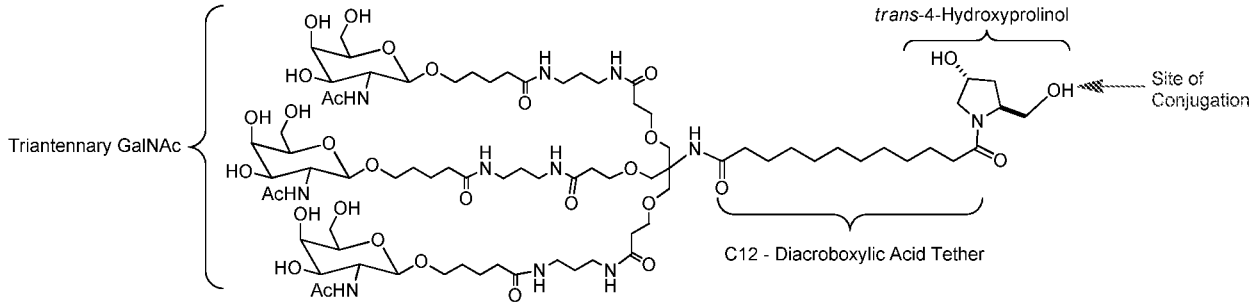
Abbreviation	Nucleotide(s)/ Nucleosides
A	Adenosine-3'-phosphate, 2'-deoxy-2'-fluorouridine-5'-phosphate or adenosine
Ab	beta-L-adenosine-3`-phosphate, beta-L-adenosine-5`-phosphate or beta-L-adenosine
Abs	beta-L-adenosine-3`-phosphorothioate
Af	2'-deoxy-2'-fluoroadenosine-3'-phosphate, 2'-deoxy-2'-fluoroadenosine-5'-phosphate or 2'-deoxy-2'-fluoroadenosine
Afs	2'-deoxy-2'-fluoroadenosine-3'-phosphorothioate
As	adenosine-3'-phosphorothioate
C	cytidine-3'-phosphate, cytidine-5'-phosphate or cytidine
Cb	beta-L-cytidine-3'-phosphate or beta-L-cytidine
Cbs	beta-L-cytidine-3'-phosphorothioate
Cf	2'-deoxy-2'-fluorocytidine-3'-phosphate, 2'-deoxy-2'-fluorocytidine-5'-phosphate or 2'-deoxy-2'-fluorocytidine
Cfs	2'-deoxy-2'-fluorocytidine-3'-phosphorothioate
(Chd)	2'-O-hexadecyl-cytidine-3'-phosphate or 2'-O-hexadecyl-cytidine
(Chds)	2'-O-hexadecyl-cytidine-3'-phosphorothioate
Cs	cytidine-3'-phosphorothioate
G	guanosine-3'-phosphate, guanosine-5'-phosphate or guanosine
Gb	beta-L-guanosine-3`-phosphate, beta-L-guanosine-5`-phosphate or beta-L-guanosine
Gbs	beta-L-guanosine-3`-phosphorothioate
Gf	2'-deoxy-2'-fluoroguanosine-3'-phosphate, 2'-deoxy-2'-fluoroguanosine-5'-phosphate or 2'-deoxy-2'-fluoroguanosine
Gfs	2'-deoxy-2'-fluoroguanosine-3'-phosphorothioate
Gs	guanosine-3'-phosphorothioate
T	5'-methyluridine-3'-phosphate, 5'-methyluridine-5'-phosphate or 5'-methyluridine
Tb	beta-L-thymidine-3'-phosphate, beta-L-thymidine-5'-phosphate or beta-L-thymidine
Tbs	beta-L-thymidine-3'-phosphorothioate

Tf	2’-deoxy-2’-fluoro-5-methyluridine-3’-phosphate, 2’-deoxy-2’-fluoro-5-methyluridine-3’-phosphate or 2’-deoxy-2’-fluoro-5-methyluridine
Tfs	2’-deoxy-2’-fluoro-5-methyluridine-3’-phosphorothioate
Ts	5-methyluridine-3’-phosphorothioate
U	Uridine-3’-phosphate, uridine-5’-phosphate or uridine-
Ub	beta-L-uridine-3`-phosphate, beta-L-uridine-5`-phosphate or beta-L-uridine
Ubs	beta-L-uridine-3`-phosphorothioate
Uf	2’-deoxy-2’-fluorouridine-3’-phosphate, 2’-deoxy-2’-fluorouridine or 2’-deoxy-2’-fluorouridine-3’-phosphate
Ufs	2’-deoxy-2’-fluorouridine -3’-phosphorothioate
(Uhd)	2'-O-hexadecyl-uridine-3'-phosphate, 2'-O-hexadecyl-uridine-6'-phosphate or 2'-O-hexadecyl-uridine
(Uhds)	2'-O-hexadecyl-uridine-3'-phosphorothioate
Us	uridine -3’-phosphorothioate
N	any nucleotide (G, A, C, T or U)
a	2'-O-methyladenosine-3’-phosphate, 2'-O-methyladenosine-5’-phosphate or 2'-O-methyladenosine
as	2'-O-methyladenosine-3’- phosphorothioate
c	2'-O-methylcytidine-3’-phosphate, 2'-O-methylcytidine-5’-phosphate or 2'-O-methylcytidine
cs	2'-O-methylcytidine-3’- phosphorothioate
g	2'-O-methylguanosine-3’-phosphate, 2'-O-methylguanosine-5’-phosphate or 2'-O-methylguanosine
gs	2'-O-methylguanosine-3’- phosphorothioate
t	2’-O-methyl-5-methyluridine-3’-phosphate, 2’-O-methyl-5-methyluridine-5’-phosphate or 2’-O-methyl-5-methyluridine
ts	2’-O-methyl-5-methyluridine-3’-phosphorothioate
u	2'-O-methyluridine-3’-phosphate, 2'-O-methyluridine-5’-phosphate or 2'-O-methyluridine
us	2'-O-methyluridine-3’-phosphorothioate
dA	2`-deoxyadenosine-3`-phosphate, 2`-deoxyadenosine-5`-phosphate or 2`-deoxyadenosine
dAs	2`-deoxyadenosine-3`-phosphorothioate
dC	2`-deoxycytidine-3`-phosphate, 2`-deoxycytidine-5`-phosphate or 2`-deoxycytidine
dCs	2`-deoxycytidine-3`-phosphorothioate

dG	2`-deoxyguanosine-3`-phosphate, 2`-deoxyguanosine-5`-phosphate or 2`-deoxyguanosine
dGs	2`-deoxyguanosine-3`-phosphorothioate or 2`-deoxyguanosine
dT	2'-deoxythymidine-3'-phosphate, 2'-deoxythymidine-5'-phosphate or 2'-deoxythymidine
dTs	2`-deoxythymidine-3`-phosphorothioate
dU	2`-deoxyuridine-3'-phosphate, 2`-deoxyuridine-5'-phosphate or 2'-deoxyuridine
s	phosphorothioate linkage
L96[1]	N-[tris(GalNAc-alkyl)-amidodecanoyl)]-4-hydroxyprolinol Hyp-(GalNAc-alkyl)3
(Aeo)	2'-O-methoxyethyladenosine-3'-phosphate, 2'-O-methoxyethyladenosine-5'-phosphate or 2'-O-methoxyethyladenosine
(Aeos)	2'-O-methoxyethyladenosine-3'-phosphorothioate
(Ceo)	2'-O-methoxyethylcytidine-3'-phosphate, 2'-O-methoxyethylcytidine-5'-phosphate or 2'-O-methoxyethylcytidine
(Ceos)	2'-O-methoxyethylcytidine-3'-phosphorothioate
(Geo)	2'-O-methoxyethylguanosine-3'-phosphate, 2'-O-methoxyethylguanosine-5'-phosphate or 2'-O-methoxyethylguanosine
(Geos)	2'-O-methoxyethylguanosine-3'- phosphorothioate
(Teo)	2'-O-methoxyethyl-5-methyluridine-3'-phosphate, 2'-O-methoxyethyl-5-methyluridine-5'-phosphate or 2'-O-methoxyethyl-5-methyluridine
(Teos)	2'-O-methoxyethyl-5-methyluridine-3'- phosphorothioate
(m5Ceo)	2'-O-methoxyethyl-5-methylcytidine-3'-phosphate, 2'-O-methoxyethyl-5-methylcytidine-5'-phosphate or 2'-O-methoxyethyl-5-methylcytidine
(m5Ceos)	2'-O-methoxyethyl-5-methylcytidine-3'- phosphorothioate
(Agn)	1-(2,3-Dihydroxypropyl)adenine-2-phosphate, 1-(2,3-Dihydroxypropyl)adenine-3-phosphate or 1-(2,3-Dihydroxypropyl) adenine
(Agns)	1-(2,3-Dihydroxypropyl)adenine-2-phosphorothioate
(Cgn)	1-(2,3-Dihydroxypropyl)cytosine-2-phosphate, 1-(2,3-Dihydroxypropyl)cytosine-3-phosphate or 1-(2,3-Dihydroxypropyl) cytosine
(Cgns)	1-(2,3-Dihydroxypropyl)cytosine-2-phosphorothioate

(Ggn)	1-(2,3-Dihydroxypropyl)guanine-2-phosphate, 1-(2,3-Dihydroxypropyl)guanine-3-phosphate or 1-(2,3-Dihydroxypropyl) guanine
(Ggns)	1-(2,3-Dihydroxypropyl)guanine-2-phosphorothiaote
(Tgn)	1-(2,3-Dihydroxypropyl)thymine-2-phosphate, 1-(2,3-Dihydroxypropyl)thymine-3-phosphate or 1-(2,3-Dihydroxypropyl) thymine
(Tgns)	1-(2,3-Dihydroxypropyl)thymine-2-phosphorothioate
(Ugn)	1-(2,3-Dihydroxypropyl)uracil-2-phosphate, 1-(2,3-Dihydroxypropyl)uracil-3-phosphate or 1-(2,3-Dihydroxypropyl) thymine
(Ugns)	1-(2,3-Dihydroxypropyl)uracil-2-phosphorothioate

[1]The chemical structure of L96 is as follows:



Example 2. ALAS1 siRNA Design and Synthesis

Experimental Methods

Bioinformatics

Transcripts

siRNA design was carried out to identify siRNAs targeting human, rhesus (*Macaca mulatta*), mouse, and rat ALAS1 transcripts annotated in the NCBI Gene database (http://www.ncbi.nlm.nih.gov/gene/). Design used the following transcripts from the NCBI RefSeq collection: Human -NM_000688.4 (see FIG.3), NM_199166.1; Rhesus - XM_001090440.2, XM_001090675.2; Mouse - NM_020559.2; Rat -NM_024484.2. Due to high primate/ rodent sequence divergence, siRNA duplexes were designed in several separate batches,

including but not limited to batches containing duplexes matching human and rhesus transcripts only; human, rhesus, mouse, and rat transcripts only; and mouse and rat transcripts only. Most siRNA duplexes were designed that shared 100% identity the listed human transcript and other species transcripts considered in each design batch (above). In some instances, (see Table 8) mismatches between duplex and mRNA target were allowed at the first antisense (last sense) position when the antisense strand:target mRNA complementary basepair was a GC or CG pair. In these cases, duplexes were designed with UA or AU pairs at the first antisense:last sense pair. Thus the duplexes maintained complementarity but were mismatched with respect to target (U:C, U:G, A:C, or A:G). Eighteen of these "UA-swap" duplexes were designed as part of the human/rhesus/mouse/rat set (see duplexes in Table 8 with "C19U", "G19U", "C19A", or "G19A" labels in the Position column).

siRNA Design, Specificity, and Efficacy Prediction

The predicted specificity of all possible 19mers was predicted from each sequence. Candidate 19mers were then selected that lacked repeats longer than 7 nucleotides. These 1510 candidate human/rhesus, 114 human/rhesus/mouse/rat, and 717 mouse/rat siRNAs were used in comprehensive searches against the appropriate transcriptomes (defined as the set of NM_ and XM_ records within the human, rhesus, dog, mouse, or rat NCBI Refseq sets) using an exhaustive "brute-force" algorithm implemented in the python script 'BruteForce.py'. The script next parsed the transcript-oligo alignments to generate a score based on the position and number of mismatches between the siRNA and any potential 'off-target' transcript. The off-target score is weighted to emphasize differences in the 'seed' region of siRNAs, in positions 2-9 from the 5' end of the molecule. Each oligo-transcript pair from the brute-force search was given a mismatch score by summing the individual mismatch scores; mismatches in the position 2-9 were counted as 2.8, mismatches in the cleavage site positions 10-11 were counted as 1.2, and mismatches in region 12-19 counted as 1.0. An additional off-target prediction was carried out by comparing the frequency of heptamers and octomers derived from 3 distinct, seed-derived hexamers of each oligo. The hexamers from positions 2-7 relative to the 5' start is used to create 2 heptamers and one octomer. We create 'heptamer1' by adding a 3' A to the hexamer; we create heptamer2 by adding a 5' A to the hexamer; we create the octomer by adding an A to both 5' and 3' ends of the hexamer. The frequency of octomers and heptamers in the human, rhesus, mouse, or rat

3'UTRome (defined as the subsequence of the transcriptome from NCBI's Refseq database where the end of the coding region, the 'CDS', is clearly defined) was pre-calculated. The octomer frequency was normalized to the heptamer frequency using the median value from the range of octomer frequencies. A 'mirSeedScore' was then calculated by calculating the sum of ((3 X normalized octomer count) + (2 X heptamer2 count) + (1 X heptamer1 count)).

Both siRNAs strands were assigned to a category of specificity according to the calculated scores: a score above 3 qualifies as highly specific, equal to 3 as specific and between 2.2 and 2.8 as moderately specific. We sorted by the specificity of the antisense strand. We then selected duplexes whose antisense oligos lacked GC at the first position, lacked G at both positions 13 and 14, and had 3 or more Us or As in the seed region (characteristics of duplexes with high predicted efficacy)

Candidate GalNac-conjugated duplexes, 21 and 23 nucleotides long on the sense and antisense strands respectively, were designed by extending antisense 19mers 4 additional nucleotides in the 3' direction (preserving perfect complementarity with the target transcript). The sense strand was specified as the reverse complement of the first 21 nucleotides of the antisense 23mer. Duplexes were selected that maintained perfect matches to all selected species transcripts across all 23 nucleotides.

siRNA sequence selection

A total of 90 sense and 90 antisense derived human/rhesus, 40 sense and 40 antisense derived human/rhesus/mouse/mouse/rat, and 40 sense and 40 antisense derived mouse/rat siRNA 19mer oligos were synthesized and formed into duplexes. A total of 45 sense and 45 antisense derived human/rhesus 21/23mer oligos were synthesized to yield 45 GalNac-conjugated duplexes.

The sequences of the sense and antisense strands of the modified duplexes are shown in Table 2, and the sequences of the sense and antisense strands of the unmodified duplexes are shown in Table 3.

Synthesis of ALAS1 Sequences

ALAS1 sequences were synthesized on MerMade 192 synthesizer at either 1 or 0.2umol scale. Single strands were made with 2'O-methyl modifications for in vitro screening using

transfection reagents. 3' GalNAc conjugates were made with sequences containing 2'F and 2'-O-methyl modifications on the sense strand in the 21-23 mer designs for free uptake in cells. For all the 21mer sequences in the list, 'endolight' chemistry was 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 were replaced with their corresponding 2-O-Methyl nucleosides
- A two base dTsdT extension at 3' end of both sense and anti sense sequences was introduced
- The sequence file was converted to a text file to make it compatible for loading in the MerMade 192 synthesis software

For GalNAc conjugated sense strands and complementary antisense sequences, 2'F and other modified nucleosides were introduced in combination with ribo with 2'O-Methyl nucleosides. The synthesis was performed on a GalNAc modified CPG support for the sense strand and CPG modified with universal support on the antisense sequence.

Synthesis, Cleavage and deprotection:

The synthesis of ALAS1 sequences used solid supported oligonucleotide synthesis using phosphoramidite chemistry. For 21 mer endolight sequences, a deoxy thymidine CPG was used as the solid support while for the GalNAc conjugates, GalNAc solid support for sense strand and an universal CPG for the antisesense strand were used.

The synthesis of the above sequences was performed at either 1 or 0.2um scale in 96 well plates. The amidite solutions were prepared at 0.1M concentration and ethyl thio tetrazole (0.6M in Acetonitrile) was used as activator.

The synthesized sequences were cleaved and deprotected in 96 well plates, using methylamine in the first step and fluoride reagent in the second step. For GalNAc and 2'F nucleoside containing sequences, deprotection conditions were modified. Sequences after cleavage and deprotection were precipitated using acetone: ethanol (80:20) mix and the pellet were re-suspended in 0.2M sodium acetate buffer. Samples from each sequence were analyzed

by LC-MS to confirm the identity, UV for quantification and a selected set of samples by IEX chromatography to determine purity.

Purification and desalting:

ALAS1 sequences were precipitated and purified on AKTA Purifier system using Sephadex column. The ALAS1ess was run at ambient temperature. Sample injection and collection was performed in 96 well (1.8mL -deep well) plates. A single peak corresponding to the full length sequence was collected in the eluent. The desalted ALAS1 sequences were analyzed for concentration (by UV measurement at A260) and purity (by ion exchange HPLC). The complementary single strands were then combined in a 1:1 stoichiometric ratio to form siRNA duplexes.

Table 2: Human ALAS1 Modified Single Strands and Duplex Sequences

SEQ ID NO: (sense)	SEQ ID NO: (anti-sense)	Position on transcript NM_ 000688.4	Duplex Name	Sense Sequence (5'-3')	Antisense Sequence (5'-3')
2	3	522-540	AD-55078.2	cuccGGccAGuGAGAAAGAdTsdT	UCUUUCUcACUGGCCGGAGdTsdT
4	5	669-687	AD-55084.2	uGGcAGcAcAGAuGAAucAdTsdT	UGAUUcAUCUGUGCUGCcAdTsdT
6	7	790-808	AD-55090.2	cAGuGuGGuuAGuGuGAAAdTsdT	UUUcAcACuAACcAcACUGdTsdT
8	9	853-871	AD-55096.2	cAucAuGcAAAAGcAAAGAdTsdT	UCUUUGCUUUUGcAUGAUGdTsdT
10	11	876-894	AD-55102.2	AAAGAGuGucucAucuucudTsdT	AGAAGAUGAGAcACUCUUUdTsdT
12	13	877-895	AD-55106.2	AAGAGuGucucAucuucuudTsdT	AAGAAGAUGAGAcACUCUUdTsdT
14	15	914-932	AD-55111.2	ucuGuuuccAcuuuucAGudTsdT	ACUGAAAAGUGGAAAcAGAdTsdT
16	17	923-941	AD-55073.2	AcuuuucAGuAuGAucGuudTsdT	AACGAUcAuACUGAAAAGUdTsdT
18	19	926-944	AD-55079.2	uuucAGuAuGAucGuuucudTsdT	AGAAACGAUcAuACUGAAAdTsdT
20	21	927-945	AD-55085.2	uucAGuAuGAucGuuucuudTsdT	AAGAAACGAUcAuACUGAAdTsdT
22	23	928-946	AD-55091.2	ucAGuAuGAucGuuucuuudTsdT	AAAGAAACGAUcAuACUGAdTsdT
24	25	932-950	AD-55097.2	uAuGAucGuuucuuuGAGAdTsdT	UCUcAAAGAAACGAUcAuAdTsdT
26	27	973-991	AD-55103.2	uGAccAcAccuAucGAGuudTsdT	AACUCGAuAGGUGUGGUcAdTsdT
28	29	975-993	AD-55107.2	AccAcAccuAucGAGuuuudTsdT	AAAACUCGAuAGGUGUGGUdTsdT
30	31	1029-1047	AD-55112.2	uGGcAGAuGAcuAuucAGAdTsdT	UCUGAAuAGUcAUCUGCcAdTsdT
32	33	1077-1095	AD-55074.2	ucuGGuGcAGuAAuGAcuAdTsdT	uAGUcAUuACUGcACcAGAdTsdT
34	35	1124-1142	AD-55080.2	uGuGGGGcAGuuAuGGAcAdTsdT	UGUCcAuAACUGCCCcAcAdTsdT

36	37	1137-1155	AD-55086.2	uGGAcAcuuuGAAAcAAcAdTsdT	UGUUGUUUcAAAGUGUCcAdTsdT
38	39	1182-1200	AuAuuucuGGAAcuAGuAAdTsdT	UuACuAGUUCcAGAAAuAUdTsdT	
40	41	1184-1202	AuuucuGGAAcuAGuAAAudTsdT	AUUuACuAGUUCcAGAAAUdTsdT	
42	43	1185-1203	uuucuGGAAcuAGuAAAuudTsdT	AAUUuACuAGUUCcAGAAAdTsdT	
44	45	1188-1206	cuGGAAcuAGuAAAuuccAdTsdT	UGGAAUUuACuAGUUCcAGdTsdT	
46	47	1325-1343	uGuGAGAuuuAcucuGAuudTsdT	AAUcAGAGuAAAUCUcAcAdTsdT	
48	49	1364-1382	AuccAAGGGAuucGAAAcAdTsdT	UGUUUCGAAUCCCUUGGAUdTsdT	
50	51	1382-1400	AGccGAGuGccAAAGuAcAdTsdT	UGuACUUUGGcACUCGGCUdTsdT	
52	53	1478-1496	uuuGAAAcuGuccAuucAAdTsdT	UUGAAUGGAcAGUUUcAAAdTsdT	
54	55	1531-1549	uGAuGuGGcccAuGAGuuudTsdT	AAACUcAUGGGCcAcAUcAdTsdT	
56	57	1631-1649	GucAuGccAAAAAuGGAcAdTsdT	UGUCcAUUUUUGGcAUGACdTsdT	
58	59	1637-1655	ccAAAAAuGGAcAucAuuudTsdT	AAAUGAUGUCcAUUUUUGGdTsdT	
60	61	1706-1724	AcGAGuucucuGAuuGAcAdTsdT	UGUcAAUcAGAGAACUCGUdTsdT	
62	63	1962-1980	AAGucuGuGAuGAAcuAAudTsdT	AUuAGUUcAUcAcAGACUUdTsdT	
64	65	1967-1985	uGuGAuGAAcuAAuGAGcAdTsdT	UGCUcAUuAGUUcAUcAcAdTsdT	
66	67	1977-1995	uAAuGAGcAGAcAuAAcAudTsdT	AUGUuAUGUCUGCUcAUuAdTsdT	
68	69	2189-2207	uuuGAAGuGAuGAGuGAAAdTsdT	UUUcACUcAUcACUUcAAAdTsdT	
70	71	2227-2245	AGGcuuGAGcAAGuuGGuAdTsdT	uACcAACUUGCUcAAGCCUdTsdT	
72	73	2313-2331	ucuucAGAGuuGucuuuAudTsdT	AuAAAGAcAACUCUGAAGAdTsdT	
74	75	2317-2335	cAGAGuuGucuuuAuAuGudTsdT	AcAuAuAAAGAcAACUCUGdTsdT	
76	77	2319-2337	GAGuuGucuuuAuAuGuGAdTsdT	UcAcAuAuAAAGAcAACUCdTsdT	
78	79	2320-2338	AGuuGucuuuAuAuGuGAAdTsdT	UUcAcAuAuAAAGAcAACUdTsdT	
80	81	2344-2362	uuAuAuuAAAuuuuAAucudTsdT	AGAUuAAAAUUuAAuAuAAdTsdT	
82	83	2352-2370	AAuuuuAAucuAuAGuAAAdTsdT	UUuACuAuAGAUuAAAAUUdTsdT	
84	85	2353-2371	AuuuuAAucuAuAGuAAAAdTsdT	UUUuACuAuAGAUuAAAAUdTsdT	
86	87	2376-2394	AGuccuGGAAAuAAAuucudTsdT	AGAAUUuAUUUCcAGGACUdTsdT	
88	89	358-376	cuGcccAuucuuAucccGAdTsdT	UCGGGAuAAGAAUGGGcAGdTsdT	
90	91	789-807	AD-53512.1	ccAGuGuGGuuAGuGuGAAdTsdT	UUcAcACuAACcAcACUGGdTsdT
92	93	1076-1094	AD-53513.1	GucuGGuGcAGuAAuGAcudTsdT	AGUcAUuACUGcACcAGACdTsdT
94	95	1253-1271	AD-53514.1	GcAcucuuGuuuuccucGudTsdT	ACGAGGAAAAcAAGAGUGCdTsdT
96	97	1544-1562	AD-53515.1	GAGuuuGGAGcAAucAccudTsdT	AGGUGAUUGCUCcAAACUCdTsdT
98	99	2228-2246	AD-53516.1	GGcuuGAGcAAGuuGGuAudTsdT	AuACcAACUUGCUcAAGCCdTsdT
100	101	404-422	AD-53517.1	GGcAAAucucuGuuGuucudTsdT	AGAAcAAcAGAGAUUUGCCdTsdT
102	103	404-422	AD-53517.1	GGcAAAucucuGuuGuucudTsdT	AGAAcAAcAGAGAUUUGCCdTsdT
104	105	866-884	AD-53518.1	cAAAGAccAGAAAGAGuGudTsdT	AcACUCUUUCUGGUCUUUGdTsdT
106	107	1080-1098	AD-53519.1	GGuGcAGuAAuGAcuAccudTsdT	AGGuAGUcAUuACUGcACCdTsdT
108	109	1258-1276	AD-53520.1	cuuGuuuuccucGuGcuuudTsdT	AAAGcACGAGGAAAAcAAGdTsdT
110	111	1616-1634	AD-53521.1	GGGGAucGGGAuGGAGucAdTsdT	UGACUCcAUCCCGAUCCCCdTsdT
112	113	2230-2248	AD-53522.1	cuuGAGcAAGuuGGuAucudTsdT	AGAuACcAACUUGCUcAAGdTsdT

114	115	436-454	AD-53523.1	ccccAAGAuGAuGGAAGuudTsdT	AACUUCcAUcAUCUUGGGGdTsdT
116	117	436-454	AD-53523.1	ccccAAGAuGAuGGAAGuudTsdT	AACUUCcAUcAUCUUGGGGdTsdT
118	119	885-903	AD-53524.1	cucAucuucuucAAGAuAAdTsdT	UuAUCUUGAAGAAGAUGAGdTsdT
120	121	1127-1145	AD-53525.1	GGGGcAGuuAuGGAcAcuudTsdT	AAGUGUCcAuAACUGCCCCdTsdT
122	123	1315-1333	AD-53526.1	GAuGccAGGcuGuGAGAuudTsdT	AAUCUcAcAGCCUGGcAUCdTsdT
124	125	1870-1888	AD-53527.1	GAGAcAGAuGcuAAuGGAudTsdT	AUCcAUuAGcAUCUGUCUCdTsdT
126	127	2286-2304	AD-53528.1	ccccAGGccAuuAucAuAudTsdT	AuAUGAuAAUGGCCUGGGGdTsdT
128	129	489-507	AD-53529.1	cAGcAGuAcAcuAccAAcAdTsdT	UGUUGGuAGUGuACUGCUGdTsdT
130	131	489-507	AD-53529.1	cAGcAGuAcAcuAccAAcAdTsdT	UGUUGGuAGUGuACUGCUGdTsdT
132	133	915-933	AD-53530.1	cuGuuuccAcuuuucAGuAdTsdT	uACUGAAAAGUGGAAAcAGdTsdT
134	135	1138-1156	AD-53531.1	GGAcAcuuuGAAAcAAcAudTsdT	AUGUUGUUUcAAAGUGUCCdTsdT
136	137	1324-1342	AD-53532.1	cuGuGAGAuuuAcucuGAudTsdT	AUcAGAGuAAAUCUcAcAGdTsdT
138	139	1927-1945	AD-53533.1	cccuGuGcGGGuuGcAGAudTsdT	AUCUGcAACCCGcAcAGGGdTsdT
140	141	2312-2330	AD-53534.1	GucuucAGAGuuGucuuuAdTsdT	uAAAGAcAACUCUGAAGACdTsdT
142	143	646-664	AD-53535.1	cAcuGcAAGcAAAuGcccudTsdT	AGGGcAUUUGCUUGcAGUGdTsdT
144	145	922-940	AD-53536.1	cAcuuuucAGuAuGAucGudTsdT	ACGAUcAuACUGAAAAGUGdTsdT
146	147	1163-1181	AD-53537.1	GGGGcAGGuGGuAcuAGAAdTsdT	UUCuAGuACcACCUGCCCCdTsdT
148	149	1347-1365	AD-53538.1	GGAAccAuGccuccAuGAudTsdT	AUcAUGGAGGcAUGGUUCCdTsdT
150	151	1964-1982	AD-53539.1	GucuGuGAuGAAcuAAuGAdTsdT	UcAUuAGUUcAUcAcAGACdTsdT
152	153	2321-2339	AD-53540.1	GuuGucuuuAuAuGuGAAudTsdT	AUUcAcAuAuAAAGAcAACdTsdT
154	155	671-689	AD-53541.1	GcAGcAcAGAuGAAucAGAdTsdT	UCUGAUUcAUCUGUGCUGCdTsdT
156	157	924-942	AD-53542.1	cuuuucAGuAuGAucGuuudTsdT	AAACGAUcAuACUGAAAAGdTsdT
158	159	1164-1182	AD-53543.1	GGGcAGGuGGuAcuAGAAAdTsdT	UUUCuAGuACcACCUGCCCdTsdT
160	161	1460-1478	AD-53544.1	GuccccAAGAuuGuGGcAudTsdT	AUGCcAcAAUCUUGGGGACdTsdT
162	163	1976-1994	AD-53545.1	cuAAuGAGcAGAcAuAAcAdTsdT	UGUuAUGUCUGCUcAUuAGdTsdT
164	165	786-804	AD-53546.1	GccccAGuGuGGuuAGuGudTsdT	AcACuAACcAcACUGGGGCdTsdT
166	167	935-953	AD-53547.1	GAucGuuucuuuGAGAAAAdTsdT	UUUUCUcAAAGAAACGAUCdTsdT
168	169	1165-1183	AD-53548.1	GGcAGGuGGuAcuAGAAAudTsdT	AUUUCuAGuACcACCUGCCdTsdT
170	171	1530-1548	AD-53549.1	GuGAuGuGGcccAuGAGuudTsdT	AACUcAUGGGCcAcAUcACdTsdT
172	173	2003-2021	AD-53550.1	cAAGcAAucAAuuAcccuAdTsdT	uAGGGuAAUUGAUUGCUUGdTsdT
174	175	788-806	AD-53551.1	cccAGuGuGGuuAGuGuGAdTsdT	UcAcACuAACcAcACUGGGdTsdT
176	177	974-992	AD-53552.1	GAccAcAccuAucGAGuuudTsdT	AAACUCGAuAGGUGUGGUCdTsdT
178	179	1191-1209	AD-53553.1	GAAcuAGuAAAuuccAuGudTsdT	AcAUGGAAUUuACuAGUUCdTsdT
180	181	1541-1559	AD-53554.1	cAuGAGuuuGGAGcAAucAdTsdT	UGAUUGCUCcAAACUcAUGdTsdT
182	183	2075-2093	AD-53555.1	ccccAGAuGAuGAAcuAcudTsdT	AGuAGUUcAUcAUCUGGGGdTsdT
184	185	360-378	AD-53561.1	GcccAuucuuAucccGAGudTsdT	ACUCGGGAuAAGAAUGGGCdTsdT
186	187	1356-1374	AD-53567.1	ccuccAuGAuccAAGGGAudTsdT	AUCCCUUGGAUcAUGGAGGdTsdT
188	189	1631-1649	AD-53573.1	GucAuGccAAAAAuGGAcAdTsdT	UGUCcAUUUUUGGcAUGACdTsdT
190	191	1634-1652	AD-53579.1	AuGccAAAAAuGGAcAucAdTsdT	UGAUGUCcAUUUUUGGcAUdTsdT

Table 3: Human ALAS1 Unmodified Single Strands and Duplex Sequences

SEQ ID NO: (sense)	SEQ ID NO: (anti-sense)	Position on transcript NM_ 000688.4	Duplex Name	Sense Sequence (5'-3')	Antisense Sequence (5'-3')
192	193	522-540	AD-55078.2	CUCCGGCCAGUGAGAAAGA	UCUUUCUCACUGGCCGGAG
194	195	669-687	AD-55084.2	UGGCAGCACAGAUGAAUCA	UGAUUCAUCUGUGCUGCCA
196	197	790-808	AD-55090.2	CAGUGUGGUUAGUGUGAAA	UUUCACACUAACCACACUG
198	199	853-871	AD-55096.2	CAUCAUGCAAAAGCAAAGA	UCUUUGCUUUUGCAUGAUG
200	201	876-894	AD-55102.2	AAAGAGUGUCUCAUCUUCU	AGAAGAUGAGACACUCUUU
202	203	877-895	AD-55106.2	AAGAGUGUCUCAUCUUCUU	AAGAAGAUGAGACACUCUU
204	205	914-932	AD-55111.2	UCUGUUUCCACUUUUCAGU	ACUGAAAAGUGGAAACAGA
206	207	923-941	AD-55073.2	ACUUUUCAGUAUGAUCGUU	AACGAUCAUACUGAAAAGU
208	209	926-944	AD-55079.2	UUUCAGUAUGAUCGUUUCU	AGAAACGAUCAUACUGAAA
210	211	927-945	AD-55085.2	UUCAGUAUGAUCGUUUCUU	AAGAAACGAUCAUACUGAA
212	213	928-946	AD-55091.2	UCAGUAUGAUCGUUUCUUU	AAAGAAACGAUCAUACUGA
214	215	932-950	AD-55097.2	UAUGAUCGUUUCUUUGAGA	UCUCAAAGAAACGAUCAUA
216	217	973-991	AD-55103.2	UGACCACACCUAUCGAGUU	AACUCGAUAGGUGUGGUCA
218	219	975-993	AD-55107.2	ACCACACCUAUCGAGUUUU	AAAACUCGAUAGGUGUGGU
220	221	1029-1047	AD-55112.2	UGGCAGAUGACUAUUCAGA	UCUGAAUAGUCAUCUGCCA
222	223	1077-1095	AD-55074.2	UCUGGUGCAGUAAUGACUA	UAGUCAUUACUGCACCAGA
224	225	1124-1142	AD-55080.2	UGUGGGGCAGUUAUGGACA	UGUCCAUAACUGCCCCACA
226	227	1137-1155	AD-55086.2	UGGACACUUUGAAACAACA	UGUUGUUUCAAAGUGUCCA
228	229	1182-1200	AD-55098.2	AUAUUUCUGGAACUAGUAA	UUACUAGUUCCAGAAAUAU
230	231	1184-1202	AD-55104.2	AUUUCUGGAACUAGUAAAU	AUUUACUAGUUCCAGAAAU
232	233	1185-1203	AD-55108.2	UUUCUGGAACUAGUAAAUU	AAUUUACUAGUUCCAGAAA
234	235	1188-1206	AD-55113.2	CUGGAACUAGUAAAUUCCA	UGGAAUUUACUAGUUCCAG
236	237	1325-1343	AD-55075.2	UGUGAGAUUUACUCUGAUU	AAUCAGAGUAAAUCUCACA
238	239	1364-1382	AD-55081.2	AUCCAAGGGAUUCGAAACA	UGUUUCGAAUCCCUUGGAU
240	241	1382-1400	AD-55087.2	AGCCGAGUGCCAAAGUACA	UGUACUUUGGCACUCGGCU
242	243	1478-1496	AD-55093.2	UUUGAAACUGUCCAUUCAA	UUGAAUGGACAGUUUCAAA
244	245	1531-1549	AD-55099.2	UGAUGUGGCCCAUGAGUUU	AAACUCAUGGGCCACAUCA
246	247	1631-1649	AD-53573.3	GUCAUGCCAAAAAUGGACA	UGUCCAUUUUUGGCAUGAC
248	249	1637-1655	AD-55109.2	CCAAAAAUGGACAUCAUUU	AAAUGAUGUCCAUUUUUGG
250	251	1706-1724	AD-55114.2	ACGAGUUCUCUGAUUGACA	UGUCAAUCAGAGAACUCGU
252	253	1962-1980	AD-55076.2	AAGUCUGUGAUGAACUAAU	AUUAGUUCAUCACAGACUU
254	255	1967-1985	AD-55082.2	UGUGAUGAACUAAUGAGCA	UGCUCAUUAGUUCAUCACA

256	257	1977-1995	AD-55088.2	UAAUGAGCAGACAUAACAU	AUGUUAUGUCUGCUCAUUA
258	259	2189-2207	AD-55094.2	UUUGAAGUGAUGAGUGAAA	UUUCACUCAUCACUUCAAA
260	261	2227-2245	AD-55100.2	AGGCUUGAGCAAGUUGGUA	UACCAACUUGCUCAAGCCU
262	263	2313-2331	AD-55105.2	UCUUCAGAGUUGUCUUUAU	AUAAAGACAACUCUGAAGA
264	265	2317-2335	AD-55110.2	CAGAGUUGUCUUUAUAUGU	ACAUAUAAAGACAACUCUG
266	267	2319-2337	AD-55115.2	GAGUUGUCUUUAUAUGUGA	UCACAUAUAAAGACAACUC
268	269	2320-2338	AD-55077.2	AGUUGUCUUUAUAUGUGAA	UUCACAUAUAAAGACAACU
270	271	2344-2362	AD-55083.2	UUAUAUUAAAUUUUAAUCU	AGAUUAAAAUUUAAUAUAA
272	273	2352-2370	AD-55089.2	AAUUUUAAUCUAUAGUAAA	UUUACUAUAGAUUAAAAUU
274	275	2353-2371	AD-55095.2	AUUUUAAUCUAUAGUAAAA	UUUUACUAUAGAUUAAAAU
276	277	2376-2394	AD-55101.2	AGUCCUGGAAAUAAAUUCU	AGAAUUUAUUUCCAGGACU
278	279	358-376	AD-53511.1	CUGCCCAUUCUUAUCCCGA	UCGGGAUAAGAAUGGGCAG
280	281	789-807	AD-53512.1	CCAGUGUGGUUAGUGUGAA	UUCACACUAACCACACUGG
282	283	1076-1094	AD-53513.1	GUCUGGUGCAGUAAUGACU	AGUCAUUACUGCACCAGAC
284	285	1253-1271	AD-53514.1	GCACUCUUGUUUUCCUCGU	ACGAGGAAAACAAGAGUGC
286	287	1544-1562	AD-53515.1	GAGUUUGGAGCAAUCACCU	AGGUGAUUGCUCCAAACUC
288	289	2228-2246	AD-53516.1	GGCUUGAGCAAGUUGGUAU	AUACCAACUUGCUCAAGCC
290	291	404-422	AD-53517.1	GGCAAAUCUCUGUUGUUCU	AGAACAACAGAGAUUUGCC
292	293	404-422	AD-53517.1	GGCAAAUCUCUGUUGUUCU	AGAACAACAGAGAUUUGCC
294	295	866-884	AD-53518.1	CAAAGACCAGAAAGAGUGU	ACACUCUUUCUGGUCUUUG
296	297	1080-1098	AD-53519.1	GGUGCAGUAAUGACUACCU	AGGUAGUCAUUACUGCACC
298	299	1258-1276	AD-53520.1	CUUGUUUUCCUCGUGCUUU	AAAGCACGAGGAAAACAAG
300	301	1616-1634	AD-53521.1	GGGGAUCGGGAUGGAGUCA	UGACUCCAUCCCGAUCCCC
302	303	2230-2248	AD-53522.1	CUUGAGCAAGUUGGUAUCU	AGAUACCAACUUGCUCAAG
304	305	436-454	AD-53523.1	CCCCAAGAUGAUGGAAGUU	AACUUCCAUCAUCUUGGGG
306	307	436-454	AD-53523.1	CCCCAAGAUGAUGGAAGUU	AACUUCCAUCAUCUUGGGG
308	309	885-903	AD-53524.1	CUCAUCUUCUUCAAGAUAA	UUAUCUUGAAGAAGAUGAG
310	311	1127-1145	AD-53525.1	GGGGCAGUUAUGGACACUU	AAGUGUCCAUAACUGCCCC
312	313	1315-1333	AD-53526.1	GAUGCCAGGCUGUGAGAUU	AAUCUCACAGCCUGGCAUC
314	315	1870-1888	AD-53527.1	GAGACAGAUGCUAAUGGAU	AUCCAUUAGCAUCUGUCUC
316	317	2286-2304	AD-53528.1	CCCCAGGCCAUUAUCAUAU	AUAUGAUAAUGGCCUGGGG
318	319	489-507	AD-53529.1	CAGCAGUACACUACCAACA	UGUUGGUAGUGUACUGCUG
320	321	489-507	AD-53529.1	CAGCAGUACACUACCAACA	UGUUGGUAGUGUACUGCUG
322	323	915-933	AD-53530.1	CUGUUUCCACUUUUCAGUA	UACUGAAAAGUGGAAACAG
324	325	1138-1156	AD-53531.1	GGACACUUUGAAACAACAU	AUGUUGUUUCAAAGUGUCC
326	327	1324-1342	AD-53532.1	CUGUGAGAUUUACUCUGAU	AUCAGAGUAAAUCUCACAG
328	329	1927-1945	AD-53533.1	CCCUGUGCGGGUUGCAGAU	AUCUGCAACCCGCACAGGG
330	331	2312-2330	AD-53534.1	GUCUUCAGAGUUGUCUUUA	UAAAGACAACUCUGAAGAC
332	333	646-664	AD-53535.1	CACUGCAAGCAAAUGCCCU	AGGGCAUUUGCUUGCAGUG

334	335	922-940	AD-53536.1	CACUUUUCAGUAUGAUCGU	ACGAUCAUACUGAAAAGUG
336	337	1163-1181	AD-53537.1	GGGGCAGGUGGUACUAGAA	UUCUAGUACCACCUGCCCC
338	339	1347-1365	AD-53538.1	GGAACCAUGCCUCCAUGAU	AUCAUGGAGGCAUGGUUCC
340	341	1964-1982	AD-53539.1	GUCUGUGAUGAACUAAUGA	UCAUUAGUUCAUCACAGAC
342	343	2321-2339	AD-53540.1	GUUGUCUUUAUAUGUGAAU	AUUCACAUAUAAAGACAAC
344	345	671-689	AD-53541.1	GCAGCACAGAUGAAUCAGA	UCUGAUUCAUCUGUGCUGC
346	347	924-942	AD-53542.1	CUUUUCAGUAUGAUCGUUU	AAACGAUCAUACUGAAAAG
348	349	1164-1182	AD-53543.1	GGGCAGGUGGUACUAGAAA	UUUCUAGUACCACCUGCCC
350	351	1460-1478	AD-53544.1	GUCCCCAAGAUUGUGGCAU	AUGCCACAAUCUUGGGGAC
352	353	1976-1994	AD-53545.1	CUAAUGAGCAGACAUAACA	UGUUAUGUCUGCUCAUUAG
354	355	786-804	AD-53546.1	GCCCCAGUGUGGUUAGUGU	ACACUAACCACACUGGGGC
356	357	935-953	AD-53547.1	GAUCGUUUCUUUGAGAAAA	UUUUCUCAAAGAAACGAUC
358	359	1165-1183	AD-53548.1	GGCAGGUGGUACUAGAAAU	AUUUCUAGUACCACCUGCC
360	361	1530-1548	AD-53549.1	GUGAUGUGGCCCAUGAGUU	AACUCAUGGGCCACAUCAC
362	363	2003-2021	AD-53550.1	CAAGCAAUCAAUUACCCUA	UAGGGUAAUUGAUUGCUUG
364	365	788-806	AD-53551.1	CCCAGUGUGGUUAGUGUGA	UCACACUAACCACACUGGG
366	367	974-992	AD-53552.1	GACCACACCUAUCGAGUUU	AAACUCGAUAGGUGUGGUC
368	369	1191-1209	AD-53553.1	GAACUAGUAAAUUCCAUGU	ACAUGGAAUUUACUAGUUC
370	371	1541-1559	AD-53554.1	CAUGAGUUUGGAGCAAUCA	UGAUUGCUCCAAACUCAUG
372	373	2075-2093	AD-53555.1	CCCCAGAUGAUGAACUACU	AGUAGUUCAUCAUCUGGGG
374	375	360-378	AD-53561.1	GCCCAUUCUUAUCCCGAGU	ACUCGGGAUAAGAAUGGGC
376	377	1356-1374	AD-53567.1	CCUCCAUGAUCCAAGGGAU	AUCCCUUGGAUCAUGGAGG
378	379	1631-1649	AD-53573.1	GUCAUGCCAAAAAUGGACA	UGUCCAUUUUUGGCAUGAC
380	381	1634-1652	AD-53579.1	AUGCCAAAAAUGGACAUCA	UGAUGUCCAUUUUUGGCAU

Example 3. *In vitro* screening of ALAS1 siRNA duplexes for ALAS1 knockdown activity.

ALAS1 siRNA duplexes were screened for the ability to knockdown ALAS1 expression *in vitro*.

***In vitro* screening**

Cell culture and transfections

Hep3B cells (ATCC, Manassas, VA) were grown to near confluence at 37°C in an atmosphere of 5% CO_2 in MEM (ATCC) supplemented with 10% FBS, 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 containing ~2 x10^4 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, 1.67, 0.27, 0.046, 0.0077, 0.0013, 0.00021, 0.00004 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 Lysis/Binding Buffer mixture were added to a round bottom plate and mixed for 1 minute. Magnetic beads were captured using magnetic stand and the supernatant was removed without disturbing the beads. After removing supernatant, the lysed cells were added to the remaining beads and mixed for 5 minutes. After removing supernatant, magnetic beads were washed 2 times with 150µl Wash Buffer A and mixed for 1 minute. Beads were captured again and supernatant removed. Beads were then washed with 150µl Wash Buffer B, captured and supernatant was removed. Beads were next washed with 150µl Elution Buffer, captured and supernatant removed. Beads were allowed to dry for 2 minutes. After drying, 50µl of Elution Buffer was added and mixed for 5 minutes at 70°C. Beads were captured on magnet for 5 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 10X Buffer, 0.8µl 25X dNTPs, 2µl Random primers, 1µl Reverse Transcriptase, 1µl RNase inhibitor and 3.2µl of H_2O 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 ALAS1 TaqMan probe (Applied Biosystems cat # Hs00167441_m1) and 5µl Lightcycler 480 probe master mix (Roche Cat #04887301001) per well in a 384 well plates (Roche cat # 04887301001). Real time PCR was done in a Roche LC480 Real Time PCR system (Roche) using the ΔΔCt(RQ) assay. Each duplex was tested in two independent transfections with two biological replicates each, 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 or naïve cells over the same dose range, or to its own lowest dose.

In vitro knockdown of endogenous ALAS1 expression by ALAS1 siRNA duplexes

Table 4 illustrates the knockdown of ALAS1 in Hep3B cells by ALAS1 modified siRNA duplexes (See Table 2). Silencing is expressed as the fraction RNA message remaining relative to the negative (luciferase) control siRNA AD-1955. Data were generated as described above following transfection of 10 nM or 0.1 nM of each siRNA. qPCR was run using the ALAS1 TaqMan probe Hs00167441_m1.

Table 4: ALAS1 expression in Hep3B cells following transfection with ALAS1 siRNA

Duplex ID	10nM Avg	0.1nM Avg	10nM STDEV	0.1nM STDEV
AD-55078.2	0.7	0.87	0.001	0.089
AD-55084.2	0.08	0.3	0	0.04
AD-55090.2	0.06	0.08	0.002	0.003
AD-55096.2	0.61	0.92	0.171	0.34
AD-55102.2	0.63	0.62	0.005	0.069
AD-55106.2	0.07	0.08	0.004	0.027
AD-55111.2	0.06	0.23	0.013	0.062
AD-55073.2	0.21	0.4	0.018	0.061
AD-55079.2	0.17	0.43	0.033	0.089
AD-55085.2	0.13	0.21	0.011	0.019
AD-55091.2	0.27	0.55	0.033	0.009
AD-55097.2	0.31	0.38	0.051	0.059
AD-55103.2	0.05	0.11	0.017	0.006
AD-55107.2	0.12	0.24	0.007	0.008
AD-55112.2	0.15	0.2	0.036	0.025
AD-55074.2	0.16	0.45	0.008	0.002
AD-55080.2	0.79	0.99	0.095	0.304
AD-55086.2	0.09	0.22	0.005	0.035
AD-55098.2	0.25	0.51	0.03	0.07
AD-55104.2	0.06	0.1	0.017	0.001
AD-55108.2	0.47	0.65	0.03	0.015
AD-55113.2	0.38	0.62	0.068	0.039
AD-55075.2	0.12	0.28	0.007	0.051
AD-55081.2	0.21	0.51	0.036	0.066
AD-55087.2	0.1	0.19	0.017	0.02
AD-55093.2	0.24	0.56	0.029	0.053
AD-55099.2	0.05	0.18	0.001	0.038
AD-53573.3	0.67	1.07	0.16	0.153
AD-55109.2	0.07	0.23	0.006	0.052
AD-55114.2	0.08	0.16	0.004	0.017
AD-55076.2	0.05	0.14	0.007	0.035
AD-55082.2	0.08	0.3	0.019	0.016
AD-55088.2	0.06	0.12	0.008	0.02
AD-55094.2	0.06	0.18	0.005	0.023

AD-55100.2	0.45	0.83	0.02	0.05
AD-55105.2	0.02	0.05	0.005	0.004
AD-55110.2	0.15	0.19	0.031	0.016
AD-55115.2	0.35	0.58	0.045	0.052
AD-55077.2	0.14	0.14	0.006	0.019
AD-55083.2	0.56	0.98	0.24	0.188
AD-55089.2	0.62	0.79	0.036	0.094
AD-55095.2	0.59	0.92	0.12	0.079
AD-55101.2	0.71	0.97	0.074	0.097
AD-1955	1.00	1.01	0.03	0.04
AD-53511.1	0.84	1.08	0.028	0.0515
AD-53512.1	0.15	0.65	0.062	0.023
AD-53513.1	0.34	0.86	0.055	0.011
AD-53514.1	0.12	0.61	0.003	0.008
AD-53515.1	0.25	0.66	0.005	0.004
AD-53516.1	1.05	1.02	0.032	0.011
AD-53517.1	0.145	0.725	0.025	0.0155
AD-53518.1	0.72	0.85	0.045	0.028
AD-53519.1	0.18	0.66	0.061	0.004
AD-53520.1	0.18	0.9	0.041	0.001
AD-53521.1	0.97	1.07	0.01	0.003
AD-53522.1	0.87	1.1	0.065	0.112
AD-53523.1	0.48	0.96	0.0305	0.0255
AD-53524.1	0.11	0.66	0.02	0.006
AD-53525.1	0.71	1.03	0.016	0.01
AD-53526.1	0.23	0.85	0.075	0.01
AD-53527.1	0.25	0.83	0.015	0.017
AD-53528.1	0.44	0.93	0.037	0.006
AD-53529.1	0.185	0.73	0.015	0.014
AD-53530.1	0.1	0.62	0.02	0.003
AD-53531.1	0.48	0.93	0.019	0.045
AD-53532.1	0.06	0.17	0	0.003
AD-53533.1	0.36	0.93	0.025	0.034
AD-53534.1	0.1	0.36	0.014	0.012
AD-53535.1	0.58	1.05	0.036	0.071
AD-53536.1	0.12	0.45	0.009	0.026
AD-53537.1	0.73	0.96	0.101	0.015

AD-53538.1	0.74	1.07	0	0.046
AD-53539.1	0.52	0.97	0.057	0.032
AD-53540.1	0.1	0.47	0.017	0.012
AD-53541.1	0.11	0.29	0.026	0.015
AD-53542.1	0.08	0.23	0.008	0.006
AD-53543.1	0.62	1.01	0.027	0.014
AD-53544.1	0.8	1.04	0.002	0.001
AD-53545.1	0.17	0.73	0.007	0.007
AD-53546.1	0.27	0.93	0.058	0.019
AD-53547.1	0.12	0.28	0.008	0.01
AD-53548.1	0.1	0.34	0.022	0.002
AD-53549.1	0.8	1.04	0.011	0.026
AD-53550.1	0.05	0.54	0.02	0.003
AD-53551.1	0.96	1.16	0.029	0.044
AD-53552.1	0.13	0.5	0.002	0.009
AD-53553.1	0.92	1.1	0.027	0.02
AD-53554.1	0.76	0.67	0.005	0.004
AD-53555.1	0.11	0.53	0.009	0.007
AD-53561.1	0.72	0.94	0.014	0.001
AD-53567.1	0.16	0.66	0.019	0.003
AD-53573.1	1.06	1.10	0.019	0.037
AD-53579.1	0.19	0.76	0.036	0.019

IC_{50}s of select ALAS1 siRNA duplexes in *in vitro* screen

Table 5 illustrates the IC_{50}s of select ALAS1 siRNA duplexes determined from the knockdown of endogenously expressed ALAS1 in the Hep3B cell line, by ALAS1 modified siRNA duplexes (see Table 2). Data were generated as described above, at 24 or 120 hours following transfection of each siRNA duplex. In this example, silencing of ALAS1 is expressed as the fraction mRNA message remaining relative to the siRNA AD-1955, a non-targeting siRNA that was used as a negative control. Data from replicate transfection experiments were used to fit a single line to determine the IC_{50}. Several of the duplexes (e.g., AD-53541.1, AD-53542.1, and AD-53547.1) had an IC_{50} as low as about 0.03 nM at 24 hours. Numerous duplexes had an IC_{50} of less than 0.1 nM (e.g., AD-53534.1, AD-53536.1, AD-53540.1, AD-

53541.1, AD-53542.1, AD-53547.1, AD-53548.1, AD-53550.1, AD-53552.1) at 24 hours, and some of these also had an IC_{50} of less than 0.1 nM (e.g., AD-53534.1, AD-53540.1, AD-53541.1, AD-53542.1, AD-53547.1, AD-53552.1) at 120 hours.

Table 5: IC_{50}s of select ALAS1 siRNA duplexes normalized to AD-1955

	IC50 (nM)	
DUPLEX ID	24hrs	120hrs
AD-53534.1	0.045	0.076
AD-53536.1	0.049	0.105
AD-53540.1	0.054	0.077
AD-53541.1	0.032	0.062
AD-53542.1	0.028	0.093
AD-53547.1	0.03	0.062
AD-53548.1	0.044	0.101
AD-53550.1	0.085	0.152
AD-53552.1	0.077	0.063
AD-53567.1	0.219	0.357
AD-53579.1	0.217	0.566

Example 4. *In Vivo* Silencing using a mouse/rat ALAS1 siRNA formulated as a LNP

The sequences of the modified duplex AD-53558 are shown in Table 6 below.

Table 6: Sequences of ALAS1 siRNA Duplex AD-53558.4

SEQ ID NO: (sense)	SEQ ID NO: (anti-sense)	Start Position on transcript of NM_020559.2	Duplex Name	Sense Sequence (5'-3')	Antisense Sequence (5'-3')
383	384	1184	AD-53558	cuGuGAAAuuuAcucuGAudTsdT	AUcAGAGuAAAUUUcAcAGdTsdT

This duplex was formulated as a LNP11 formulation (see Table 10 above). The LNP-formulated AD-53558 siRNA was tested in *in vivo* in mice (N=25 animals; 5 animals per group) and rats (N=20 animals; 4 animals per group) and was confirmed to silence ALAS1 mRNA *in vivo*. The results are shown in FIG. 5 and FIG. 6.

FIG. 5 shows that the siRNA demonstrated a dose-response effect in mice. The expression of mouse ALAS1 (mALAS1) mRNA was reduced by about 78% when the siRNA was administered at 1mg/kg; mouse ALAS1 mRNA was reduced by about 60% when the siRNA was administered at 0.3 mg/kg; and mouse ALAS1 mRNA was reduced by about 49% when the siRNA was administered at 0.1 mg/kg. These reductions are expressed relative to a PBS control. An AD-1955 LUC control was also employed, as shown in FIG. 5.

Similarly, FIG. 6 shows that the siRNA demonstrated a dose-response effect in rats. The expression of ALAS1 RNA was reduced by about 70% when the when the siRNA was administered at 1mg/kg; ALAS1 mRNA was reduced by about 62% when the siRNA was administered at 0.3 mg/kg; and ALAS1 mRNA was reduced by about 34% when the siRNA was administered at 0.1 mg/kg.

The durability of silencing was also tested in mice (N=15; 3 animals per timepoint. The results are shown in FIG. 7, which shows that AD-53558 suppressed mALAS1 mRNA by about 80% for at least 9 days. Suppression of at least about 50% persisted for at least 14 days.

Example 5. Efficacy of ALAS1 siRNA in an Animal Model of AIP

The effects of the AD-53558 LNP11 formulation (a mouse/rat ALAS1 siRNA described in the previous example) were investigated in a mouse model of AIP. The PBGD knockout is not viable (-/-, 0% activity). Heterozygous PBGD knockout mice (+/-, ~50% activity) are available but do not have the full biochemical phenotype and thus do not recapitulate the human disease phenotype. Thus, a mouse model of AIP has been developed that is a compound heterozygote with T1/T2 alleles, including T1 (+/-) promoter disruption and T2 (-/-) splice-site alteration. These mice have been shown to have hepatic residual PBGD activity that is about ~30% of the wild-type level and normal or slightly elevated baseline plasma ALA and PBG levels. The mice have been found to appear normal early in life and to become slightly slower and ataxic with age. By six months of age, the mice have been documented to develop impaired

motor coordination and muscular performance and axonal degeneration on pathological examination. Investigation of the pathology of the mouse model has shown axonal degeneration, impaired motor coordination and muscular performance in older mice. Urinary and plasma ALA and PBG have been found to markedly increase with serial i.p. administration of phenobarbital (see Lindberg et al., (1996), Nature Genetics, 12:195-219 and Lindberg et al., (1999), Journal of Clinical Investigation, 103:1127-34). The mice were rescued by AAV-mediated expression of PBGD in the liver (Yasuda et al. (2010), Molecular Medicine, 1:17-22 and Unzu et al. (2011), Molecular Medicine, 2:243-50).

On day 1, the mice were administered 1 mg/kg ALAS1 siRNA (n=5) or LUC AD-1955 control (n=3) by i.v. injection. Three phenobarbital injections were given (1 injection per day on days 2, 3, and 4) to induce hepatic ALAS1 and the porphyrin precursors, ALA and PBG. Plasma and overnight urine specimens were collected on day 5 and metabolite levels were measured by LC-MS. Metabolite levels were measured in plasma by LC-MS and were also measured in urine. Baseline levels of metabolites were measured prior to the first treatment on day 1. The results are shown in FIGs. 8-12 and in Tables 12 and 13.

FIG. 8 and FIG. 9 show the plasma ALA levels in μM. Baseline ALA levels were low, (n=4), and phenobarbital treatment induced significant increases in plasma ALA levels in the control LUC siRNA treated animals (n=3). Treatment with ALAS1 siRNA inhibited the induction of plasma ALA (n=5), as shown in FIG. 8. The ALAS1 siRNA was consistently effective in blocking the induction of plasma ALA in each of the individual animals studied (see FIG. 9). These results indicate that ALAS1 siRNA treatment was effective in preventing the increases in plasma ALA associated with the phenobarbital-induced acute attacks in this AIP animal model.

FIG. 10 and FIG. 11 show the plasma PBG levels in μM. Baseline PBG levels were low (n=4), and phenobarbital treatment induced significant increases in plasma PBG levels in the control LUC siRNA treated animals (n=3) Treatment with ALAS1 siRNA inhibited the induction of plasma PBG (n=5), as shown in FIG. 10. The ALAS1 siRNA was consistently effective in blocking the induction of plasma PBG in each of the individual animals studied (see FIG. 11). These results indicate that ALAS1 siRNA treatment was effective in preventing the

increases in plasma PBG associated with the phenobarbital-induced acute attacks in this AIP animal model.

Tables 12 and 13 shows urine ALA and PBG levels at baseline and after phenobarbital treatment in LUC siRNA (n=2) control (CTR, which refers to a PBS buffer treated animal, n=1) and ALAS1 siRNA (n=5) treated animals.

Table 12: Urine data from individual animals showing prevention of induced acute attack

Mouse ID	ALA (micro M/l)	PBG (micro M/L)	Creatinine (mg/dl)	ALA (microM/mg creatinine)	PBG (microM/mg creatinine)	siRNA	PB
Ha-17-4-6				29.7	7.9	Baseline	-
Ha-19-5-4/2				15.7	5.1	Baseline	-
Ha-20-39-4/3				28.6	6.7	Baseline	-
Ha-20-38-4				21.4	4.7	Baseline	-
Ha-21-33-4	934.92	483.71	0.4205	222.33	115.03	Luc	+
Ha-21-36-9	944.08	563.53	0.5055	186.76	111.48	Luc	+
Ha-21-18-8	32.88	8.69	0.133	24.72	6.53	ALAS1; 1mg/kg	+
Ha-21-33-7	83.07	23.28	0.426	19.50	5.46	ALAS1; 1mg/kg	+
Ha-21-34-5	59.15	18.41	0.263	22.49	7.00	ALAS1; 1mg/kg	+

PB stands for phenobarbital. A "+" indicates that phenobarbital was administered.

Table 13: Average Urine Data

Condition	Mean ALA (microM/mg creatinine)	Mean PBG (microM/mg creatinine)
AIP Baseline	23.8	6.1
Luc-siRNA	204.55	113.26
ALAS1-siRNA	22.24	6.33

Phenobarbital treatment induced strong increases (~25-30 fold increases) in urine ALA (~9-fold over baseline levels) and PBG (~19-fold over baseline levels) in the LUC siRNA treated mice, control, whereas such increases were not observed in the ALAS1 siRNA treated animals. Thus, ALAS1 siRNA blocked phenobarbital-induced increases in urinary ALA and PBG. These

results are consistent with the plasma measurements and show that ALAS1 siRNA treatment was effective in preventing increases in urinary metabolites (ALA and PBG) associated with the phenobarbital-induced acute attacks in this AIP animal model.

In further experiments (FIG. 12), it was found that phenobarbital treatment induced large increases (~25 fold) in ALAS1 mRNA expression in the liver of the mouse model. Administration of ALAS1 siRNA completely blocked this ALAS1 mRNA induction. These results provide further evidence that ALAS1 siRNA is effective in an animal model of AIP.

Collectively, the results provided in this Example show that ALAS1 siRNA was effective in treating acute attacks in an animal model of the acute hepatic porphyria AIP. Multiple outcome measures support this conclusion, including plasma ALA levels, plasma PBG levels, urine ALA levels, urine PBG levels, and liver ALAS1 mRNA expression levels.

Example 6. *In Vivo* Silencing using GalNAc-Conjugated Mouse ALAS1 siRNA

The experiments described in this example investigated the *in vivo* efficacy of three GalNAc-conjugated siRNAs (see Table 7). These siRNAs were designed and produced with methods such as those described in Example 2.

Table 7: Sequences AD-57929

SEQ ID NO: (sense)	SEQ ID NO: (anti-sense)	Position of sense seq. on transcript NM_020559.2	Duplex Name	Sense Sequence (5'-3')	Antisense Sequence (5'-3')	Position of antisense seq. on transcript NM_020559.2
385	386	775-795	AD-56211	AfaGfuCfuGfuUfUfCfcAfcUfuUfuCfaAfL96	uUfgAfaAfaGfuGfgaaAfcAfgAfcUfusUfsg	773-795
387	388	2168-	AD-56173	AfcAfuAfgUfaGfCfCfaGfaAfuUfgUfcUfL96	aGfaCfaAfuUfcUfggcUfaCfuAfuGfusGfsg	2166-2188

		2188				
389	390	775-795	AD-57929	AfsasGfuCfuGfuUfUfCfcAfcUfuUfuCfaAfL96	usUfsgAfaAfaGfuGfgaaAfcAfgAfcUfususg	773-795

The mice (n=40; n=4 per experimental condition) were divided into groups that received PBS or doses of 3 mg/kg, 10 mg/kg, or 30 mg/kg of siRNA administered subcutaneously. The level of mALAS1/mGAPDH mRNA, relative to the PBS control, was determined in liver cells at 72 hours post-administration. The results are shown in FIG. 13. There was not a clear dose-response effect for the siRNAs AD-56211 and AD-56173. In contrast, the ALAS1 siRNA AD-57929 showed a dose-response effect in inhibiting mALAS1 expression. These results demonstrate that an ALAS1 GalNAc conjugate was effective in inhibiting expression of ALAS1 mRNA *in vivo* and showed a dose-response effect.

Example 7. Human siRNAs

Additional human siRNAs were designed and produced as described in Example 2. The top 45 siRNAs were selected based on their predicted efficacy. The sequences of these 45 siRNAs are provided in Table 8 and the Sequence Listing attached herewith (e.g., a sense sequence corresponding to one of the odd numbered sequences identified as SEQ ID NOs: 391 to 551, and an antisense sequence corresponding to one of the even numbered sequences identified as SEQ ID NOs: 392 to 552, respectively). Table 8 is disclosed in International Publication No. WO2013/155204A2.The contents of WO 2013/155204 and the Sequence Listing, includingTable 8, are expressly incorporated by reference.

Example 8. Human siRNAs

Additional 19mer human siRNAs were generated. The sequences of these siRNAs are provided in Table 9 and the Sequence Listing attached herewith (e.g., a sense sequence corresponding to one of the odd numbered sequences identified as SEQ ID NOs: 553 to 3365, and an antisense sequence corresponding to one of the even numbered sequences identified as SEQ ID NOs: 554 to 3366, respectively). Table 9 is disclosed in International Publication No.

WO2013/155204A2. The contents of WO 2013/155204 and the Sequence Listing, includingTable 9, are expressly incorporated by reference. These siRNAs can be tested for efficacy using methods described herein and/or methods known in the art.

Example 9. Suppression of Porphyrin Precursors Using ALAS1 siRNA in an Acute Treatment Paradigm

The AIP mouse model (see Example 5) was used to investigate whether ALAS1 siRNA would work an an acute treatment paradigm to lower already elevated levels of ALA and PBG, as would be present, for example, when a human porphyria patient suffers from an acute attack. Administration of the AD-53558 LNP11 formulation siRNA at a 1mg/kg dose 12 hours after the last dose of phenobarbital rapidly decreased the levels of both ALA and PBG in mouse plasma, whereas in Luc control treated animals the levels continued to rise (FIG. 14). These results indicate that ALAS siRNA is effective for treating an acute attack. The ALAS1 siRNA was effective to lower and prevent further increases in ALA and PBG levels.

As can be observed in FIG. 14, ALAS siRNA had a rapid onset effect in reducing ALA and PBG levels. The onset of the effect occurred within hours after administration of the siRNA. The effect on plasma ALA could be observed within 4 hours of administration of the siRNA (see FIG. 14; the siRNA was administered at 12 hours after the last dose of phenobarbital, and a reduction in plasma ALA relative to control can be observed at 16 hours after the last dose of phenobarbital). The effect on plasma PBG could be observed within 8 hours of administration of the siRNA (see FIG. 14; the siRNA was administered at 12 hours after the last dose of phenobarbital, and a reduction in plasma ALA relative to control can be observed at 20 hours after the last dose of phenobarbital).

Example 10. siRNAs that target ALAS1

Further unmodified and modified siRNA sequences that target ALAS1 siRNA were designed and produced as described in Example 2. The *in vitro* activity of the modified duplexes was tested as described below.

Methods

Lipid mediated transfection

For Hep3B, PMH, and primary *Cynomolgus* hepatocytes, transfection was carried out by adding 14.8 µl of Opti-MEM plus 0.2 µl of Lipofectamine RNAiMax per well (Invitrogen, Carlsbad CA. catalog number13778-150) to 5 µl of each siRNA duplex to an individual well in a 96-well plate. The mixture was then incubated at room temperature for 20 minutes. Eighty µl of complete growth media without antibiotic containing the appropriate cell number were then added to the siRNA mixture. Cells were incubated for 24 hours prior to RNA purification.

Single dose experiments were performed at 1 uM, 500nM, 20nM, 10nM and 0.2nM final duplex concentration for GalNAc modified.

Free uptake transfection

Cryopreserved Primary *Cynomolgus* Hepatocytes (Celsis In Vitro Technologies, M003055-P) were thawed at 37°C water bath immediately prior to usage and re-suspended at $0.26x10^6$ cells/ml in InVitroGRO CP (plating) medium (Celsis In Vitro Technologies, catalog number Z99029). During transfections, cells were plated onto a BD BioCoat 96 well collagen plate (BD, 356407) at 25,000 cells per well and incubated at 37°C in an atmosphere of 5% CO_2. Free Uptake experiments were performed by adding 10µl of siRNA duplexes in PBS per well into a 96 well (96w) plate. Ninety µl of complete growth media containing appropriate cell number for the cell type was then added to the siRNA. Cells were incubated for 24 hours prior to RNA purification. Single dose experiments were performed at 1 uM, 500nM, 20nM and 10nM final duplex.

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

Cells were harvested and lysed in 150 µl of Lysis/Binding Buffer then mixed for 5 minutes at 850 rpm using an Eppendorf Thermomixer (the mixing speed was the same throughout the process). Ten microliters of magnetic beads and 80 µl 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, magnetic beads were washed 2 times with 150 µl

Wash Buffer A and mixed for 1 minute. The beads were capturedagain and the supernatant was removed. The beads were then washed with 150 µl Wash Buffer B, captured and the supernatant was removed. The beads were next washed with 150 µl Elution Buffer, captured and the supernatant removed. Finally, the beads were allowed to dry for 2 minutes. After drying, 50 µl of Elution Buffer was added and mixed for 5 minutes at 70°C. The beads were captured on magnet for 5 minutes. Forty-five µl 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 2 µl 10X Buffer, 0.8 µl 25X dNTPs, 2 µl Random primers, 1 µl Reverse Transcriptase, 1 µl RNase inhibitor and 3.2 µl of H2O per reaction as prepared. Equal volumes master mix and RNA were mixed for a final volume of 12µl for *in vitro* screened or 20µl for *in vivo* screened samples. cDNA was generated using a Bio-Rad C-1000 or S-1000 thermal cycler (Hercules, CA) through the following steps: 25°C for 10 minutes, 37°C for 120 minutes, 85°C for 5 seconds, and 4°C hold.

Real time PCR

Two µl of cDNA were added to a master mix containing 2µl of H_2O, 0.5µl GAPDH TaqMan Probe (Life Technologies catalog number 4326317E for Hep3B cells, catalog number 352339E for primary mouse hepatocytes or custom probe for cynomolgus primary hepatocytes), 0.5µl C5 TaqMan probe (Life Technologies catalog number Hs00167441_m1 for Hep3B cells or Mm00457879_m1 for Primary Mouse Hepatoctyes or custom probe for cynomolgus primary hepatocytes) and 5µl Lightcycler 480 probe master mix (Roche catalog number 04887301001) per well in a 384 well (384 w) plates (Roche catalog number 04887301001). Real time PCR was performed in an Roche LC480 Real Time PCR system (Roche) using the ΔΔCt(RQ) assay. For *in vitro* screening, each duplex was tested with two biological replicates unless otherwise noted and each Real Time PCR was performed in duplicate technical replicates. For *in vivo* screening, each duplex was tested in one or more experiments (3 mice per group) and each Real Time PCR was run in duplicate technical replicates.

To calculate relative fold change in ALAS1 mRNA levels, real time data were analyzed using the ΔΔCt method and normalized to assays performed with cells transfected with 10 nM AD-1955, or mock transfected cells. IC_{50}s 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.

The sense and antisense sequences of AD-1955 are:

SENSE: cuuAcGcuGAGuAcuucGAdTsdT (SEQ ID NO:3682)

ANTISENSE: UCGAAGuACUcAGCGuAAGdTsdT (SEQ ID NO:3683).

The single strand and duplex sequences of the modified and unmodified siRNAs are provided in Table 14 and Table 15, respectively.

Table 14: Human ALAS1 Modified Single Strands and Duplex Sequences

SEQ ID NO: (sense)	SEQ ID NO: (anti-sense)	Duplex Name	Sense Sequence (5'-3')	Antisense Sequence (5'-3')	Target sites of antisense sequence on NM_ 000688.4
3371	3372	AD-58848	CfsasUfgCfcAfaAfAfAfuGfgAfcAfuCfaUfL96	asUfsgAfuGfuCfcAfuuuUfuGfgCfaUfgsAfsc	1635-1657
3373	3374	AD-58849	AfsusUfuUfgAfaGfUfGfaUfgAfgUfgAfaAfL96	usUfsuCfaCfuCfaUfcacUfuCfaAfaAfusGfsc	2189-2211
3375	3376	AD-58850	AfsgsUfuAfuAfuUfAfAfaUfuUfuAfaUfcUfL96	asGfsaUfuAfaAfaUfuuaAfuAfuAfaCfusUfsa	2344-2366
3377	3378	AD-58851	GfscsAfuUfuUfgAfAfGfuGfaUfgAfgUfgAfL96	usCfsaCfuCfaUfcAfcuuCfaAfaAfuGfcsAfsg	2187-2209
3379	3380	AD-58852	GfsasAfcUfaAfuGfAfGfcAfgAfcAfuAfaCfL96	gsUfsuAfuGfuCfuGfcucAfuUfaGfuUfcsAfsu	1975-1997
3381	3382	AD-58853	AfsasUfgAfcCfaCfAfCfcUfaUfcGfaGfuUfL96	asAfscUfcGfaUfaGfgugUfgGfuCfaUfusCfsu	973-995
3383	3384	AD-58854	UfsasAfaUfuUfuAfAfUfcUfaUfaGfuAfaAfL96	usUfsuAfcUfaUfaGfauuAfaAfaUfuUfasAfsu	2352-2374
3385	3386	AD-58855	UfsusCfaGfuAfuGfAfUfcGfuUfuCfuUfuGfL96	csAfsaAfgAfaAfcGfaucAfuAfcUfgAfasAfsa	929-951
3387	3388	AD-58856	CfsasCfuUfuUfcAfGfUfaUfgAfuCfgUfuUfL96	asAfsaCfgAfuCfaUfacuGfaAfaAfgUfgsGfsa	924-946
3389	3390	AD-58857	AfsasAfuCfuGfuUfUfCfcAfcUfuUfuCfaGfL96	csUfsgAfaAfaGfuGfgaaAfcAfgAfuUfusUfsg	913-935

3391	3392	AD-58858	CfsasUfuUfgAfaAfCfUfgUfcCfaUfuCfaAfL96	usUfsgAfaUfgGfaCfaguUfuCfaAfaUfgsCfsc	1478-1500
3393	3394	AD-58859	CfscsUfaUfcGfaGfUfUfuUfuAfaAfaCfuGfL96	csAfsgUfuUfuAfaAfaacUfcGfaUfaGfgsUfsg	983-1005
3395	3396	AD-58861	GfsasCfcAfgAfaAfGfAfgUfgUfcUfcAfuCfL96	gsAfsuGfaGfaCfaCfucuUfuCfuGfgUfcsUfsu	872-894
3397	3398	AD-58862	AfscsCfaGfaAfaGfAfGfuGfuCfuCfaUfcUfL96	asGfsaUfgAfgAfcAfcucUfuUfcUfgGfusCfsu	873-895
3399	3400	AD-58863	AfscsUfaAfuGfaGfCfAfgAfcAfuAfaCfaUfL96	asUfsgUfuAfuGfuCfugcUfcAfuUfaGfusUfsc	1977-1999
3401	3402	AD-58864	UfsasGfuAfaAfaAfCfAfuAfgUfcCfuGfgAfL96	usCfscAfgGfaCfuAfuguUfuUfuAfcUfasUfsa	2366-2388
3403	3404	AD-58865	UfsasUfuUfcUfgGfAfAfcUfaGfuAfaAfuUfL96	asAfsuUfuAfcUfaGfuucCfaGfaAfaUfasUfsu	1185-1207
3405	3406	AD-58867	UfsusCfuGfcAfaAfGfCfcAfgUfcUfuGfaGfL96	csUfscAfaGfaCfuGfgcuUfuGfcAfgAfasGfsa	706-728
3407	3408	AD-58868	GfsasGfgAfaAfgAfGfGfuUfgCfuGfaAfaCfL96	gsUfsuUfcAfgCfaAfccuCfuUfuCfcUfcsAfsc	759-781
3409	3410	AD-58869	GfsgsUfaCfuAfgAfAfAfuAfuUfuCfuGfgAfL96	usCfscAfgAfaAfuAfuuuCfuAfgUfaCfcsAfsc	1174-1196
3411	3412	AD-58870	GfsasCfaUfcAfuGfCfAfaAfaGfcAfaAfgAfL96	usCfsuUfuGfcUfuUfugcAfuGfaUfgUfcsCfsu	853-875
3413	3414	AD-58871	AfsasAfuUfuUfaAfUfCfuAfuAfgUfaAfaAfL96	usUfsuUfaCfuAfuAfgauUfaAfaAfuUfusAfsa	2353-2375
3415	3416	AD-58873	CfsasUfgAfuCfcAfAfGfgGfaUfuCfgAfaAfL96	usUfsuCfgAfaUfcCfcuuGfgAfuCfaUfgsGfsa	1362-1384
3417	3418	AD-58874	AfsgsAfcCfaGfaAfAfGfaGfuGfuCfuCfaUfL96	asUfsgAfgAfcAfcUfcuuUfcUfgGfuCfusUfsu	871-893
3419	3420	AD-58875	AfsusCfcUfgAfaGfAfGfcGfcUfgAfgGfgAfL96	usCfscCfuCfaGfcGfcucUfuCfaGfgAfusCfsc	1810-1832
3421	3422	AD-58876	GfsusCfuGfuGfaUfGfAfaCfuAfaUfgAfgCfL96	gsCfsuCfaUfuAfgUfucaUfcAfcAfgAfcsUfsu	1966-1988
3423	3424	AD-58877	CfsasGfaAfaGfaGfUfGfuCfuCfaUfcUfuCfL96	gsAfsaGfaUfgAfgAfcacUfcUfuUfcUfgsGfsu	875-897
3425	3426	AD-58878	AfscsUfuUfuCfaGfUfAfuGfaUfcGfuUfuCfL96	gsAfsaAfcGfaUfcAfuacUfgAfaAfaGfusGfsg	925-947
3427	3428	AD-58879	UfscsAfuGfcCfaAfAfAfaUfgGfaCfaUfcAfL96	usGfsaUfgUfcCfaUfuuuUfgGfcAfuGfasCfsu	1634-1656
3429	3430	AD-58880	AfsasUfaUfuUfcUfGfGfaAfcUfaGfuAfaAfL96	usUfsuAfcUfaGfuUfccaGfaAfaUfaUfusUfsc	1183-1205
3431	3432	AD-58881	CfsusUfcUfuCfaAfGfAfuAfaCfuUfgCfcAfL96	usGfsgCfaAfgUfuAfucuUfgAfaGfaAfgsAfsu	892-914
3433	3434	AD-58882	UfsusUfcAfgUfaUfGfAfuCfgUfuUfcUfuUfL96	asAfsaGfaAfaCfgAfucaUfaCfuGfaAfasAfsg	928-950
3435	3436	AD-58883	CfscsCfaGfuGfuGfGfUfuAfgUfgUfgAfaAfL96	usUfsuCfaCfaCfuAfaccAfcAfcUfgGfgsGfsc	790-812
3437	3438	AD-58884	GfscsUfgUfgAfgAfUfUfuAfcUfcUfgAfuUfL96	asAfsuCfaGfaGfuAfaauCfuCfaCfaGfcsCfsu	1325-1347
3439	3440	AD-58885	AfsgsGfcUfuGfaGfCfAfaGfuUfgGfuAfuCfL96	gsAfsuAfcCfaAfcUfugcUfcAfaGfcCfusGfsa	2229-2251
3441	3442	AD-58886	GfsasAfaGfaGfuGfUfCfuCfaUfcUfuCfuUfL96	asAfsgAfaGfaUfgAfgacAfcUfcUfuUfcsUfsg	877-899
3443	3444	AD-58887	AfsusUfuCfuGfgAfAfCfuAfgUfaAfaUfuCfL96	gsAfsaUfuUfaCfuAfguuCfcAfgAfaAfusAfsu	1186-1208

3445	3446	AD-58888	UfsgsUfgAfuGfuGfGfCfcCfaUfgAfgUfuUfL96	asAfsaCfuCfaUfgGfgccAfcAfuCfaCfasCfsa	1531-1553
3447	3448	AD-58889	AfsasGfaGfaGfaAfGfUfcCfuAfuUfuCfuCfL96	gsAfsgAfaAfuAfgGfacuUfcUfcUfcUfusUfsc	2208-2230
3449	3450	AD-58890	UfsgsGfcAfgCfaCfAfGfaUfgAfaUfcAfgAfL96	usCfsuGfaUfuCfaUfcugUfgCfuGfcCfasGfsg	671-693
3451	3452	AD-58891	AfsusGfaUfcGfuUfUfCfuUfuGfaGfaAfaAfL96	usUfsuUfcUfcAfaAfgaaAfcGfaUfcAfusAfsc	935-957
3453	3454	AD-58892	UfscsUfgGfaAfcUfAfGfuAfaAfuUfcCfaUfL96	asUfsgGfaAfuUfuAfcuaGfuUfcCfaGfasAfsa	1189-1211
3455	3456	AD-59095	GfscsCfcAfuUfcUfUfAfuCfcCfgAfgUfL96	asCfsuCfgGfgAfuAfagaAfuGfgsgsc	360-382
3457	3458	AD-59096	GfsgsAfaCfcAfuGfCfCfuCfcAfuGfaUfL96	asUfscAfuGfgAfgGfcauGfgUfuscsc	1347-1369
3459	3460	AD-59097	UfsgsGfaGfuCfuGfUfGfcGfgAfuCfcUfL96	asGfsgAfuCfcGfcAfcagAfcUfcscsa	1794-1816
3461	3462	AD-59098	CfsasCfcCfaCfgGfGfUfgUfgUfgGfgAfL96	usCfscCfaCfaCfaCfccgUfgGfgsusg	1112-1134
3463	3464	AD-59099	GfsgsAfgUfcUfgUfGfCfgGfaUfcCfuAfL96	usAfsgGfaUfcCfgCfacaGfaCfuscsc	1795-1817
3465	3466	AD-59100	CfsasAfaAfcUfgCfCfCfcAfaGfaUfgAfL96	usCfsaUfcUfuGfgGfgcaGfuUfususg	428-450
3467	3468	AD-59101	GfscsCfuCfcAfuGfAfUfcCfaAfgGfgAfL96	usCfscCfuUfgGfaUfcauGfgAfgsgsc	1355-1377
3469	3470	AD-59102	CfsasUfcAfuCfcCfUfGfuGfcGfgGfuUfL96	asAfscCfcGfcAfcAfgggAfuGfasusg	1921-1943
3471	3472	AD-59103	AfscsCfcAfcGfgGfUfGfuGfuGfgGfgAfL96	usCfscCfcAfcAfcAfcccGfuGfgsgsu	1113-1135
3473	3474	AD-59104	CfsasCfaUfcAfuCfCfCfuGfuGfcGfgAfL96	usCfscGfcAfcAfgGfgauGfaUfgsusg	1919-1941
3475	3476	AD-59105	CfsasGfaAfaGfaGfUfGfuCfuCfaUfcUfL96	asGfsaUfgAfgAfcAfcucUfuUfcsusg	873-895
3477	3478	AD-59106	CfscsUfcCfaUfgAfUfCfcAfaGfgGfaUfL96	asUfscCfcUfuGfgAfucaUfgGfasgsg	1356-1378
3479	3480	AD-59107	UfsgsCfcCfaUfuCfUfUfaUfcCfcGfaAfL96	usUfscGfgGfaUfaAfgaaUfgGfgscsa	359-381
3481	3482	AD-59108	CfsusUfcAfcCfcUfGfGfcUfaAfgAfuAfL96	usAfsuCfuUfaGfcCfaggGfuGfasasg	1297-1319
3483	3484	AD-59109	AfsusCfaUfcCfcUfGfUfgCfgGfgUfuAfL96	usAfsaCfcCfgCfaCfaggGfaUfgsasu	1922-1944
3485	3486	AD-59110	AfsgsAfaAfgAfgUfGfUfcUfcAfuCfuUfL96	asAfsgAfuGfaGfaCfacuCfuUfuscsu	874-896
3487	3488	AD-59111	CfsusCfcAfuGfaUfCfCfaAfgGfgAfuUfL96	asAfsuCfcCfuUfgGfaucAfuGfgsasg	1357-1379
3489	3490	AD-59112	CfscsAfuUfcUfuAfUfCfcCfgAfgUfcAfL96	usGfsaCfuCfgGfgAfuaaGfaAfusgsg	362-384
3491	3492	AD-59113	CfsasCfcCfuGfgCfUfAfaGfaUfgAfuAfL96	usAfsuCfaUfcUfuAfgccAfgGfgsusg	1300-1322
3493	3494	AD-59114	UfscsAfuCfcCfuGfUfGfcGfgGfuUfgAfL96	usCfsaAfcCfcGfcAfcagGfgAfusgsa	1923-1945
3495	3496	AD-59115	AfsasGfaGfuGfuCfUfCfaUfcUfuCfuUfL96	asAfsgAfaGfaUfgAfgacAfcUfcsusu	877-899
3497	3498	AD-59116	GfsusCfaUfgCfcAfAfAfaAfuGfgAfcAfL96	usGfsuCfcAfuUfuUfuggCfaUfgsasc	1631-1653

3499	3500	AD-59117	CfsasUfuCfuUfaUfCfCfcGfaGfuCfcAfL96	usGfsgAfcUfcGfgGfauaAfgAfasusg	363-385
3501	3502	AD-59118	AfscsCfcUfgGfcUfAfAfgAfuGfaUfgAfL96	usCfsaUfcAfuCfuUfagcCfaGfgsgsu	1301-1323
3503	3504	AD-59119	CfsusCfuUfcAfcCfCfUfgGfcUfaAfgAfL96	usCfsuUfaGfcCfaGfgguGfaAfgsasg	1295-1317
3505	3506	AD-59120	AfsusGfcCfaAfaAfAfUfgGfaCfaUfcAfL96	usGfsaUfgUfcCfaUfuuuUfgGfcsasu	1634-1656
3507	3508	AD-59121	UfsgsCfcCfcAfaGfAfUfgAfuGfgAfaUfL96	asUfsuCfcAfuCfaUfcuuGfgGfgscsa	434-456
3509	3510	AD-59122	GfsasAfcCfaUfgCfCfUfcCfaUfgAfuAfL96	usAfsuCfaUfgGfaGfgcaUfgGfususc	1348-1370
3511	3512	AD-59123	UfscsUfuCfaCfcCfUfGfgCfuAfaGfaUfL96	asUfscUfuAfgCfcAfgggUfgAfasgsa	1296-1318
3513	3514	AD-59124	UfsgsCfcAfaAfaAfUfGfgAfcAfuCfaUfL96	asUfsgAfuGfuCfcAfuuuUfuGfgscsa	1635-1657
3515	3516	AD-59125	CfscsAfgAfaAfgAfGfUfgUfcUfcAfuAfL96	usAfsuGfaGfaCfaCfucuUfuCfusgsg	872-894
3517	3518	AD-59126	GfsasAfaCfuGfuCfCfAfuUfcAfaUfgAfL96	usCfsaUfuGfaAfuGfgacAfgUfususc	1481-1503
3519	3520	AD-59127	UfscsAfcCfcUfgGfCfUfaAfgAfuGfaUfL96	asUfscAfuCfuUfaGfccaGfgGfusgsa	1299-1321
3521	3522	AD-59128	CfscsCfuGfgAfgUfCfUfgUfgCfgGfaUfL96	asUfscCfgCfaCfaGfacuCfcAfgsgsg	1791-1813
3523	3524	AD-59129	GfsasAfaGfaGfuGfUfCfuCfaUfcUfuAfL96	usAfsaGfaUfgAfgAfcacUfcUfususc	875-897
3525	3526	AD-59130	UfsgsGfaGfcCfcUfGfGfaGfuCfuGfuAfL96	usAfscAfgAfcUfcCfaggGfcUfcscsa	1786-1808

Table 15: Human ALAS1 Unmodified Single Strands and Duplex Sequences

SEQ ID NO: (sense)	SEQ ID NO: (anti-sense)	Duplex Name	Sense Sequence (5'-3')	Antisense Sequence (5'-3')	Target sites of antisense sequence on NM_ 000688.4
3684	3527	AD-58848	CAUGCCAAAAAUGGACAUCAU	AUGAUGUCCAUUUUUGGCAUGAC	1635-1657
3528	3529	AD-58849	AUUUUGAAGUGAUGAGUGAAA	UUUCACUCAUCACUUCAAAAUGC	2189-2211
3530	3531	AD-58850	AGUUAUAUUAAAUUUUAAUCU	AGAUUAAAAUUUAAUAUAACUUA	2344-2366
3532	3533	AD-58851	GCAUUUUGAAGUGAUGAGUGA	UCACUCAUCACUUCAAAAUGCAG	2187-2209
3534	3535	AD-58852	GAACUAAUGAGCAGACAUAAC	GUUAUGUCUGCUCAUUAGUUCAU	1975-1997
3536	3537	AD-58853	AAUGACCACACCUAUCGAGUU	AACUCGAUAGGUGUGGUCAUUCU	973-995
3538	3539	AD-58854	UAAAUUUUAAUCUAUAGUAAA	UUUACUAUAGAUUAAAAUUUAAU	2352-2374
3540	3541	AD-58855	UUCAGUAUGAUCGUUUCUUUG	CAAAGAAACGAUCAUACUGAAAA	929-951
3542	3543	AD-58856	CACUUUUCAGUAUGAUCGUUU	AAACGAUCAUACUGAAAAGUGGA	924-946
3544	3545	AD-58857	AAAUCUGUUUCCACUUUUCAG	CUGAAAAGUGGAAACAGAUUUUG	913-935
3546	3547	AD-58858	CAUUUGAAACUGUCCAUUCAA	UUGAAUGGACAGUUUCAAAUGCC	1478-1500

3548	3549	AD-58859	CCUAUCGAGUUUUUAAAACUG	CAGUUUUAAAAACUCGAUAGGUG	983-1005
3550	3551	AD-58861	GACCAGAAAGAGUGUCUCAUC	GAUGAGACACUCUUUCUGGUCUU	872-894
3552	3553	AD-58862	ACCAGAAAGAGUGUCUCAUCU	AGAUGAGACACUCUUUCUGGUCU	873-895
3554	3555	AD-58863	ACUAAUGAGCAGACAUAACAU	AUGUUAUGUCUGCUCAUUAGUUC	1977-1999
3556	3557	AD-58864	UAGUAAAAACAUAGUCCUGGA	UCCAGGACUAUGUUUUUACUAUA	2366-2388
3558	3559	AD-58865	UAUUUCUGGAACUAGUAAAUU	AAUUUACUAGUUCCAGAAAUAUU	1185-1207
3560	3561	AD-58867	UUCUGCAAAGCCAGUCUUGAG	CUCAAGACUGGCUUUGCAGAAGA	706-728
3562	3563	AD-58868	GAGGAAAGAGGUUGCUGAAAC	GUUUCAGCAACCUCUUUCCUCAC	759-781
3564	3565	AD-58869	GGUACUAGAAAUAUUUCUGGA	UCCAGAAAUAUUUCUAGUACCAC	1174-1196
3566	3567	AD-58870	GACAUCAUGCAAAAGCAAAGA	UCUUUGCUUUUGCAUGAUGUCCU	853-875
3568	3569	AD-58871	AAAUUUUAAUCUAUAGUAAAA	UUUUACUAUAGAUUAAAAUUUAA	2353-2375
3570	3571	AD-58873	CAUGAUCCAAGGGAUUCGAAA	UUUCGAAUCCCUUGGAUCAUGGA	1362-1384
3572	3573	AD-58874	AGACCAGAAAGAGUGUCUCAU	AUGAGACACUCUUUCUGGUCUUU	871-893
3574	3575	AD-58875	AUCCUGAAGAGCGCUGAGGGA	UCCCUCAGCGCUCUUCAGGAUCC	1810-1832
3576	3577	AD-58876	GUCUGUGAUGAACUAAUGAGC	GCUCAUUAGUUCAUCACAGACUU	1966-1988
3578	3579	AD-58877	CAGAAAGAGUGUCUCAUCUUC	GAAGAUGAGACACUCUUUCUGGU	875-897
3580	3581	AD-58878	ACUUUUCAGUAUGAUCGUUUC	GAAACGAUCAUACUGAAAAGUGG	925-947
3582	3583	AD-58879	UCAUGCCAAAAAUGGACAUCA	UGAUGUCCAUUUUUGGCAUGACU	1634-1656
3584	3585	AD-58880	AAUAUUUCUGGAACUAGUAAA	UUUACUAGUUCCAGAAAUAUUUC	1183-1205
3586	3587	AD-58881	CUUCUUCAAGAUAACUUGCCA	UGGCAAGUUAUCUUGAAGAAGAU	892-914
3588	3589	AD-58882	UUUCAGUAUGAUCGUUUCUUU	AAAGAAACGAUCAUACUGAAAAG	928-950
3590	3591	AD-58883	CCCAGUGUGGUUAGUGUGAAA	UUUCACACUAACCACACUGGGGC	790-812
3592	3593	AD-58884	GCUGUGAGAUUUACUCUGAUU	AAUCAGAGUAAAUCUCACAGCCU	1325-1347
3594	3595	AD-58885	AGGCUUGAGCAAGUUGGUAUC	GAUACCAACUUGCUCAAGCCUGA	2229-2251
3596	3597	AD-58886	GAAAGAGUGUCUCAUCUUCUU	AAGAAGAUGAGACACUCUUUCUG	877-899
3598	3599	AD-58887	AUUUCUGGAACUAGUAAAUUC	GAAUUUACUAGUUCCAGAAAUAU	1186-1208
3600	3601	AD-58888	UGUGAUGUGGCCCAUGAGUUU	AAACUCAUGGGCCACAUCACACA	1531-1553
3602	3603	AD-58889	AAGAGAGAAGUCCUAUUUCUC	GAGAAAUAGGACUUCUCUCUUUC	2208-2230
3604	3605	AD-58890	UGGCAGCACAGAUGAAUCAGA	UCUGAUUCAUCUGUGCUGCCAGG	671-693
3606	3607	AD-58891	AUGAUCGUUUCUUUGAGAAAA	UUUUCUCAAAGAAACGAUCAUAC	935-957
3608	3609	AD-58892	UCUGGAACUAGUAAAUUCCAU	AUGGAAUUUACUAGUUCCAGAAA	1189-1211
3610	3611	AD-59095	GCCCAUUCUUAUCCCGAGU	ACUCGGGAUAAGAAUGGGC	360-382
3612	3613	AD-59096	GGAACCAUGCCUCCAUGAU	AUCAUGGAGGCAUGGUUCC	1347-1369
3614	3615	AD-59097	UGGAGUCUGUGCGGAUCCU	AGGAUCCGCACAGACUCCA	1794-1816
3616	3617	AD-59098	CACCCACGGGUGUGUGGGA	UCCCACACACCCGUGGGUG	1112-1134
3618	3619	AD-59099	GGAGUCUGUGCGGAUCCUA	UAGGAUCCGCACAGACUCC	1795-1817
3620	3621	AD-59100	CAAAACUGCCCCAAGAUGA	UCAUCUUGGGGCAGUUUUG	428-450
3622	3623	AD-59101	GCCUCCAUGAUCCAAGGGA	UCCCUUGGAUCAUGGAGGC	1355-1377
3624	3625	AD-59102	CAUCAUCCCUGUGCGGGUU	AACCCGCACAGGGAUGAUG	1921-1943
3626	3627	AD-59103	ACCCACGGGUGUGUGGGGA	UCCCCACACACCCGUGGGU	1113-1135
3628	3629	AD-59104	CACAUCAUCCCUGUGCGGA	UCCGCACAGGGAUGAUGUG	1919-1941
3630	3631	AD-59105	CAGAAAGAGUGUCUCAUCU	AGAUGAGACACUCUUUCUG	873-895
3632	3633	AD-59106	CCUCCAUGAUCCAAGGGAU	AUCCCUUGGAUCAUGGAGG	1356-1378
3634	3635	AD-59107	UGCCCAUUCUUAUCCCGAA	UUCGGGAUAAGAAUGGGCA	359-381
3636	3637	AD-59108	CUUCACCCUGGCUAAGAUA	UAUCUUAGCCAGGGUGAAG	1297-1319
3638	3639	AD-59109	AUCAUCCCUGUGCGGGUUA	UAACCCGCACAGGGAUGAU	1922-1944
3640	3641	AD-59110	AGAAAGAGUGUCUCAUCUU	AAGAUGAGACACUCUUUCU	874-896
3642	3643	AD-59111	CUCCAUGAUCCAAGGGAUU	AAUCCCUUGGAUCAUGGAG	1357-1379
3644	3645	AD-59112	CCAUUCUUAUCCCGAGUCA	UGACUCGGGAUAAGAAUGG	362-384
3646	3647	AD-59113	CACCCUGGCUAAGAUGAUA	UAUCAUCUUAGCCAGGGUG	1300-1322
3648	3649	AD-59114	UCAUCCCUGUGCGGGUUGA	UCAACCCGCACAGGGAUGA	1923-1945
3650	3651	AD-59115	AAGAGUGUCUCAUCUUCUU	AAGAAGAUGAGACACUCUU	877-899
3652	3653	AD-59116	GUCAUGCCAAAAAUGGACA	UGUCCAUUUUUGGCAUGAC	1631-1653

3654	3655	AD-59117	CAUUCUUAUCCCGAGUCCA	UGGACUCGGGAUAAGAAUG	363-385
3656	3657	AD-59118	ACCCUGGCUAAGAUGAUGA	UCAUCAUCUUAGCCAGGGU	1301-1323
3658	3659	AD-59119	CUCUUCACCCUGGCUAAGA	UCUUAGCCAGGGUGAAGAG	1295-1317
3660	3661	AD-59120	AUGCCAAAAAUGGACAUCA	UGAUGUCCAUUUUUGGCAU	1634-1656
3662	3663	AD-59121	UGCCCCAAGAUGAUGGAAU	AUUCCAUCAUCUUGGGGCA	434-456
3664	3665	AD-59122	GAACCAUGCCUCCAUGAUA	UAUCAUGGAGGCAUGGUUC	1348-1370
3666	3667	AD-59123	UCUUCACCCUGGCUAAGAU	AUCUUAGCCAGGGUGAAGA	1296-1318
3668	3669	AD-59124	UGCCAAAAAUGGACAUCAU	AUGAUGUCCAUUUUUGGCA	1635-1657
3670	3671	AD-59125	CCAGAAAGAGUGUCUCAUA	UAUGAGACACUCUUUCUGG	872-894
3672	3673	AD-59126	GAAACUGUCCAUUCAAUGA	UCAUUGAAUGGACAGUUUC	1481-1503
3674	3675	AD-59127	UCACCCUGGCUAAGAUGAU	AUCAUCUUAGCCAGGGUGA	1299-1321
3676	3677	AD-59128	CCCUGGAGUCUGUGCGGAU	AUCCGCACAGACUCCAGGG	1791-1813
3678	3679	AD-59129	GAAAGAGUGUCUCAUCUUA	UAAGAUGAGACACUCUUUC	875-897
3680	3681	AD-59130	UGGAGCCCUGGAGUCUGUA	UACAGACUCCAGGGCUCCA	1786-1808

The results of the *in vitro* assays are provided in Table 16. Table 16 also notes the target species of each of the siRNAs.

Table 16: Results of Functional Assays

			Cyno Free Uptake				Cyno Transfection		Hep3b Transfection	
Duplex ID	Target Species	Type	1uM Avg	500nM	20nM Avg	10nM	20nM Avg	0.2nM Avg	10nM Avg	0.1nM Avg
AD-58848	M/R/Rh/H	21/23	131.6	176.0	104.4	128.0	43.5	44.8	25.3	76.8
AD-58849	H/Rh	21/23	91.9	88.1	92.2	105.0	29.4	35.4	11.5	47.1
AD-58850	H/Rh	21/23	79.4	103.4	80.0	111.2	NA	62.2	31.3	72.0
AD-58851	H/Rh	21/23	99.7	74.7	94.8	104.7	NA	40.7	8.6	81.3
AD-58852	H/Rh	21/23	108.1	91.8	103.3	111.9	101.1	128.8	43.4	129.0
AD-58853	H/Rh	21/23	74.8	67.7	84.2	93.5	24.7	52.9	14.1	61.2
AD-58854	H/Rh	21/23	145.9	124.1	106.6	115.3	119.0	83.9	85.0	84.0
AD-58855	H/Rh	21/23	81.5	97.9	92.7	101.8	39.5	40.3	15.3	67.6
AD-58856	H/Rh	21/23	74.1	90.6	84.6	82.6	22.4	30.7	8.7	33.3
AD-58857	H/Rh	21/23	64.7	91.4	62.3	87.1	22.0	31.6	9.8	106.3
AD-58858	H/Rh	21/23	67.4	91.7	68.6	98.3	27.9	40.3	17.4	44.8
AD-58859	H/Rh	21/23	71.2	77.2	92.4	90.1	19.1	34.3	13.1	39.7
AD-58861	H/Rh	21/23	104.6	107.2	102.0	100.6	25.9	35.1	18.0	69.8
AD-58862	H/Rh	21/23	66.8	77.0	68.7	88.5	20.3	31.1	24.2	49.9
AD-58863	H/Rh	21/23	70.8	66.8	76.8	98.5	21.5	29.7	8.7	54.9
AD-58864	H/Rh	21/23	76.2	85.6	83.7	100.8	60.4	61.0	56.4	87.3
AD-58865	H/Rh	21/23	67.9	77.9	95.9	98.4	21.3	38.6	15.5	81.4
AD-58867	H/Rh	21/23	95.9	93.3	107.0	97.5	32.3	42.7	16.6	79.8
AD-58868	H/Rh	21/23	95.2	92.1	116.2	94.7	54.6	69.2	61.5	105.9
AD-58869	H/Rh	21/23	65.0	78.2	75.8	88.2	17.4	25.0	13.0	63.9
AD-58870	H/Rh	21/23	69.4	92.3	81.0	88.1	29.2	43.8	33.7	79.1
AD-58871	H/Rh	21/23	61.2	77.3	88.2	77.0	71.2	73.2	36.7	110.3
AD-58873	H/Rh	21/23	95.2	100.9	83.3	94.6	54.2	52.8	36.6	73.3
AD-58874	H/Rh	21/23	75.8	76.8	63.8	85.3	22.3	31.2	15.0	38.2

AD-58875	H/Rh	21/23	80.7	88.7	78.6	97.9	48.6	73.6	61.2	90.6
AD-58876	H/Rh	21/23	90.8	93.1	82.5	100.2	41.1	56.9	21.2	58.7
AD-58877	H/Rh	21/23	68.3	85.1	51.2	78.7	18.5	46.6	11.9	27.4
AD-58878	H/Rh	21/23	78.3	68.3	81.2	91.2	24.1	23.4	6.2	37.1
AD-58879	H/Rh	21/23	87.9	94.1	79.7	95.4	32.0	47.8	15.7	82.5
AD-58880	H/Rh	21/23	74.9	72.2	88.9	88.1	20.1	27.5	14.0	60.7
AD-58881	H/Rh	21/23	85.9	76.8	78.8	118.0	22.2	36.7	27.6	71.6
AD-58882	H/Rh	21/23	54.1	53.4	60.3	85.8	14.6	27.2	8.2	23.8
AD-58883	H/Rh	21/23	80.4	69.9	75.7	80.3	31.8	25.8	12.3	63.0
AD-58884	H/Rh	21/23	57.7	55.3	64.8	78.2	20.0	30.0	11.8	68.9
AD-58885	H/Rh	21/23	101.8	91.8	104.1	101.5	85.9	71.9	61.8	71.2
AD-58886	M/R/Rh/H	21/23	47.1	58.0	36.3	93.3	16.0	26.6	9.2	32.0
AD-58887	H/Rh	21/23	73.6	98.7	82.6	95.2	28.5	33.5	12.8	65.2
AD-58888	H/Rh	21/23	90.2	69.9	69.4	85.6	46.9	45.0	16.6	72.0
AD-58889	H/Rh	21/23	83.6	98.6	82.4	92.2	36.5	40.3	31.6	99.4
AD-58890	H/Rh	21/23	69.5	95.4	84.2	88.2	50.8	45.6	21.7	92.9
AD-58891	H/Rh	21/23	62.8	75.7	75.4	109.2	23.6	34.3	15.6	55.8
AD-58892	H/Rh	21/23	60.2	92.9	89.8	92.9	22.8	43.3	20.2	75.6
AD-59095	M/R/Rh/H	19mer	88.9	NA	132.8	NA	48.3	97.4	54.3	99.0
AD-59096	M/R/Rh/H	19mer	95.5	NA	90.5	NA	105.7	138.6	131.4	120.7
AD-59097	M/R/Rh/H	19mer	92.5	NA	84.2	NA	75.0	NA	94.7	108.5
AD-59098	M/R/Rh/H	19mer	84.0	NA	87.7	NA	109.3	NA	130.0	87.3
AD-59099	M/R/Rh/H	19mer	89.7	NA	90.0	NA	77.8	85.4	46.8	74.9
AD-59100	M/R/Rh/H	19mer	84.8	NA	144.3	NA	70.6	108.1	91.5	117.6
AD-59101	M/R/Rh/H	19mer	79.0	NA	103.8	NA	89.8	102.9	124.2	107.0
AD-59102	M/R/Rh/H	19mer	85.9	NA	100.6	NA	72.2	68.5	87.9	95.1
AD-59103	M/R/Rh/H	19mer	86.0	NA	91.1	NA	93.0	81.3	130.0	96.0
AD-59104	M/R/Rh/H	19mer	92.6	NA	96.9	NA	94.9	91.4	124.4	83.1
AD-59105	M/R/Rh/H	19mer	48.9	NA	101.7	NA	18.4	48.9	17.0	34.7
AD-59106	M/R/Rh/H	19mer	63.2	NA	76.7	NA	28.5	40.7	28.6	46.4
AD-59107	M/R/Rh/H	19mer	71.4	NA	68.7	NA	37.1	45.3	26.8	63.6
AD-59108	M/R/Rh/H	19mer	70.7	NA	85.1	NA	89.9	84.8	139.2	101.7
AD-59109	M/R/Rh/H	19mer	86.1	NA	83.4	NA	84.9	96.2	131.7	86.7
AD-59110	M/R/Rh/H	19mer	70.8	NA	119.7	NA	38.5	60.4	67.4	80.3
AD-59111	M/R/Rh/H	19mer	66.1	NA	76.5	NA	52.2	61.0	69.7	87.6
AD-59112	M/R/Rh/H	19mer	71.2	NA	80.2	NA	91.2	83.4	127.4	89.0
AD-59113	M/R/Rh/H	19mer	67.0	NA	77.8	NA	49.1	59.0	66.8	91.4
AD-59114	M/R/Rh/H	19mer	81.7	NA	79.3	NA	96.3	88.0	129.6	72.4
AD-59115	M/R/Rh/H	19mer	40.4	NA	69.6	NA	19.6	35.7	9.3	16.9
AD-59116	M/R/Rh/H	19mer	72.2	NA	78.3	NA	53.5	77.8	70.1	107.8
AD-59117	M/R/Rh/H	19mer	70.7	NA	75.6	NA	75.8	74.9	129.0	103.5
AD-59118	M/R/Rh/H	19mer	68.8	NA	75.9	NA	81.4	82.1	114.1	89.7
AD-59119	M/R/Rh/H	19mer	64.9	NA	86.5	NA	85.1	125.1	122.8	124.8
AD-59120	M/R/Rh/H	19mer	63.5	NA	75.1	NA	29.9	52.0	16.1	54.1
AD-59121	M/R/Rh/H	19mer	67.6	NA	72.0	NA	88.8	77.4	108.0	103.1
AD-59122	M/R/Rh/H	19mer	60.2	NA	62.3	NA	25.1	45.3	16.2	54.8
AD-59123	M/R/Rh/H	19mer	68.6	NA	108.2	NA	59.2	84.6	80.0	97.7
AD-59124	M/R/Rh/H	19mer	47.5	NA	56.5	NA	23.9	40.0	9.8	18.9
AD-59125	M/R/Rh/H	19mer	45.4	NA	47.2	NA	15.2	40.7	14.7	15.1
AD-59126	M/R/Rh/H	19mer	64.3	NA	74.6	NA	51.6	57.1	35.5	54.4
AD-59127	M/R/Rh/H	19mer	103.4	NA	105.8	NA	94.0	156.4	135.9	113.7
AD-59128	M/R/Rh/H	19mer	102.4	NA	81.4	NA	66.3	89.3	60.2	74.9
AD-59129	M/R/Rh/H	19mer	41.3	NA	38.8	NA	17.9	41.4	8.6	12.6

AD-59130	M/R/Rh/H	19mer	58.3	NA	80.8	NA	94.9	78.3	106.7	88.0

Table 17 illustrates the IC_{50}s of select ALAS1 siRNA duplexes. The IC_{50}s were determined from the knockdown of endogenously expressed ALAS1 in the Hep3B cell line, at 24 hours following transfection of each ALAS1 modified siRNA duplex (see Table 14). At least seven duplexes, including AD-58882, AD-58878, AD-58886, AD-58877, AD-59115, AD-58856, and AD-59129, consistently demonstrated IC_{50}s of less than 0.1 nm, indicating that these duplexes were particularly effective in suppressing ALAS1 expression.

Table 17: IC_{50}s of select ALAS1 siRNA duplexes

Duplex ID	384w IC50 (nM)	96w IC50 (nM)
AD-58882	0.008	0.014
AD-58878	0.040	0.031
AD-58886	0.037	0.033
AD-58877	0.031	0.034
AD-59115	0.093	0.052
AD-58856	0.061	0.066
AD-59129	0.085	0.071
AD-59124	0.572	0.078
AD-58874	0.140	0.102
AD-59125	0.118	0.115
AD-59105	0.511	0.144
AD-59120	180.592	0.498
AD-59122	36.646	0.646
AD-59106	7.906	0.847
AD-59126	n/a	1.014
AD-59107	n/a	1.971

Example 11. ALAS1-GalNAc activity in AIP Phenobarbital induction mouse model

The AIP mouse model was used to investigate the effect of an siRNA that was an ALAS1-GalNAc conjugate. The siRNA had the sequence of duplex AD-58632 (see Table 20)

Table 20: Sequences of ALAS1 siRNA Duplex AD-58632

SEQ ID NO: (sense)	SEQ ID NO: (anti-sense)	Target sites of antisense sequence	Duplex Name	Sense Sequence (5'-3')	Antisense Sequence (5'-3')
4149	4150	873-895	AD-58632	GfsasAfaGfaGfuGfUfCfuCfaUfcUfuCfuUfL96	asAfsgAfaGfaUfgAfgacAfcUfcUfuUfcsusg

AIP mice were untreated (baseline), or injected subcutaneously on day 1 with saline or the ALAS1-GalNAc conjugate at a dose of 20mg/kg. On Days 2, 3, and 4 they were left untreated (baseline) or they were treated with IP injections of Phenobarbital. On Day 5 plasma was taken and levels of ALA and PBG were measured using an LC-MS assay. As shown in FIG. 15, the ALAS1-GalNAc conjugate blunted the production of plasma ALA and PBG by about 84 and 80% respectively. These results indicate that treatment with an ALAS1-GalNAc conjugate was effective in preventing increases in both plasma ALA and PBG associated with phenobarbital-induced acute attacks in this AIP animal model.

Example 12. Further siRNAs that Target ALAS1 and Inhibit ALAS1 Expression

Modified siRNA sequences that target ALAS1 siRNA were designed and produced as described in Example 2. The sequences are provided in Table 18. The *in vitro* activity of the modified duplexes was tested as described below.

Table 18: Human ALAS1 Modified Single Strands and Duplex Sequences

SEQ ID NO: (sense)	SEQ ID NO: (anti-sense)	Duplex Name	Sense Sequence (5'-3')	Antisense Sequence (5'-3')	Target sites of antisense sequence on NM_ 000688.4
3685	3686	AD-59453	CAGGCAAAUCUCUGUUGUUdTdT	AACAACAGAGAUUUGCCUGdTdT	402-420
3687	3688	AD-59395	GAAAAAAAUUGAUGAGAAAdTdT	UUUCUCAUCAAUUUUUUUCdTdT	949-967
3689	3690	AD-59477	GGAAAGAUGCCGCACUCUUdTdT	AAGAGUGCGGCAUCUUUCCdTdT	1242-1260
3691	3692	AD-59492	UGUCUCAUCUUCUUCAAGAdTdT	UCUUGAAGAAGAUGAGACAdTdT	882-900
3693	3694	AD-59361	ACAUCUACGUGCAAGCAAUdTdT	AUUGCUUGCACGUAGAUGUdTdT	1992-2010
3695	3696	AD-59462	UUCUCUGAUUGACACCGUAdTdT	UACGGUGUCAAUCAGAGAAdTdT	1711-1729
3697	3698	AD-59433	GCUGCUGGCUUCAUCUUCAdTdT	UGAAGAUGAAGCCAGCAGCdTdT	1739-1757
3699	3700	AD-59424	AGCGCAACGUCAAACUCAUdTdT	AUGAGUUUGACGUUGCGCUdTdT	1851-1869
3701	3702	AD-59414	UAUUUCUGGAACUAGUAAAdTdT	UUUACUAGUUCCAGAAAUAdTdT	1183-1201
3703	3704	AD-59539	GGUUGUGUUGGAGGGUACAdTdT	UGUACCCUCCAACACAACCdTdT	1679-1697
3705	3706	AD-59400	GUGUCAGUCUGGUGCAGUAdTdT	UACUGCACCAGACUGACACdTdT	1070-1088
3707	3708	AD-59551	CUUUGUGGCCAAUGACUCAdTdT	UGAGUCAUUGGCCACAAAGdTdT	1273-1291
3709	3710	AD-59482	AGAUGCUGCUAAAAACACAdTdT	UGUGUUUUUAGCAGCAUCUdTdT	1942-1960
3711	3712	AD-59448	GAGUCAUGCCAAAAAUGGAdTdT	UCCAUUUUUGGCAUGACUCdTdT	1629-1647
3713	3714	AD-59392	CUGUGCGGAUCCUGAAGAGdTdT	CUCUUCAGGAUCCGCACAGdTdT	1800-1818
3715	3716	AD-59469	CACUUUGAAACAACAUGGUdTdT	ACCAUGUUGUUUCAAAGUGdTdT	1141-1159
3717	3718	AD-59431	AAGUGAUGAGUGAAAGAGAdTdT	UCUCUUUCACUCAUCACUUdTdT	2193-2211
3719	3720	AD-59423	AUCUGCUAGUCACAUGGAAdTdT	UUCCAUGUGACUAGCAGAUdTdT	2103-2121
3721	3722	AD-59517	UGGGGCAGGUGGUACUAGAdTdT	UCUAGUACCACCUGCCCCAdTdT	1162-1180
3723	3724	AD-59578	GCAGAUGACUAUUCAGACUdTdT	AGUCUGAAUAGUCAUCUGCdTdT	1031-1049
3725	3726	AD-59495	GCCUCAUUCCUCAGCUGAGdTdT	CUCAGCUGAGGAAUGAGGCdTdT	2143-2161
3727	3728	AD-59432	GUAUGAUCGUUUCUUUGAGdTdT	CUCAAAGAAACGAUCAUACdTdT	931-949
3729	3730	AD-59382	UAUCCAGAUGGUCUUCAGAdTdT	UCUGAAGACCAUCUGGAUAdTdT	2302-2320
3731	3732	AD-59472	UAGUGUGAAAACCGAUGGAdTdT	UCCAUCGGUUUUCACACUAdTdT	799-817
3733	3734	AD-59459	UCCCCAUGGCAGAUGACUAdTdT	UAGUCAUCUGCCAUGGGGAdTdT	1023-1041
3735	3736	AD-59413	CCACUGCAGCAGUACACUAdTdT	UAGUGUACUGCUGCAGUGGdTdT	483-501
3737	3738	AD-59478	CUGUGAACCGGCGAGCACAdTdT	UGUGCUCGCCGGUUCACAGdTdT	999-1017
3739	3740	AD-59376	GGUCCUAUGCUGCUGGCUUdTdT	AAGCCAGCAGCAUAGGACCdTdT	1731-1749
3741	3742	AD-59556	AGCCUUUGGUUGUGUUGGAdTdT	UCCAACACAACCAAAGGCUdTdT	1672-1690
3743	3744	AD-59399	AAUUCCAUGUGGACUUAGAdTdT	UCUAAGUCCACAUGGAAUUdTdT	1200-1218
3745	3746	AD-59474	CCAGGGCACUGCAAGCAAAdTdT	UUUGCUUGCAGUGCCCUGGdTdT	640-658
3747	3748	AD-53542	cuuuucAGuAuGAucGuuudTsdT	AAACGAUcAuACUGAAAAGdTsdT	924-942
3749	3750	AD-59480	GAAUCAGAGAGGCAGCAGUdTdT	ACUGCUGCCUCUCUGAUUCdTdT	682-700
3751	3752	AD-59549	GCAAAGAUCUGACCCCUCAdTdT	UGAGGGGUCAGAUCUUUGCdTdT	1441-1459
3753	3754	AD-59515	GGAGAAGAGCUCCUACGGAdTdT	UCCGUAGGAGCUCUUCUCCdTdT	2033-2051
3755	3756	AD-59427	CCAUGAGUUUGGAGCAAUCdTdT	GAUUGCUCCAAACUCAUGGdTdT	1540-1558
3757	3758	AD-59390	CUUUGAGAAAAAAAUUGAUdTdT	AUCAAUUUUUUUCUCAAAGdTdT	943-961
3759	3760	AD-59511	UGAGCAGACAUAACAUCUAdTdT	UAGAUGUUAUGUCUGCUCAdTdT	1980-1998

3761	3762	AD-59532	CGUGCAAGCAAUCAAUUACdTdT	GUAAUUGAUUGCUUGCACGdTdT	1999-2017
3763	3764	AAAGCAAAGACCAGAAAGAdTdT	UCUUUCUGGUCUUUGCUUUdTdT	862-880
3765	3766	GGAUGUGCAGGAAAUGAAUdTdT	AUUCAUUUCCUGCACAUCCdTdT	733-751
3767	3768	CAGCAUACUUCCUGAACAUdTdT	AUGUUCAGGAAGUAUGCUGdTdT	321-339
3769	3770	AD-53541	GcAGcAcAGAuGAAucAGAdTsdT	UCUGAUUcAUCUGUGCUGCdTsdT	671-689
3771	3772	AD-59490	UCUGUUGUUCUAUGCCCAAdTdT	UUGGGCAUAGAACAACAGAdTdT	412-430
3773	3774	AD-59422	UGAGACAGAUGCUAAUGGAdTdT	UCCAUUAGCAUCUGUCUCAdTdT	1869-1887
3775	3776	AD-59467	GCCAAUGACUCAACCCUCUdTdT	AGAGGGUUGAGUCAUUGGCdTdT	1280-1298
3777	3778	AD-59579	GAGUGCAACUUCUGCAGGAdTdT	UCCUGCAGAAGUUGCACUCdTdT	2159-2177
3779	3780	AD-59426	GUGAAAGAGAGAAGUCCUAdTdT	UAGGACUUCUCUCUUUCACdTdT	2202-2220
3781	3782	AD-59363	UAACUUGCCAAAAUCUGUUdTdT	AACAGAUUUUGGCAAGUUAdTdT	901-919
3783	3784	AD-59436	AAGCCAGUCUUGAGCUUCAdTdT	UGAAGCUCAAGACUGGCUUdTdT	711-729
3785	3786	AD-53536	cAcuuuucAGuAuGAucGudTsdT	ACGAUcAuACUGAAAAGUGdTsdT	922-940
3787	3788	AD-59491	GCAGCAGUGUCUUCUGCAAdTdT	UUGCAGAAGACACUGCUGCdTdT	693-711
3789	3790	AD-59500	UCCUGAACAUGGAGAGUGUdTdT	ACACUCUCCAUGUUCAGGAdTdT	330-348
3791	3792	AD-59394	AUUUCUGGAACACUUGGCAdTdT	UGCCAAGUGUUCCAGAAAUdTdT	1652-1670
3793	3794	AD-59441	CAGUACACUACCAACAGAUdTdT	AUCUGUUGGUAGUGUACUGdTdT	492-510
3795	3796	AD-59365	GCAUGACCUCAAUUAUUUCdTdT	GAAAUAAUUGAGGUCAUGCdTdT	2261-2279
3797	3798	AD-59411	AGAACUGCUGCAAAGAUCUdTdT	AGAUCUUUGCAGCAGUUCUdTdT	1432-1450
3799	3800	AD-59544	CACCCCAGAUGAUGAACUAdTdT	UAGUUCAUCAUCUGGGGUGdTdT	2073-2091
3801	3802	AD-59428	GAUCCAAGGGAUUCGAAACdTdT	GUUUCGAAUCCCUUGGAUCdTdT	1363-1381
3803	3804	AD-59471	CUCAUCACCAAAAAGCAAGdTdT	CUUGCUUUUUGGUGAUGAGdTdT	1052-1070
3805	3806	AD-59518	ACAACAUGGUGCUGGGGCAdTdT	UGCCCCAGCACCAUGUUGUdTdT	1150-1168
3807	3808	AD-53547	GAucGuuucuuuGAGAAAAdTsdT	UUUUCUcAAAGAAACGAUCdTsdT	935-953
3809	3810	AD-59573	CAGCACGAGUUCUCUGAUUdTdT	AAUCAGAGAACUCGUGCUGdTdT	1702-1720
3811	3812	AD-59473	AAUGAUGUCAGCCACCUCAdTdT	UGAGGUGGCUGACAUCAUUdTdT	1412-1430
3813	3814	AD-59412	AGUUAUGGACACUUUGAAAdTdT	UUUCAAAGUGUCCAUAACUdTdT	1132-1150
3815	3816	AD-59522	GAUGAUGAACUACUUCCUUdTdT	AAGGAAGUAGUUCAUCAUCdTdT	2080-2098
3817	3818	AD-59502	GCAGGAAAUGAAUGCCGUGdTdT	CACGGCAUUCAUUUCCUGCdTdT	739-757
3819	3820	AD-59499	UCUUCAAGAUAACUUGCCAdTdT	UGGCAAGUUAUCUUGAAGAdTdT	892-910
3821	3822	AD-59520	CGAUGGAGGGGAUCCCAGUdTdT	ACUGGGAUCCCCUCCAUCGdTdT	811-829
3823	3824	AD-59581	CCAAAAAGCAAGUGUCAGUdTdT	ACUGACACUUGCUUUUUGGdTdT	1059-1077
3825	3826	AD-59461	GAUUGGGGAUCGGGAUGGAdTdT	UCCAUCCCGAUCCCCAAUCdTdT	1612-1630
3827	3828	AD-59370	CCCUGGAGUCUGUGCGGAUdTdT	AUCCGCACAGACUCCAGGGdTdT	1791-1809
3829	3830	AD-53540	GuuGucuuuAuAuGuGAAudTsdT	AUUcAcAuAuAAAGAcAACdTsdT	2321-2339
3831	3832	AD-59574	CGGGCAUUGUCCACUGCAGdTdT	CUGCAGUGGACAAUGCCCGdTdT	473-491
3833	3834	AD-59375	UAUUCAGACUCCCUCAUCAdTdT	UGAUGAGGGAGUCUGAAUAdTdT	1040-1058
3835	3836	AD-59387	CACUGCAUUUUGAAGUGAUdTdT	AUCACUUCAAAAUGCAGUGdTdT	2181-2199
3837	3838	AD-59397	CCAGAAAGAGUGUCUCAUCdTdT	GAUGAGACACUCUUUCUGGdTdT	872-890
3839	3840	AD-59396	AGGCGGAGGGAUUGGGGAUdTdT	AUCCCCAAUCCCUCCGCCUdTdT	1603-1621
3841	3842	AD-59393	AGACCUCCAUGGGAAAGAUdTdT	AUCUUUCCCAUGGAGGUCUdTdT	1231-1249
3843	3844	AD-59483	GCAGGAGGCCACUGCAUUUdTdT	AAAUGCAGUGGCCUCCUGCdTdT	2172-2190
3845	3846	AD-59430	AUCUGUUUCCACUUUUCAGdTdT	CUGAAAAGUGGAAACAGAUdTdT	913-931
3847	3848	AD-59463	AGAGAAGUCCUAUUUCUCAdTdT	UGAGAAAUAGGACUUCUCUdTdT	2209-2227
3849	3850	AD-53534	GucuucAGAGuuGucuuuAdTsdT	uAAAGAcAACUCUGAAGACdTsdT	2312-2330
3851	3852	AD-59514	GGCUGGAACUGAAGCCUCAdTdT	UGAGGCUUCAGUUCCAGCCdTdT	2130-2148
3853	3854	AD-59575	GCCAUUAUCAUAUCCAGAUdTdT	AUCUGGAUAUGAUAAUGGCdTdT	2292-2310
3855	3856	AD-59364	AGCAGGCCCCAGUGUGGUUdTdT	AACCACACUGGGGCCUGCUdTdT	781-799
3857	3858	AD-59402	UCAGCUGAGUGCAACUUCUdTdT	AGAAGUUGCACUCAGCUGAdTdT	2153-2171
3859	3860	AD-59479	GAGCACACAUCUUCCCCAUdTdT	AUGGGGAAGAUGUGUGCUCdTdT	1011-1029
3861	3862	AD-59481	ACUUCCAGGACAUCAUGCAdTdT	UGCAUGAUGUCCUGGAAGUdTdT	843-861
3863	3864	AD-59530	CCUAUCGAGUUUUUAAAACdTdT	GUUUUAAAAACUCGAUAGGdTdT	981-999
3865	3866	AD-59582	CUUCCUUGAGAAUCUGCUAdTdT	UAGCAGAUUCUCAAGGAAGdTdT	2092-2110

3867	3868	AD-59506	ACCAACAGAUCAAAGAAACdTdT	GUUUCUUUGAUCUGUUGGUdTdT	501-519
3869	3870	AD-59567	UAACCCCAGGCCAUUAUCAdTdT	UGAUAAUGGCCUGGGGUUAdTdT	2283-2301
3871	3872	AD-59485	CCAUGCCUCCAUGAUCCAAdTdT	UUGGAUCAUGGAGGCAUGGdTdT	1351-1369
3873	3874	AD-59525	UGAUGAACUAAUGAGCAGAdTdT	UCUGCUCAUUAGUUCAUCAdTdT	1969-1987
3875	3876	AD-59566	CCUGAAGAGCGCUGAGGGAdTdT	UCCCUCAGCGCUCUUCAGGdTdT	1810-1828
3877	3878	AD-59580	AACACUUGGCAAAGCCUUUdTdT	AAAGGCUUUGCCAAGUGUUdTdT	1660-1678
3879	3880	AD-59512	UCUGCAGAAAGCAGGCAAAdTdT	UUUGCCUGCUUUCUGCAGAdTdT	391-409
3881	3882	AD-59475	CCGGCCUCCCUGUUGUCCAdTdT	UGGACAACAGGGAGGCCGGdTdT	1890-1908
3883	3884	AD-59438	CAUCAUCCCUGUGCGGGUUdTdT	AACCCGCACAGGGAUGAUGdTdT	1921-1939
3885	3886	AD-59442	UGUGCGGGUUGCAGAUGCUdTdT	AGCAUCUGCAACCCGCACAdTdT	1930-1948
3887	3888	AD-59516	GGAAAGAGGUUGCUGAAACdTdT	GUUUCAGCAACCUCUUUCCdTdT	759-777
3889	3890	AD-59429	AGGUCCACGCAGUGGGGCUdTdT	AGCCCCACUGCGUGGACCUdTdT	1572-1590
3891	3892	AD-59510	UGCCGUGAGGAAAGAGGUUdTdT	AACCUCUUUCCUCACGGCAdTdT	751-769
3893	3894	AD-59457	GCUAAUGGAUGCCGGCCUCdTdT	GAGGCCGGCAUCCAUUAGCdTdT	1879-1897
3895	3896	AD-59434	GAAGCAAGUGGGGCUGGAAdTdT	UUCCAGCCCCACUUGCUUCdTdT	2119-2137
3897	3898	AD-59454	CAUCUUCCGCCACAAUGAUdTdT	AUCAUUGUGGCGGAAGAUGdTdT	1399-1417
3899	3900	AD-59468	AUUUCUCAGGCUUGAGCAAdTdT	UUGCUCAAGCCUGAGAAAUdTdT	2220-2238
3901	3902	AD-59565	CCCGAGUCCCCCAGGCCUUdTdT	AAGGCCUGGGGGACUCGGGdTdT	372-390
3903	3904	AD-59416	CAAGCAAAUGCCCUUUCCUdTdT	AGGAAAGGGCAUUUGCUUGdTdT	651-669
3905	3906	AD-59420	CCCCUCAGUCCCCAAGAUUdTdT	AAUCUUGGGGACUGAGGGGdTdT	1453-1471
3907	3908	AD-59552	CUACGGUGCCCCGGGGAGAdTdT	UCUCCCCGGGGCACCGUAGdTdT	2019-2037
3909	3910	AD-59558	AAAACUGCCCCAAGAUGAUdTdT	AUCAUCUUGGGGCAGUUUUdTdT	429-447
3911	3912	AD-59404	ACAAAACUGCUAAGGCCAAdTdT	UUGGCCUUAGCAGUUUUGUdTdT	540-558
3913	3914	AD-59455	GAUUCUGGGAACCAUGCCUdTdT	AGGCAUGGUUCCCAGAAUCdTdT	1340-1358
3915	3916	AD-59496	CCAGAUGGCACACAGCUUCdTdT	GAAGCUGUGUGCCAUCUGGdTdT	593-611
3917	3918	AD-59446	AGGGAUUCGAAACAGCCGAdTdT	UCGGCUGUUUCGAAUCCCUdTdT	1369-1387
3919	3920	AD-59435	CUCUGCAGUCCUCAGCGCAdTdT	UGCGCUGAGGACUGCAGAGdTdT	109-127
3921	3922	AD-59419	CCGCCGCCUCUGCAGUCCUdTdT	AGGACUGCAGAGGCGGCGGdTdT	102-120
3923	3924	AD-59533	CUGGCUGGAGCCCUGGAGUdTdT	ACUCCAGGGCUCCAGCCAGdTdT	1781-1799
3925	3926	AD-59366	GACAUCAUGCAAAAGCAAAdTdT	UUUGCUUUUGCAUGAUGUCdTdT	851-869
3927	3928	AD-59521	GCUUGAGCAAGUUGGUAUCdTdT	GAUACCAACUUGCUCAAGCdTdT	2229-2247
3929	3930	AD-59563	CAGGCUGUGAGAUUUACUCdTdT	GAGUAAAUCUCACAGCCUGdTdT	1320-1338
3931	3932	AD-59534	AGAGCUGUGUGAUGUGGCCdTdT	GGCCACAUCACACAGCUCUdTdT	1522-1540
3933	3934	AD-59407	GGAGCUGGCAGACCUCCAUdTdT	AUGGAGGUCUGCCAGCUCCdTdT	1222-1240
3935	3936	AD-59445	AUCCCAGUGGACUGCUGAAdTdT	UUCAGCAGUCCACUGGGAUdTdT	822-840
3937	3938	AD-59546	GUCAAACUCAUGAGACAGAdTdT	UCUGUCUCAUGAGUUUGACdTdT	1859-1877
3939	3940	AD-59456	CUUUCCUGGCAGCACAGAUdTdT	AUCUGUGCUGCCAGGAAAGdTdT	663-681
3941	3942	AD-59503	CCCUCCGGCCAGUGAGAAAdTdT	UUUCUCACUGGCCGGAGGGdTdT	520-538
3943	3944	AD-59536	CUACCUAGGAAUGAGUCGCdTdT	GCGACUCAUUCCUAGGUAGdTdT	1093-1111
3945	3946	AD-59385	CCCAAGAUUGUGGCAUUUGdTdT	CAAAUGCCACAAUCUUGGGdTdT	1463-1481
3947	3948	AD-59367	GAGCAAUCACCUUCGUGGAdTdT	UCCACGAAGGUGAUUGCUCdTdT	1551-1569
3949	3950	AD-59458	UGCCCAUUCUUAUCCCGAGdTdT	CUCGGGAUAAGAAUGGGCAdTdT	359-377
3951	3952	AD-59381	AAGGCCAAGGUCCAACAGAdTdT	UCUGUUGGACCUUGGCCUUdTdT	551-569
3953	3954	AD-59538	CACACAGCUUCCGUCUGGAdTdT	UCCAGACGGAAGCUGUGUGdTdT	601-619
3955	3956	AD-59421	UUAUGGGGCUCGAGGCGGAdTdT	UCCGCCUCGAGCCCCAUAAdTdT	1591-1609
3957	3958	AD-59388	UGUCUUCUGCAAAGCCAGUdTdT	ACUGGCUUUGCAGAAGACAdTdT	700-718
3959	3960	AD-59444	AGGCCUGAGCAUGACCUCAdTdT	UGAGGUCAUGCUCAGGCCUdTdT	2253-2271
3961	3962	AD-59528	AUGUGAAUUAAGUUAUAUUdTdT	AAUAUAACUUAAUUCACAUdTdT	2332-2350
3963	3964	AD-59498	ACUGCUGAAGAACUUCCAGdTdT	CUGGAAGUUCUUCAGCAGUdTdT	832-850
3965	3966	AD-59497	UGAGAAAGACAAAACUGCUdTdT	AGCAGUUUUGUCUUUCUCAdTdT	532-550
3967	3968	AD-59384	UCAGCCACCUCAGAGAACUdTdT	AGUUCUCUGAGGUGGCUGAdTdT	1419-1437
3969	3970	AD-59452	GGCAACGAGCGUUUCGUUUdTdT	AAACGAAACGCUCGUUGCCdTdT	51-69
3971	3972	AD-59379	CCUGAUGGAUCCCAGCAGAdTdT	UCUGCUGGGAUCCAUCAGGdTdT	572-590

3973	3974	AD-59529	UGUGCCCACUGGAAGAGCUdTdT	AGCUCUUCCAGUGGGCACAdTdT	1509-1527
3975	3976	AD-59389	CCACAGGAGCCAGCAUACUdTdT	AGUAUGCUGGCUCCUGUGGdTdT	311-329
3977	3978	AD-59585	GUGGUACUAGAAAUAUUUCdTdT	GAAAUAUUUCUAGUACCACdTdT	1170-1188
3979	3980	AD-59570	UUCGCCGCUGCCCAUUCUUdTdT	AAGAAUGGGCAGCGGCGAAdTdT	351-369
3981	3982	AD-59415	CCGCCAGCACCAGCGCAACdTdT	GUUGCGCUGGUGCUGGCGGdTdT	1840-1858
3983	3984	AD-59505	CGCUGAGGGACGGGUGCUUdTdT	AAGCACCCGUCCCUCAGCGdTdT	1819-1837
3985	3986	AD-59557	UGGACUUCUCGACUUGAGUdTdT	ACUCAAGUCGAGAAGUCCAdTdT	69-87
3987	3988	AD-59548	AAAGAAACCCCUCCGGCCAdTdT	UGGCCGGAGGGGUUUCUUUdTdT	512-530
3989	3990	AD-59487	UUGACACCGUACGGUCCUAdTdT	UAGGACCGUACGGUGUCAAdTdT	1719-1737
3991	3992	AD-59550	CCCUCUUCACCCUGGCUAAdTdT	UUAGCCAGGGUGAAGAGGGdTdT	1293-1311
3993	3994	AD-59572	CCCCCAGGCCUUUCUGCAGdTdT	CUGCAGAAAGGCCUGGGGGdTdT	379-397
3995	3996	AD-59554	AUGCCCAAAACUGCCCCAAdTdT	UUGGGGCAGUUUUGGGCAUdTdT	423-441
3997	3998	AD-59437	CUUGAGUGCCCGCCUCCUUdTdT	AAGGAGGCGGGCACUCAAGdTdT	81-99
3999	4000	AD-59584	GGGUACAUCGCCAGCACGAdTdT	UCGUGCUGGCGAUGUACCCdTdT	1691-1709
4001	4002	AD-59373	GUGUGGGGCAGUUAUGGACdTdT	GUCCAUAACUGCCCCACACdTdT	1123-1141
4003	4004	AD-59545	ACAUAGUCCUGGAAAUAAAdTdT	UUUAUUUCCAGGACUAUGUdTdT	2372-2390
4005	4006	AD-59547	AUCCCAGCAGAGUCCAGAUdTdT	AUCUGGACUCUGCUGGGAUdTdT	580-598
4007	4008	AD-59470	CUAGAUUCUUUCCACAGGAdTdT	UCCUGUGGAAAGAAUCUAGdTdT	300-318
4009	4010	AD-59417	UUGUUUUCCUCGUGCUUUGdTdT	CAAAGCACGAGGAAAACAAdTdT	1259-1277
4011	4012	AD-59535	CCUCCUUCGCCGCCGCCUCdTdT	GAGGCGGCGGCGAAGGAGGdTdT	93-111
4013	4014	AD-59507	UGAGGCUGCUCCCGGACAAdTdT	UUGUCCGGGAGCAGCCUCAdTdT	31-49
4015	4016	AD-59519	CCAACAGACUCCUGAUGGAdTdT	UCCAUCAGGAGUCUGUUGGdTdT	562-580
4017	4018	AD-59391	UCACAUGGAAGCAAGUGGGdTdT	CCCACUUGCUUCCAUGUGAdTdT	2112-2130
4019	4020	AD-59537	CAUUCAAUGGAUGGGGCGGdTdT	CCGCCCCAUCCAUUGAAUGdTdT	1490-1508
4021	4022	AD-59450	AGGAAUGAGUCGCCACCCAdTdT	UGGGUGGCGACUCAUUCCUdTdT	1099-1117
4023	4024	AD-59449	UGGACUUAGAGCGGGAGCUdTdT	AGCUCCCGCUCUAAGUCCAdTdT	1209-1227
4025	4026	AD-59418	CUAAAAACACAGAAGUCUGdTdT	CAGACUUCUGUGUUUUUAGdTdT	1950-1968
4027	4028	AD-59561	CCCUCACCACACACCCCAGdTdT	CUGGGGUGUGUGGUGAGGGdTdT	2062-2080
4029	4030	AD-59460	AAUCCUUGCUUCAGGGACUdTdT	AGUCCCUGAAGCAAGGAUUdTdT	171-189
4031	4032	AD-59409	UUGUGGCAUUUGAAACUGUdTdT	ACAGUUUCAAAUGCCACAAdTdT	1470-1488
4033	4034	AD-59476	UCAAUUACCCUACGGUGCCdTdT	GGCACCGUAGGGUAAUUGAdTdT	2010-2028
4035	4036	AD-59406	CAAGCCAGCCCCUCGGGCAdTdT	UGCCCGAGGGGCUGGCUUGdTdT	460-478
4037	4038	AD-59569	GAGUCUUCCCUGCCUGGAUdTdT	AUCCAGGCAGGGAAGACUCdTdT	259-277
4039	4040	AD-59451	UGGAGAGUGUUGUUCGCCGdTdT	CGGCGAACAACACUCUCCAdTdT	339-357
4041	4042	AD-59553	ACCCCUUGCCUGCCACAAGdTdT	CUUGUGGCAGGCAAGGGGUdTdT	621-639
4043	4044	AD-59372	CUGGAUGGAUGAGUGGCUUdTdT	AAGCCACUCAUCCAUCCAGdTdT	272-290
4045	4046	AD-59377	CAAGAUGAUGGAAGUUGGGdTdT	CCCAACUUCCAUCAUCUUGdTdT	439-457
4047	4048	AD-59531	UUUCGUUUGGACUUCUCGAdTdT	UCGAGAAGUCCAAACGAAAdTdT	62-80
4049	4050	AD-59560	UCAUCUUCACCACCUCUCUdTdT	AGAGAGGUGGUGAAGAUGAdTdT	1749-1767
4051	4052	AD-59489	UGCCCAGUUCUUCCCGCUGdTdT	CAGCGGGAAGAACUGGGCAdTdT	132-150
4053	4054	AD-59540	AAAAAUGGACAUCAUUUCUdTdT	AGAAAUGAUGUCCAUUUUUdTdT	1639-1657
4055	4056	AD-59378	CUUGAGCUUCAGGAGGAUGdTdT	CAUCCUCCUGAAGCUCAAGdTdT	719-737
4057	4058	AD-59403	CCUCUCUGCCACCCAUGCUdTdT	AGCAUGGGUGGCAGAGAGGdTdT	1761-1779
4059	4060	AD-59493	AAAGUCAGGAUCCCUAAGAdTdT	UCUUAGGGAUCCUGACUUUdTdT	242-260
4061	4062	AD-59374	CGACCACGGAGGAAUCCUUdTdT	AAGGAUUCCUCCGUGGUCGdTdT	159-177
4063	4064	AD-59380	UUCCGUCUGGACACCCCUUdTdT	AAGGGGUGUCCAGACGGAAdTdT	609-627
4065	4066	AD-59576	CCACCCAUGCUGCUGGCUGdTdT	CAGCCAGCAGCAUGGGUGGdTdT	1769-1787
4067	4068	AD-59425	UGAGAAAAAGAAUGACCACdTdT	GUGGUCAUUCUUUUUCUCAdTdT	961-979
4069	4070	AD-59509	UAAGAUGAUGCCAGGCUGUdTdT	ACAGCCUGGCAUCAUCUUAdTdT	1309-1327
4071	4072	AD-59488	AGUUAUAUUAAAUUUUAAUdTdT	AUUAAAAUUUAAUAUAACUdTdT	2342-2360
4073	4074	AD-59486	UCUUCCCGCUGUGGGGACAdTdT	UGUCCCCACAGCGGGAAGAdTdT	140-158
4075	4076	AD-59465	UGCCACAAGCCAGGGCACUdTdT	AGUGCCCUGGCUUGUGGCAdTdT	631-649
4077	4078	AD-59484	AGCGCAGUUAUGCCCAGUUdTdT	AACUGGGCAUAACUGCGCUdTdT	122-140

4079	4080	AD-59368	GGACCAGGAGAAAGUCAGGdTdT	CCUGACUUUCUCCUGGUCCdTdT	232-250
4081	4082	AD-59464	UGUCCACUGCCCCAGCCACdTdT	GUGGCUGGGGCAGUGGACAdTdT	1903-1921
4083	4084	AD-59386	AUCGCGGCCUGAGGCUGCUdTdT	AGCAGCCUCAGGCCGCGAUdTdT	22-40
4085	4086	AD-59439	GGGGAUGUGGGGACCAGGAdTdT	UCCUGGUCCCCACAUCCCCdTdT	222-240
4087	4088	AD-59440	CUGGAAAUAAAUUCUUGCUdTdT	AGCAAGAAUUUAUUUCCAGdTdT	2380-2398
4089	4090	AD-59542	UUGAAACUGUCCAUUCAAUdTdT	AUUGAAUGGACAGUUUCAAdTdT	1479-1497
4091	4092	AD-59559	GUGGGGACACGACCACGGAdTdT	UCCGUGGUCGUGUCCCCACdTdT	150-168
4093	4094	AD-59586	CGCAGUGGGGCUUUAUGGGdTdT	CCCAUAAAGCCCCACUGCGdTdT	1579-1597
4095	4096	AD-59408	UUGUCUUUAUAUGUGAAUUdTdT	AAUUCACAUAUAAAGACAAdTdT	2322-2340
4097	4098	AD-59568	UCACCCUGGCUAAGAUGAUdTdT	AUCAUCUUAGCCAGGGUGAdTdT	1299-1317
4099	4100	AD-59398	GUAUCUGCUCAGGCCUGAGdTdT	CUCAGGCCUGAGCAGAUACdTdT	2243-2261
4101	4102	AD-59508	AUGAGUGGCUUCUUCUCCAdTdT	UGGAGAAGAAGCCACUCAUdTdT	280-298
4103	4104	AD-59523	GAAGUUGGGGCCAAGCCAGdTdT	CUGGCUUGGCCCCAACUUCdTdT	449-467
4105	4106	AD-59410	UCAGGGACUCGGGACCCUGdTdT	CAGGGUCCCGAGUCCCUGAdTdT	181-199
4107	4108	AD-59541	UCCUACGGAUUGCCCCCACdTdT	GUGGGGGCAAUCCGUAGGAdTdT	2043-2061
4109	4110	AD-59524	UUACUCUGAUUCUGGGAACdTdT	GUUCCCAGAAUCAGAGUAAdTdT	1333-1351
4111	4112	AD-59501	AUCCCUAAGAGUCUUCCCUdTdT	AGGGAAGACUCUUAGGGAUdTdT	251-269
4113	4114	AD-59383	UGCCAAAGUACAUCUUCCGdTdT	CGGAAGAUGUACUUUGGCAdTdT	1389-1407
4115	4116	AD-59577	UCCUCGGGUUUAGGGGAUGdTdT	CAUCCCCUAAACCCGAGGAdTdT	210-228
4117	4118	AD-59447	UGCUGAAACCUCAGCAGGCdTdT	GCCUGCUGAGGUUUCAGCAdTdT	769-787
4119	4120	AD-59555	CCACCCACGGGUGUGUGGGdTdT	CCCACACACCCGUGGGUGGdTdT	1111-1129
4121	4122	AD-59405	UGGUGCAGUAAUGACUACCdTdT	GGUAGUCAUUACUGCACCAdTdT	1079-1097
4123	4124	AD-59371	UUCUCCACCUAGAUUCUUUdTdT	AAAGAAUCUAGGUGGAGAAdTdT	292-310
4125	4126	AD-59443	UAAGGCGCCGGCGAUCGCGdTdT	CGCGAUCGCCGGCGCCUUAdTdT	9-27
4127	4128	AD-59401	UGGAACUAGUAAAUUCCAUdTdT	AUGGAAUUUACUAGUUCCAdTdT	1189-1207
4129	4130	AD-59494	GGACCCUGCUGGACCCCUUdTdT	AAGGGGUCCAGCAGGGUCCdTdT	192-210
4131	4132	AD-59504	UCAAUUAUUUCACUUAACCdTdT	GGUUAAGUGAAAUAAUUGAdTdT	2269-2287
4133	4134	AD-59369	CCCGGACAAGGGCAACGAGdTdT	CUCGUUGCCCUUGUCCGGGdTdT	41-59
4135	4136	AD-59571	UUUUAAAACUGUGAACCGGdTdT	CCGGUUCACAGUUUUAAAAdTdT	991-1009
4137	4138	AD-59527	GUGCUUCGCCGCCAGCACCdTdT	GGUGCUGGCGGCGAAGCACdTdT	1832-1850
4139	4140	AD-59466	UGGACCCCUUCCUCGGGUUdTdT	AACCCGAGGAAGGGGUCCAdTdT	201-219
4141	4142	AD-59526	CUGUAUAUUAAGGCGCCGGdTdT	CCGGCGCCUUAAUAUACAGdTdT	1-19
4143	4144	AD-59543	UUGCCCCCACCCCUCACCAdTdT	UGGUGAGGGGUGGGGGCAAdTdT	2052-2070
4145	4146	AD-59564	AUGGGGCGGUGUGCCCACUdTdT	AGUGGGCACACCGCCCCAUdTdT	1500-1518
4147	4148	AD-59583	CUAUAGUAAAAACAUAGUCdTdT	GACUAUGUUUUUACUAUAGdTdT	2361-2379

The *in vitro* activity of the siRNAs in suppressing ALAS1 mRNA was tested in a single dose screen in Hep3B cells that were transfected using Lipofectamine2000 as a transfection reagent. Single dose experiments were performed at 10nM duplex concentration and analyzed by branched DNA (bDNA) assay. The results are shown in Table 19 and are expressed as percent remaining mRNA.

Table 19: Suppression of ALAS1 mRNA as assessed by bDNA assay

Duplex	% remaining mRNA	SD
AD-59453	11.2	1.5
AD-59395	12.7	1.1

AD-59477	14.5	2.0
AD-59492	14.8	2.1
AD-59361	15.1	4.9
AD-59462	15.4	2.6
AD-59433	15.8	2.7
AD-59424	16.0	1.7
AD-59414	16.1	1.3
AD-59539	16.2	2.6
AD-59400	16.2	1.8
AD-59551	16.3	2.3
AD-59482	16.6	2.1
AD-59448	16.6	3.7
AD-59392	16.9	3.5
AD-59469	16.9	2.2
AD-59431	17.0	2.0
AD-59423	17.1	3.8
AD-59517	17.2	1.5
AD-59578	17.3	3.1
AD-59495	17.7	3.7
AD-59432	17.7	2.8
AD-59382	17.9	3.2
AD-59472	18.6	3.5
AD-59459	18.7	3.8
AD-59413	18.8	2.4
AD-59478	18.9	3.0
AD-59376	18.9	3.2
AD-59556	18.9	2.4
AD-59399	19.0	4.1
AD-59474	19.4	1.6
AD-53542	19.4	1.7
AD-59480	19.6	1.6
AD-59549	19.7	2.1
AD-59515	19.8	4.4
AD-59427	19.9	3.2
AD-59390	19.9	3.4
AD-59511	19.9	2.2
AD-59532	20.0	2.4
AD-59562	20.2	2.6
AD-59513	20.3	3.9
AD-59362	20.6	2.5

AD-53541	20.6	2.2
AD-59490	20.7	2.3
AD-59422	20.8	4.5
AD-59467	21.2	2.3
AD-59579	21.2	3.3
AD-59426	21.7	2.3
AD-59363	21.7	2.7
AD-59436	21.7	2.7
AD-53536	21.9	1.5
AD-59491	21.9	2.6
AD-59500	22.2	2.8
AD-59394	22.3	10.1
AD-59441	22.3	2.6
AD-59365	22.4	4.2
AD-59411	22.5	2.9
AD-59544	22.5	2.1
AD-59428	22.7	4.7
AD-59471	22.9	5.0
AD-59518	22.9	2.3
AD-53547	22.9	1.5
AD-59573	23.0	4.2
AD-59473	23.2	1.8
AD-59412	23.4	2.5
AD-59522	23.4	3.3
AD-59502	23.6	2.7
AD-59499	23.6	1.6
AD-59520	23.8	3.8
AD-59581	23.9	6.0
AD-59461	24.3	4.2
AD-59370	24.3	5.6
AD-53540	24.4	2.1
AD-59574	24.5	2.0
AD-59375	24.6	2.3
AD-59387	24.8	7.2
AD-59397	24.9	9.6
AD-59396	25.0	10.2
AD-59393	25.3	11.6
AD-59483	25.4	3.8
AD-59430	25.5	1.8
AD-59463	25.6	4.8

AD-53534	25.9	3.1
AD-59514	26.2	5.7
AD-59575	26.2	3.2
AD-59364	26.2	4.5
AD-59402	26.3	3.1
AD-59479	26.3	2.5
AD-59481	26.4	2.2
AD-59530	26.4	4.4
AD-59582	26.6	3.9
AD-59506	27.0	4.1
AD-59567	27.3	1.1
AD-59485	27.7	4.7
AD-59525	28.3	3.1
AD-59566	28.5	0.6
AD-59580	28.7	7.1
AD-59512	29.5	2.5
AD-59475	29.6	4.2
AD-59438	29.6	3.3
AD-59442	29.9	2.8
AD-59516	30.4	3.8
AD-59429	30.8	4.3
AD-59510	31.3	1.9
AD-59457	31.4	1.2
AD-59434	31.6	3.5
AD-59454	32.0	1.9
AD-59468	32.2	3.2
AD-59565	32.4	1.5
AD-59416	32.7	1.7
AD-59420	33.2	3.1
AD-59552	33.2	2.2
AD-59558	33.8	3.8
AD-59404	34.0	5.4
AD-59455	34.8	1.3
AD-59496	34.9	5.2
AD-59446	35.5	1.7
AD-59435	35.9	1.2
AD-59419	36.0	1.4
AD-59533	36.7	3.7
AD-59366	36.7	6.0
AD-59521	36.9	4.3

AD-59563	36.9	4.1
AD-59534	36.9	3.3
AD-59407	37.1	4.7
AD-59445	37.2	3.2
AD-59546	37.9	4.9
AD-59456	38.3	4.0
AD-59503	38.8	5.0
AD-59536	39.8	4.2
AD-59385	39.9	13.7
AD-59367	40.0	3.6
AD-59458	40.0	3.4
AD-59381	40.3	9.9
AD-59538	40.8	4.9
AD-59421	40.9	6.4
AD-59388	41.0	9.1
AD-59444	41.1	2.7
AD-59528	41.9	3.3
AD-59498	42.2	3.3
AD-59497	42.4	4.9
AD-59384	42.7	17.6
AD-59452	42.7	3.1
AD-59379	43.6	2.6
AD-59529	43.8	4.8
AD-59389	44.1	6.4
AD-59585	44.3	3.2
AD-59570	45.1	4.0
AD-59415	46.6	2.3
AD-59505	47.5	6.2
AD-59557	48.1	4.4
AD-59548	49.9	4.0
AD-59487	50.7	3.2
AD-59550	50.8	5.8
AD-59572	51.1	4.0
AD-59554	51.3	6.0
AD-59437	52.2	4.8
AD-59584	54.9	2.7
AD-59373	55.3	20.1
AD-59545	55.4	3.4
AD-59547	55.9	4.7
AD-59470	56.0	2.7

AD-59417	56.4	7.7
AD-59535	57.6	5.1
AD-59507	58.8	4.7
AD-59519	59.1	5.6
AD-59391	60.1	12.5
AD-59537	60.6	9.1
AD-59450	60.7	7.2
AD-59449	61.6	6.8
AD-59418	61.8	8.4
AD-59561	62.2	7.2
AD-59460	62.8	4.7
AD-59409	64.4	9.0
AD-59476	65.2	5.6
AD-59406	65.6	3.5
AD-59569	66.7	7.6
AD-59451	66.9	2.9
AD-59553	67.2	8.8
AD-59372	67.3	25.6
AD-59377	68.7	5.1
AD-59531	68.7	9.0
AD-59560	68.7	12.7
AD-59489	69.6	8.9
AD-59540	70.1	10.1
AD-59378	70.6	14.1
AD-59403	71.4	3.3
AD-59493	72.3	3.5
AD-59374	75.9	5.1
AD-59380	76.4	11.1
AD-59576	77.5	16.2
AD-59425	77.9	10.6
AD-59509	78.0	3.2
AD-59488	78.6	7.1
AD-59486	79.4	5.0
AD-59465	79.5	5.1
AD-59484	79.8	3.2
AD-59368	80.0	11.9
AD-59464	80.2	9.3
AD-59386	80.6	33.2
AD-59439	80.9	4.0
AD-59440	82.2	1.9

AD-59542	83.3	10.6
AD-59559	83.7	9.1
AD-59586	83.8	11.5
AD-59408	86.3	2.8
AD-59568	86.8	4.2
AD-59398	87.4	24.9
AD-59508	87.5	2.5
AD-59523	87.6	11.8
AD-59410	88.8	8.3
AD-59541	88.9	10.8
AD-59524	89.5	12.1
AD-59501	89.9	5.1
AD-59383	90.8	27.4
AD-59577	91.1	2.3
AD-59447	91.3	12.9
AD-59555	91.7	3.4
AD-59405	92.5	5.7
AD-59371	93.5	31.7
AD-59443	93.8	9.0
AD-59401	94.5	7.1
AD-59494	95.1	9.1
AD-59504	96.8	11.7
AD-59369	96.8	4.8
AD-59571	97.4	7.0
AD-59527	98.6	7.8
AD-59466	99.7	14.0
AD-59526	102.9	4.6
AD-59543	103.7	3.0
AD-59564	103.7	12.1
AD-59583	112.4	13.2

The two hundred thirty-two duplexes that were tested suppressed ALAS1 mRNA to varying extents in this single dose assay. According to this assay, at least four of the duplexes (AD-59453, AD-59395, AD-59477, and AD-59492) suppressed ALAS1 mRNA by 85% or more, 39 of the duplexes suppressed ALAS1 mRNA by 80% or more, 101 of the duplexes suppressed ALAS1 mRNA by 70% or more, and 152 of the duplexes suppressed ALAS1 mRNA by 50% or more. In contrast, some duplexes did not show appreciable suppression in this assay.

Example 13: Dose responsive inhibition of porphyrin precursors ALA and PBG using ALAS1 siRNA

The dose response effects of an ALAS1 siRNA were investigated in a mouse model of AIP (see Example 5). This model shows ~30% residual PBGD activity, ~2 fold increase in basal ALA and PBG levels, ~30-100 fold increase in ALA and PBG levels following induction by injections of Phenobarbital once per day for 3-4 days. Older animals have axonal degeneration and impaired motor function.

The ALAS1 siRNA used in this example was the AD-53558 duplex in the AF11 formulation. On day 1, the mice were administered 1 mg/kg, 0.5 mg/kg, 0.1 mg/kg, or 0.05 mg/kg of ALAS1 siRNA or LUC AD-1955 control by i.v. injection. Three phenobarbital injections were given (1 injection per day on days 2, 3, and 4) to induce hepatic ALAS1 and the porphyrin precursors, ALA and PBG. Plasma and overnight urine specimens were collected on day 5 and metabolite levels were measured by LC-MS. Baseline levels of ALA and PBG were measured prior to the first treatment on day 1. The results are shown in FIG. 16. The ALAS1 siRNA inhibited ALA and PBG levels in a dose dependent manner. The inhibitory effect on plasma ALA levels was observed at ALAS1 siRNA doses as low as 0.05 mg/kg, and the inhibitory effect on plasma PBG levels was seen at siRNA doses as low as 0.1 mg/kg.

Example 14: Durable inhibition of porphyrin precursors ALA and PBG using ALAS1 siRNA

The durability of the effects of an ALAS1 siRNA were investigated in a mouse model of AIP (see Example 5). The ALAS1 siRNA used in this example was the AD-53558 duplex in the AF11 formulation. The experimental design and results of this experiment are shown in FIG. 17. On day 1, mice were administered 1 mg/kg of ALAS1 siRNA or LUC AD-1955 control by i.v. injection. Three phenobarbital injections were given in week 0 (1 injection per day on days 2, 3, and 4), week 2 (1 injection per day on days 15, 16, and 17), and week 4 (1 injection per day on days 29, 30, and 31) to induce hepatic ALAS1 and porphyrin precursors ALA and PBG. Plasma and overnight urine specimens were collected on days 5, 18, and 32 and metabolite levels were

measured by LC-MS. Baseline levels of ALA and PBG were measured prior to the first treatment on day 1.

As is shown in FIG. 17, the ALAS1 siRNA had a durable effect in reducing plasma ALA and PBG levels. Administration of the ALAS1 siRNA suppressed plasma ALA and PBG levels for at least 2 weeks. These results indicate that ALAS1 siRNA is an effective treatment for lowering elevated levels of ALA and PBG and thus can be used in prophylaxis, e.g., to lower chronically elevated ALA and PBG levels and to prevent recurrent porphyric attacks.

Example 15: ALAS1 siRNA provides more rapid onset of action compared with hemin treatment

The effects of treatment with an ALAS1 siRNA were compared with the effects of hemin treatment in a mouse model of AIP (see Example 5). The ALAS1 siRNA used in this example was the AD-53558 duplex in the AF11 formulation. The experimental design and results of this experiment are shown in FIG. 18. Phenobarbital (PB) and diethyldithiocarbamate (DDC) were administered on days 1, 2, and 3. DDC is another p450 inducer that, like Phenobarbital, increases the demand for heme and helps extend the induction of ALA/PBG metabolites.

Hemin at a dose of 4 mg/kg, ALAS1 siRNA at a dose of 2 mg/kg, or control treatment was administered intravenously at 8 hours after the last administration of PB and DDC.

As is shown in FIG. 18, the onset of treatment effects was faster with ALAS1 siRNA treatment compared with hemin treatment. The rapid reduction of ALA and PBG with siRNA treatment indicates that siRNA is an effective treatment for acute attacks, because a rapid improvement in clinical symptoms is expected to accompany the reduction in ALA and PBG levels.

Example 16: Effects of ALAS1 siRNA GalNAc conjugate AD-58632

AD-58632 is a 21/23mer disclosed in Example 11. AD-58632 targets the human transcript NM_ 000688.4 and is also cross reactive with mouse, rat, and cynomolgous monkey

mRNA transcripts. AD-58632 was the only cross reactive 21/23mer identified from a screen of about 45 compounds. Further experiments with this duplex are described in this example.

Dose dependent effects of AD-58632 in suppressing ALAS1 mRNA

The dose response effect of AD-58632 in suppressing ALAS1 mRNA, relative to GAPDH mRNA, was investigated in rats. Doses of 30 mg/kg, 10 mg/kg, and 3 mg/kg were tested. The levels of ALAS1 mRNA were measured in liver at 72 hours after the last dose. AD-58632, compared with PBS control, suppressed ALAS1 mRNA in a dose dependent manner (see FIG. 19). AD-58632 had a single dose ED50 of about 10 mg/kg.

Effects of AD-58632 in Rat AIP Model

The dose response effect of the AD-58632 ALAS1 GalNAc conjugate siRNA was further investigated in a rat AIP model. In this model, siRNA in an LNP is used to knock down the level of PBGD specifically in liver prior to inducing heme demaind with phenobarbitol. The rat AIP model shows transient PBGD siRNA knockdown in the liver, has ~15% residual PBGD mRNA, and shows about a 10-50 fold increase in ALA and PBG levels upon induction by daily Phenobarbital injection for three days.

The experimental design is depicted in FIG. 20. Four groups of rats were studied. One group was treated with phenobarbital (PB) only at the indicated timepoints. A second group was treated with phenobarbital and porphobilinogen deaminase (PBGD) siRNA. A third group received phenobarbital, PBGD siRNA, and a dose of 30 mg/kg of the ALAS1 siRNA. A fourth group received phenobarbital, PBGD siRNA, and a dose of 10 mg/kg of the ALAS1 siRNA. As is shown in FIG. 20, the PBGD siRNA was administered intravenously on day 1. The ALAS1 GalNAc siRNA was administered on day 4. Phenobarbital injections were given on days 4, 5, 6, and 7. Urine was collected for a 24 hour period starting on day 7 and ending on day 8. Levels of liver PBGD mRNA, GAPDH mRNA, and ALAS-1 mRNA were assessed on day 8 using a bDNA assay. PBG and ALA levels in urine were determined using LC-MS.

The mRNA results are shown in FIG. 21. PBGD siRNA decreased PBGD mRNA level but did not decrease ALAS1 mRNA level. The ALAS1 siRNA decreased ALAS1 mRNA levels

in a dose-dependent manner (see FIG. 21). The ALA and PBG results are shown in FIG. 22. ALAS1 siRNA decreased ALA and PBG levels in a dose-dependent manner (see FIG. 22).

Example 17: Split dosing with AD-58632

The efficacy of the ALAS1 siRNA GalNAc conjugate AD-58632 was investigated in two separate split dosing paradigms. For each of these studies, female Sprague Dawley rats were used. The rats were housed in SCLR (a light cycle room that is 12 hours light on and 12 hours light off) and were sacrificed at 72 hours following the last injection. ALAS1 and GAPDH mRNA levels in the liver were measured using branched DNA (bDNA) assay.

Five daily doses versus one bolus dose paradigm

In the first paradigm, groups of rats were given either five doses of siRNA (one dose each day) or a single bolus dose that had the same total concentration as the sum of the five individual doses. Specifically, rats were assigned to one of the following treatment conditions: (1) subcutaneous injection of 6 mg/kg siRNA once per day for five days (2) subcutaneous injection of 2 mg/kg siRNA once per day for five days, (3) subcutaneous injection of 1 mg/kg siRNA once per day for five days, (4) subcutaneous injection of a single bolus dose of 30 mg/kg siRNA (5) subcutaneous injection of a single bolus dose of 10 mg/kg siRNA, (6) subcutaneous injection of a single bolus dose of 5 mg/kg siRNA, or (7) PBS control treatment.

The results are shown in FIG. 23. In this paradigm, a single bolus dose of siRNA provided greater suppression of ALAS1 mRNA than did repeated dosing of the same concentration of siRNA over the course of five days. This was true for all doses studied.

Once per week dosing for four weeks

In the second paradigm, rats were given subcutaneous injections of siRNA at one of three doses (10 mg/kg, 5 mg/kg, or 2.5 mg/kg) once per week for four weeks. A control group received PBS injections.

The results are shown in FIG. 24. Compared with single dosing, providing four weekly doses at 10 mg/kg improved the maximal knockdown achieved (ED50 is 10 mg/kg at single

dose). In contrast, multiple dosing at 5 and 2.5 mg/kg per week did not improve silencing in this paradigm.

Example 18: Identification and testing of ALAS1 siRNAs with shorter sense and antisense strands

Further experiments were conducted to explore the effects of shortening the siRNA duplexes to 19-19mers. Five more new cross-reactive 19-19mer duplexes that bind to human (h) (NM_000688.4), rhesus monkey (rh) (XM_001090440.2), mouse (m) (NM_020559.2), and rat (r) (NM_024484.2) ALAS1 mRNA transcripts were identified. None of these duplexes showed results as good as the 21/23 mer AD-58632 (see FIG. 25).

The effects of modifying the length and overhangs on the best two 19-19mers (AD-59115 and AD-59125) were investigated (FIG. 26 and 27). The modified sequences are shown in Table 21.

Table 21: Sequences for length/overhang evaluation of best two 19-19mers

SEQ ID NO: (sense)	SEQ ID NO: (anti-sense)	Target sites of anti-sense sequence on NM_000688.4	Cross reactivity	Over-hang	Duplex Name	Sense Sequence (5'-3')	Antisense (AS) Sequence (5'-3')
4172	4173	877-895	h/rh/m/r	19/19	AD-59115	AfsasGfaGfuGfuCfUfCfaUfcUfuCfuUfL96	asAfsgAfaGfaUfgAfgacAfcUfcsusu
4174	4175	875-895	h/rh/m/r	19/21	AD-60090	AfsasGfaGfuGfuCfUfCfaUfcUfuCfuUfL96	asAfsgAfaGfaUfgAfgacAfcUfcUfususc
4176	4177	877-895	NC OH*	19/21	AD-60091	AfsasGfaGfuGfuCfUfCfaUfcUfuCfuUfL96	asAfsgAfaGfaUfgAfgacAfcUfcUfusasa
4178	4179	873-895	h/rh/m/r	21/23	AD-58632	GfsasAfaGfaGfuGfUfCfuCfaUfcUfuCfuUfL96	asAfsgAfaGfaUfgAfgacAfcUfcUfuUfcsusg

4180	4181	875-895	NC OH*	21/23	AD-60092	GfsasAfaGfaGfuGfUfCfuCfaUfcUfuCfuUfL96	asAfsgAfaGfaUfgAfgacAfcUfcUfuUfcsasa
4182	4183	875-893	h/rh/m/r	19/19	AD-59129	GfsasAfaGfaGfuGfUfCfuCfaUfcUfuAfL96	usAfsaGfaUfgAfgAfcacUfcUfususc
4184	4185	873-893	h/rh/m/r	19/21	AD-60093	GfsasAfaGfaGfuGfUfCfuCfaUfcUfuAfL96	usAfsaGfaUfgAfgAfcacUfcUfuUfcsusg
4186	4187	875-893	NC OH*	19/21	AD-60094	GfsasAfaGfaGfuGfUfCfuCfaUfcUfuAfL96	usAfsaGfaUfgAfgAfcacUfcUfuUfcsasa
4188	4189	871-893	h/rh	21/23	AD-60095	CfsasGfaAfaGfaGfuGfUfCfuCfaUfcUfuAfL96	usAfsaGfaUfgAfgAfcacUfcUfuUfcUfgsgsu
4190	4191	871-893	m/r	21/23	AD-60096	CfsasGfaAfaGfaGfuGfUfCfuCfaUfcUfuAfL96	usAfsaGfaUfgAfgAfcacUfcUfuUfcUfgsgsc

*Non-complementary overhang

Overhangs improved potency. They also provided a further derivative sequence (AD-60489, which was based on AD-60095) for further structure activity relationship (SAR) studies (1 mismatch at pos23 to rodent).

Example 19: Effects of ALAS1 siRNA GalNAc conjugates AD-60489 and AD-58632

The effects of a further GalNAc conjugate ALAS1 siRNA duplex AD-60489 were investigated and compared with the effects of AD-58632. The sequences of these duplexes are shown in Table 22A. AD-60489 has a single mismatch to rodent ALAS1 mRNA at the 3' end of the antisense sequence. Thus, whereas AD-58632 is fully complementary with human, cynomolgous monkey, mouse, and rat sequences, AD-60489 is fully complementary only with human and cynomolgous monkey sequences.

Table 22A: Sequences of ALAS1 siRNA Duplexes AD-58632 and AD-60489

SEQ ID NO: (sense)	SEQ ID NO: (anti-sense)	Target sites of antisense sequence on NM_ 000688.4	Duplex Name	Sense Sequence (5'-3')	Antisense Sequence (5'-3')
4149	4150	873-895	AD-58632	GfsasAfaGfaGfuGfUfCfuCfaUfcUfuCfuUf L96	asAfsgAfaGfaUfgAfgacAfcUfcUfuUfcs usg
4151	4152	871-893	AD-60489	CfsasGfaAfaGfaGfUfGfuCfuCfaUfcUfuAf L96	usAfsaGfaUfgAfgAfcacUfcUfuUfcUfgs gsu

The suppression of ALAS1 mRNA is shown in FIG. 28. Compared with AD-58632, AD-60489 provided more effective suppression at 3 mg/kg and 10 mg/kg and exhibited about a two fold improvement in ED50. The single dose ED50 of AD-60489 was about 5 mg/kg.

Example 20: Effects of ALAS1 siRNA GalNAc conjugates AD-60489 and AD-58632 in non-human primate studies

The effectiveness of AD-58632 and AD-60489 in suppressing liver mRNA was investigated in non-human primates. The experimental design is shown in FIG. 29. Doses of siRNA (5 mg/kg, 2.5 mg/kg, or 1.25 mg/kg) or PBS control in a volume of 2 mL/kg were administered subcutaneously every day for 5 days, then every 2 days for 3 weeks. ALAS1 mRNA silencing was evaluated in liver tissue obtained from a liver biopsy taken on day 15. The biopsy was taken after a serum draw and prior to the administration of dose 10 (see FIG. 29). Serum samples for the circulating extracellular RNA detection (cERD) method (see Example 21) were collected on days -10, -3, 7, 15, 23, 31, and 43. Serum was collected for a clinical chemistry panel on days -3, 6, 30, and 43. The clinical chemistry panel included assessment of the levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP).

AD-58632 and AD-60489 suppressed ALAS1 mRNA in liver in a dose-dependent manner (see FIG. 30). AD-60489 showed greater efficacy than did AD-58632. For example, at the lowest dose studied (1.25 mg/kg), AD-60489 suppressed the relative ALAS1 message to

about 42% of the control level, whereas AD-58632 showed little suppression at this dose. At 2.5 mg/kg, AD-60489 suppressed the relative ALAS1 message to about 26% of the control level, whereas AD-58632 suppressed the relative ALAS1 message to about 64% of the control level. At 5 mg/kg, AD-60489 suppresed the relative ALAS1 message to about 21% of the control level, and AD-58632 suppressed the relative ALAS1 message to about 55% of the control level.

Clinical chemistry results indicated that the sustained knockdown of ALAS1 using the ALAS1 siRNAs was safe and well tolerated. No elevations in ALT, AST, or ALP were observed.

Example 21: Effects of ALAS1 siRNA GalNAc conjugates AD-60489 and AD-58632 in non-human primate studies as assessed using the cERD assay

The effects of ALAS1 siRNA GalNAc conjugates AD-60489 and AD-58632 were assessed in non-numan primates using the circulating extracellular RNA detection (cERD) method. This method is described, *e.g.*, in Sehgal, A. et al. Quantitation of tissue-specific target gene modulation using circulating RNA (Poster presented on February 9, 2012 at the Keystone Gene Silencing by small RNAs symposium (Vancouver, February 7-12, 2012) and in Sehgal, A. et al. Tissue-specific gene silencing monitored in circulating RNA, *RNA*, 20: 1-7, published online December 19, 2013. As is shown in FIG. 29, serum samples for the circulating extracellular RNA detection (cERD) method were collected on days -10, -3, 7, 15, 23, 31, and 43.

For the cERD assay, serum samples were thawed on ice. 375-400 μL of 8M LiCl was added to 3-3.5 mL of serum in ultracentrifuge (UC) tubes, and incubated at a temperature of 4°C for at least 1 hour. PBS was added to the top of each UC tube, leaving about 1 cm of dry space at the top of the tube to prevent walls of tubes from collapsing during spin. The tubes were dried to remove any condensation from being incubated on ice. Samples were loaded into an MC 55 Rotor under a hood, and the samples were spun at 150,000-200,000g for 100-120 minutes. The supernatant was discarded from the pellet. 1 mL Trizol was added to the pellet in the UC tube, the tube was vortexed, and the contents were transferred to a 1.5 mL microcentrifuge tube. To each tube, 200 μL of chloroform was added, and the tube was inverted several times to mix. One sample was prepared at a time. The samples were spun at 13,000 RPM for 10-20 minutes at 4°C.

The upper aqueous phase was transferred to a fresh 1.5 mL tube (~500 µL volume). An equal volume of 100% isopropanol, 1 µL of linear acrylamind (4°), and 1/10th volume of 3M NaoAc pH 5.5 or less was added to each sample (typically 500 µL of isopropanol and 50 µL NaoAc). The sample was spun at 13,000 RPM for 10 min at 4°C. Supernatants were reserved. The pellet was washed twice with ice cold 70% EtOH (500 µL each wash) and spun at 13,000 RPM for ~5 min. at 4°C after each wash. The pellet was allowed to air dry for ~5 minutes and then resuspended in 20µL NF H2O. 10 µL was used in cDNA reaction. The resuspended RNA was stored at -80°C.

Results

The serum mRNA knockdown as assessed using the cERD assay correlated with the results obtained from the liver biopsy. See FIG. 31. This is a surprising result, because ALAS1 is not a serum protein. The cERD assay provided herein allows monitoring of circulating ALAS1 mRNA. This has the advantage, for example, that the levels of ALAS1 mRNA can be measured over time without doing serial liver biopsies, which would be technically difficult and expensive.

The kinetics of mRNA knockdown were determined using the cERD assay results. See FIG. 32. AD-60489 achieved greater than 50% knockdown, even at a dose of only 1.25 mg/kg.

Example 22: Safety studies of ALAS1 siRNAs

The following safety studies indicate that sustained knockdown of ALAS1 is safe and well tolerated.

Non-human primate studies

As described above (see Example 20), in non-human primate studies, no ALT, AST, or ALP elevations were observed after administration of AD-60489 and AD-58632.

Rat studies

In rats, a four week study was carried out with AD-58632. The siRNA was administered every day for 5 days at 10 mg/kg in the first week, then every other day at 10 mg/kg for weeks 2-4 of the study. The total exposure was 140 mg. No adverse clinical signs or body weight changes were observed. No test article related changes in hematology or coagulation parameters

were observed. Furthermore, no adverse histopathology was observed. There was minimal vacuolation in spleen and minimal subcapsular fibrosis in kidney.

Mouse studies

In mice, P450 mRNAs were assessed after ALAS1 knockdown. Minor dose dependent increases in Cyp2b10 were observed at 48 hours after administration of an ALAS1 LNP formulation. This resolved by 168 hours.

Example 23: Identification of further effective ALAS1 siRNAs using structure activity relationship studies

Structure activity relationship (SAR) studies, including studies described in other examples herein, were carried out to identify further effective ALAS1 siRNAs derived from those that have already been identified, e.g., AD-58632 and AD-60489. Effects of chemical modifications were investigated. Chemical modifications include 1) 2'-O-methyl versus 2'-fluoro modifications, 2) Decrease in 2'Uf (2'fluoro modifications), 3) Add PS (phosphorothioate), 4) Use internal dTs, and/or 5) glycol nucleic acids (GNAs). Without wishing to be bound by theory, modifications can enhance potency, e.g., through 1) better unwinding or enhanced RISC loading, or 2) better catalytic target engagement. Modifications can also enhance stability so that compounds can accumulate and perform better when multiple doses are administered.

Improved activity relative to other duplexes (e.g., AD-58632 and/or AD-60489) was observed in some instances (see Table 22B), whereas similar activity (see Table 23) or reduced activity (Table 24) was observed in other instances. These instances are merely presented as examples based on the screening of more than 150 siRNAs. Further exemplification of SAR studies is provided herein.

Table 22B: Improved Activity Relative to Parent

Duplex*	IC50	Sense (5' to 3')	Antisense (5' to 3')
AD-58632.10 (parent)	0.017	GfsasAfaGfaGfuGfUfCfuCfaUfcUfuCfuUfL96 (SEQ ID NO: 4192)	asAfsgAfaGfaUfgAfgacAfcUfcUfuUfcsusg (SEQ ID NO: 4193)
AD-80643.1	0.004	GfsasAfaGfAfGfuGfdTcucaucuucuuL96 (SEQ ID NO: 4194)	asAfsgAfaGfaugAfgAfcAfcucuuucsusg (SEQ ID NO: 4195)
AD-60489.3 (parent)	0.010	CfsasGfaAfaGfaGfUfGfuCfuCfaUfcUfuAfL96 (SEQ ID NO: 4196)	usAfsaGfaUfgAfgAfcacUfcUfuUfcUfgsgsu (SEQ ID NO: 4197)
AD-60879.1	0.001	CfsasGfaAfaGfaGfdTGfuCfuCf(Agn)UfscUfsuAfsL96 (SEQ ID NO: 4198)	usAfsaGfaUfgAfgAfcacUfcdTuUfcUfgsgsu (SEQ ID NO: 4199)

*The number following the decimal point in a duplex name in this and other tables merely refers to a batch production number.

Table 23: Similar Activity Relative to Parent but Increased Stability

Duplex	IC50	Sense (5' to 3')	Antisense (5' to 3')
AD-58632.10 (parent)	0.017	GfsasAfaGfaGfuGfUfCfuCfaUfcUfuCfuUfL96 (SEQ ID NO: 4200)	asAfsgAfaGfaUfgAfgacAfcUfcUfuUfcsusg (SEQ ID NO: 4201)
AD-60839.1	0.014	gsasaagaGfuGfuCfucaucuucuuL96 (SEQ ID NO: 4202)	asAfsgAfaGfaugAfgacAfcucuuucsusg (SEQ ID NO: 4203)

Table 24: Reduced Activity Relative to Parent

Duplex	IC50	Sense (5' to 3')	Antisense (5' to 3')
AD-58632.10 (parent)	0.017	GfsasAfaGfaGfuGfUfCfuCfaUfcUfuCfuUfL96 (SEQ ID NO: 4204)	asAfsgAfaGfaUfgAfgacAfcUfcUfuUfcsusg (SEQ ID NO: 4205)
AD-60886.1	0.801	GfsasAfaGfAfGfuGfdTcucaucuucuuL96 (SEQ ID NO: 4206)	asAfsgAf(Agn)GfaUfgAfgacAfcUfcUfuUfcsusg (SEQ ID NO: 4207)

Example 24: In vitro structure activity relationship studies of AD-58632

AD-58632 and siRNA derivatives of AD-58632 were generated, and some siRNAs were screened *in vitro* for activity. Abbreviations for chemical modifications are provided in Table 1.

In vitro activity at 10 nM and 0.1 nM siRNA

The *in vitro* activity of the siRNAs in suppressing ALAS1 mRNA was tested in in Hep3B cells that were transfected using Lipofectamine2000 as a transfection reagent. Experiments were performed at the indicated siRNA concentrations (e.g., 0.1 nM, 10 nM) and analyzed by branched DNA (bDNA) assay at 24 hours post-transfection. The results are expressed as percent remaining mRNA relative to the siRNA AD-1955, a non-targeting siRNA that was used as a negative control.

Sequences of siRNAs and results of in vitro testing are provided in Table 25, Table 26, and Table 27.

Table 25: Sequences and in vitro screen results for AD-58632 and AD-58632 derivative siRNAs

SEQ ID NO: (sense)	SEQ ID NO: (anti-sense)	Target sites of antisense seq on NM_000688.4	Duplex	Sense Sequence (5'-3')	Antisense (AS) Sequence (5'-3')	Avg 10 nM	SD 10 nM	Avg 0.1 nM	SD 0.1 nM
4208	4209	873-895	AD-58632.8	GfsasAfaGfaGfuGfUfCfuCfaUfcUfuCfuUfL96	asAfsgAfaGfaUfgAfgacAfcUfcUfuUfcsusg	11.79	2.70	46.65	4.21
4210	4211	873-895	AD-60405.1	GfsasAfaGfaGfuGfuCfuCfaucuuCfuuL96	asAfsgAfaGfaUfgAfgacAfcUfcUfuUfcsusg	15.61	4.49	63.49	10.51
4212	4213	873-895	AD-60411.1	GfsasAfaGfaGfuGfuCfuCfaucuucuuL96	asAfsGfAfaGfaUfgAfgAfcAfcUfcUfuUfcsusg	15.04	6.13	62.59	10.05
4214	4215	873-895	AD-60417.1	GfsasaagaGfuGfuCfuCfaucuucuuL96	asAfsGfAfaGfaUfgAfgAfcAfcUfcUfuUfcsusg	13.96	5.47	66.10	8.21
4216	4217	873-895	AD-60423.1	gsasaagaGfuGfuCfuCfaucuucuuL96	asAfsGfAfaGfaUfgAfgAfcAfcUfcUfuUfcsusg	12.59	3.03	41.47	3.77
4218	4219	873-895	AD-60423.2	gsasaagaGfuGfuCfuCfaucuucuuL96	asAfsGfAfaGfaUfgAfgAfcAfcUfcUfuUfcsusg	13.79	3.38	55.93	7.90
4220	4221	873-895	AD-60434.1	gsasaagaGfuGfuCfucaucuucuuL96	asAfsGfAfaGfaUfgAfgAfcAfcUfcUfuUfcsusg	14.74	2.76	48.68	6.64
4222	4223	873-895	AD-60440.1	gsasaagaGfuGfucucaucuucuuL96	asAfsGfAfaGfaUfgAfgAfcAfcUfcUfuUfcsusg	28.31	8.68	77.01	3.99
4224	4225	873-895	AD-60400.1	gsasaagaguGfucucaucuucuuL96	asAfsGfAfaGfaUfgAfgAfcAfcUfcUfuUfcsusg	39.90	5.67	99.64	8.58
4226	4227	873-895	AD-60406.1	gsasaagagugucucaucuucuuL96	asAfsGfAfaGfaUfgAfgAfcAfcUfcUfuUfcsusg	56.06	2.08	95.83	17.01
4228	4229	873-895	AD-60412.1	GfsasaagaGfuGfucucaucuucuuL96	asAfsGfAfaGfaUfgAfgAfcAfcUfcUfuUfcsusg	43.09	2.23	87.52	8.10
4230	4231	873-895	AD-60418.1	gsasaagaGfuGfucucaucuucuuL96	asAfsGfAfaGfaUfgAfgAfcAfcucUfuUfcsusg	65.84	7.75	108.07	21.88
4232	4233	873-895	AD-60424.1	gsasaagaGfuGfucucaucuucuuL96	asAfsGfAfaGfaUfgAfgAfcAfcucuuUfcsusg	45.51	11.82	84.40	10.69
4234	4235	873-895	AD-60429.1	gsasaagaGfuGfucucaucuucuuL96	asAfsGfAfaGfaUfgAfgAfcAfcucuuucsusg	63.96	13.25	81.21	1.96
4236	4237	873-895	AD-60435.1	gsasaagaGfuGfucucaucuucuuL96	asAfsGfAfaGfaugAfgAfcAfcucuuucsusg	80.12	10.02	95.33	23.09
4238	4239	873-895	AD-60441.1	gsasaaGfaGfuGfucucaucuucuuL96	asAfsGfAfaGfaugAfgAfcAfcucuuucsusg	63.29	17.48	97.07	8.04

4240	4241	873-895	AD-60401.1	gsasaaGfAfGfuGfucucaucuucuuL96	asAfsGfAfaGfaugAfgAfcAfcucuuucsusg	55.27	10.26	109.06	4.23
4242	4243	873-895	AD-60407.1	GfsasaaGfAfGfuGfucucaucuucuuL96	asAfsGfAfaGfaugAfgAfcAfcucuuucsusg	47.39	1.88	98.04	22.58
4244	4245	873-895	AD-60413.1	GfsasAfaGfAfGfuGfucucaucuucuuL96	asAfsGfAfaGfaugAfgAfcAfcucuuucsusg	55.60	11.65	92.88	5.65
4246	4247	873-895	AD-60419.1	GfsasAfaGfAfGfuGfdTcucaucuucuuL96	asAfsGfAfaGfaugAfgAfcAfcucuuucsusg	20.82	15.07	57.82	8.31
4248	4249	873-895	AD-60425.1	GfsasAfaGfAfGfuGfudCucaucuucuuL96	asAfsGfAfaGfaugAfgAfcAfcucuuucsusg	35.58	13.30	73.46	10.91
4250	4251	873-895	AD-60430.1	GfsasAfaGfAfGfuGfucdTcaucuucuuL96	asAfsGfAfaGfaugAfgAfcAfcucuuucsusg	40.54	1.41	81.87	11.23
4252	4253	873-895	AD-60436.1	GfsasAfaGfAfGfuGfucudCaucuucuuL96	asAfsGfAfaGfaugAfgAfcAfcucuuucsusg	60.12	11.74	81.51	7.41
4254	4255	873-895	AD-60442.1	GfsasAfaGfAfGfuGfucucdAucuucuuL96	asAfsGfAfaGfaugAfgAfcAfcucuuucsusg	40.82	12.61	83.06	1.05
4256	4257	873-895	AD-60402.1	GfsasAfaGfAfGfuGfucucadTcuucuuL96	asAfsGfAfaGfaugAfgAfcAfcucuuucsusg	62.16	11.24	123.60	6.71
4258	4259	873-895	AD-60408.1	GfsasAfaGfAfGfuGfucucaudCuucuuL96	asAfsGfAfaGfaugAfgAfcAfcucuuucsusg	45.39	15.50	86.69	6.12
4260	4261	873-895	AD-60414.1	GfsasAfaGfAfGfuGfucucaucudTcuuL96	asAfsGfAfaGfaugAfgAfcAfcucuuucsusg	32.56	3.52	84.21	0.24
4262	4263	873-895	AD-60420.1	GfsasAfaGfAfGfuGfucucaucuudCuuL96	asAfsGfAfaGfaugAfgAfcAfcucuuucsusg	52.57	10.77	94.45	3.43
4264	4265	873-895	AD-60426.1	GfsasAfaGfAfGfuGfucucaucuucudTL96	asAfsGfAfaGfaugAfgAfcAfcucuuucsusg	52.49	1.91	91.15	14.49
4266	4267	873-895	AD-60431.1	gsasaaGfaGfuGfudCudCaucuucuuL96	asAfsGfAfaGfaugAfgAfcAfcucuuucsusg	26.66	1.16	73.09	6.83
4268	4269	873-895	AD-60437.1	gsasaaGfaGfuGfudCucadTcuucuuL96	asAfsGfAfaGfaugAfgAfcAfcucuuucsusg	32.80	4.58	69.03	3.02
4270	4271	873-895	AD-60443.1	gsasaaGfaGfuGfudCucaucdTucuuL96	asAfsGfAfaGfaugAfgAfcAfcucuuucsusg	35.10	7.10	69.92	17.93
4272	4273	873 - 895	AD-60403.1	gsasaaGfaGfuGfudCudCaucdTucuuL96	asAfsGfAfaGfaugAfgAfcAfcucuuucsusg	25.28	1.68	105.23	23.99
4274	4275	873 - 895	AD-60409.1	gsasaaGfaGfuGfudCudCadTcdTucuuL96	asAfsGfAfaGfaugAfgAfcAfcucuuucsusg	30.48	1.88	72.34	3.34
4276	4277	873 - 895	AD-60415.1	gsasaaGfaGfuGfudCudCadTcdTudCuuL96	asAfsGfAfaGfaugAfgAfcAfcucuuucsusg	25.28	7.10	69.53	10.72
4278	4279	873 - 895	AD-60421.1	gsasaaGfaGfuGfucucaucuucuuL96	asAfsGfAfaGfadTgAfgAfcAfcucuuucsusg	59.28	5.83	66.88	0.63
4280	4281	873 - 895	AD-60427.1	gsasaaGfaGfuGfucucaucuucuuL96	asAfsGfAfaGfaugAfgAfcAfcdTcuuucsusg	34.80	8.13	79.65	11.25

4282	4283	873 - 895	AD-60432.1	gsasaaGfaGfuGfucucaucuucuuL96	asAfsGfAfaGfaugAfgAfcAfcucdTuucsusg	46.78	6.42	79.19	16.72
4284	4285	873 - 895	AD-60438.1	gsasaaGfaGfuGfucucaucuucuuL96	asAfsGfAfaGfadTgAfgAfcAfcdTcuuucsusg	32.07	9.46	57.87	10.18
4286	4287	873 - 895	AD-60444.1	gsasaaGfaGfuGfucucaucuucuuL96	asAfsGfAfaGfadTgAfgAfcAfcucdTuucsusg	55.55	10.17	89.52	3.91
4288	4289	873 - 895	AD-60404.1	gsasaaGfaGfuGfucucaucuucuuL96	asAfsGfAfaGfa(Tgn)gAfgAfcAfcucuuucsusg	50.06	9.17	93.46	2.56
4290	4291	873 - 895	AD-60410.1	gsasaaGfaGfuGfucucaucuucuuL96	asAfsGfAfaGfaugAfgAfcAfc(Tgn)cuuucsusg	44.40	13.93	88.96	6.06
4292	4293	873 - 895	AD-60416.1	gsasaaGfaGfuGfucucaucuucuuL96	asAfsGfAfaGfaugAfgAfcAfcuc(Tgn)uucsusg	28.56	7.82	76.36	19.47
4294	4295	873 - 895	AD-60422.1	GfsasAfaGfAfGfuGf(Tgn)cucaucuucuuL96	asAfsGfAfaGfaugAfgAfcAfcucuuucsusg	40.37	10.86	84.06	12.08
4296	4297	873 - 895	AD-60428.1	GfsasAfaGfAfGfuGfuc(Tgn)caucuucuuL96	asAfsGfAfaGfaugAfgAfcAfcucuuucsusg	56.81	22.64	92.15	0.26
4298	4299	873 - 895	AD-60433.1	GfsasAfaGfAfGfuGfucuc(Agn)ucuucuuL96	asAfsGfAfaGfaugAfgAfcAfcucuuucsusg	36.78	5.31	67.92	12.55
4300	4301	873 - 895	AD-60439.1	GfsasAfaGfAfGfuGfucuca(Tgn)cuucuuL96	asAfsGfAfaGfaugAfgAfcAfcucuuucsusg	32.81	6.72	77.93	13.33
4302	4303	873 - 895	AD-60445.1	GfsasAfaGfAfGfuGfucucauc(Tgn)ucuuL96	asAfsGfAfaGfaugAfgAfcAfcucuuucsusg	13.25	3.28	78.08	4.05
4304	4305	873 - 895	AD-60451.1	GfsasAfaGfAfGfuGfucucaucu(Tgn)cuuL96	asAfsGfAfaGfaugAfgAfcAfcucuuucsusg	34.74	11.06	88.93	5.19
4306	4307	873 - 895	AD-60457.1	GfsasAfaGfAfGfuGfucucaucuuc(Tgn)uL96	asAfsGfAfaGfaugAfgAfcAfcucuuucsusg	41.26	6.16	92.15	0.64
4308	4309	873 - 895	AD-60463.1	GfsasAfaGfaGfuGfUfCfuCfaUfcUfuCfuAfL96	usAfsgAfaGfaUfgAfgacAfcUfcUfuUfcsusg	19.58	7.98	69.67	4.64
4310	4311	873 - 895	AD-60469.1	CfsasAfaGfaGfuGfUfCfuCfaUfcUfuCfuUfL96	asAfsgAfaGfaUfgAfgacAfcUfcUfuUfgsasc	19.35	9.30	72.30	0.50
4312	4313	873 - 895	AD-60474.1	CfsusAfaGfaGfuGfUfCfuCfaUfcUfuCfuUfL96	asAfsgAfaGfaUfgAfgacAfcUfcUfuAfgsasc	21.60	4.27	76.35	11.71

4314	4315	873 - 895	AD-60479.1	CfsusUfaGfaGfuGfUfCfuCfaUfcUfuCfuUfL96	asAfsgAfaGfaUfgAfgacAfcUfcUfaAfgsasc	28.01	4.45	76.55	27.32
4316	4317	873 - 895	AD-60484.1	CfsusUfaGfaGfuGfUfCfuCfaUfcUfuCfuUfL96	asAfsgAfaGfaUfgAfgacAfcUfcUfaAfgsusg	20.31	3.08	71.99	9.53
4318	4319	873 - 895	AD-60446.1	GfsusUfuGfaGfuGfUfCfuCfaUfcUfuCfuUfL96	asAfsgAfaGfaUfgAfgacAfcUfcAfaAfcsusg	18.65	5.11	73.52	17.87
4320	4321	873 - 895	AD-60452.1	GfsasUfuCfaGfuGfUfCfuCfaUfcUfuCfuUfL96	asAfsgAfaGfaUfgAfgacAfcUfgAfaUfcsusg	28.72	1.21	83.09	15.75
4322	4323	873 - 895	AD-60458.1	GfsasAfuCfuGfuGfUfCfuCfaUfcUfuCfuUfL96	asAfsgAfaGfaUfgAfgacAfcAfgAfuUfcsusg	50.15	13.02	114.93	11.58
4324	4325	873 - 895	AD-60464.1	GfsasAfaGfaGfuGfUfCfuCfaUfcUfuCfuUfL96	(Agn)AfsgAfaGfaUfgAfgacAfcUfcUfuUfcsusg	35.71	5.07	103.88	3.01
4326	4327	873 - 895	AD-60470.1	GfsasAfaGfaGfuGfUfCfuCfaUfcUfuCfuUfL96	asAfs(Ggn)AfaGfaUfgAfgacAfcUfcUfuUfcsusg	17.59	0.78	73.15	11.75
4328	4329	873 - 895	AD-60475.1	GfsasAfaGfaGfuGfUfCfuCfaUfcUfuCfuUfL96	asAfsg(Agn)aGfaUfgAfgacAfcUfcUfuUfcsusg	22.07	4.57	68.64	25.12
4330	4331	873 - 895	AD-60480.1	GfsasAfaGfaGfuGfUfCfuCfaUfcUfuCfuUfL96	asAfsgAf(Agn)GfaUfgAfgacAfcUfcUfuUfcsusg	15.54	1.22	66.39	14.34
4332	4333	873 - 895	n/a	GfsasAfaGfaGfuGfUfCfuCfaUfcUfuCfuUfL96	asAfsgAfa(Ggn)aUfgAfgacAfcUfcUfuUfcsusg				
4334	4335	873 - 895	AD-60447.1	GfsasAfaGfaGfuGfUfCfuCfaUfcUfuCfuUfL96	asAfsgAfaGf(Agn)UfgAfgacAfcUfcUfuUfcsusg	31.33	4.75	104.36	7.71
4336	4337	873 - 895	AD-60453.1	GfsasAfaGfaGfuGfUfCfuCfaUfcUfuCfuUfL96	asAfsgAfaGfa(Tgn)gAfgacAfcUfcUfuUfcsusg	15.42	0.90	76.29	0.41
4338	4339	873 - 895	AD-60459.1	GfsasAfaGfaGfuGfUfCfuCfaUfcUfuCfuUfL96	asAfsgAfaGfaUf(Ggn)AfgacAfcUfcUfuUfcsusg	27.70	10.91	89.20	3.46
4340	4341	873 - 895	AD-60465.1	GfsasAfaGfaGfuGfUfCfuCfaUfcUfuCfuUfL96	asAfsgAfaGfaUfg(Agn)gacAfcUfcUfuUfcsusg	28.44	6.84	87.28	5.73
4342	4343	873 - 895	AD-60471.1	GfsasAfaGfaGfuGfUfCfuCfaUfcUfuCfuUfL96	asAfsgAfaGfaUfgAf(Ggn)acAfcUfcUfuUfcsusg	24.03	8.87	85.86	14.62
4344	4345	873 - 895	AD-60476.1	GfsasAfaGfaGfuGfUfCfuCfaUfcUfuCfuUfL96	asAfsgAfaGfaUfgAfg(Agn)cAfcUfcUfuUfcsusg	21.48	5.53	88.73	25.48

4346	4347	873 - 895	AD-60481.1	GfsasAfaGfaGfuGfUfCfuCfaUfcUfuCfu(Tgn)L96	asAfsgAfaGfaUfgAfgacAfcUfcUfuUfcsusg	15.18	4.19	68.10	6.86
4348	4349	873 - 895	AD-60486.1	GfsasAfaGfaGfuGfUfCfuCfaUfcUfuCf(Tgn)UfL96	asAfsgAfaGfaUfgAfgacAfcUfcUfuUfcsusg	15.31	0.31	74.06	8.48
4350	4351	873 - 895	AD-60448.1	GfsasAfaGfaGfuGfUfCfuCfaUfcUfu(Cgn)uUfL96	asAfsgAfaGfaUfgAfgacAfcUfcUfuUfcsusg	14.89	1.35	59.22	6.64
4352	4353	873 - 895	AD-60454.1	GfsasAfaGfaGfuGfUfCfuCfaUfcUf(Tgn)CfuUfL96	asAfsgAfaGfaUfgAfgacAfcUfcUfuUfcsusg	19.24	5.72	75.90	1.27
4354	4355	873 - 895	AD-60460.1	GfsasAfaGfaGfuGfUfCfuCfaUfc(Tgn)uCfuUfL96	asAfsgAfaGfaUfgAfgacAfcUfcUfuUfcsusg	14.91	5.84	60.11	6.01
4356	4357	873 - 895	AD-60466.1	GfsasAfaGfaGfuGfUfCfuCfaUf(Cgn)UfuCfuUfL96	asAfsgAfaGfaUfgAfgacAfcUfcUfuUfcsusg	13.99	7.53	56.83	11.59
4358	4359	873 - 895	AD-60472.1	GfsasAfaGfaGfuGfUfCfuCfa(Tgn)cUfuCfuUfL96	asAfsgAfaGfaUfgAfgacAfcUfcUfuUfcsusg	12.35	0.60	64.36	11.74
4360	4361	873 - 895	AD-60477.1	GfsasAfaGfaGfuGfUfCfuCf(Agn)UfcUfuCfuUfL96	asAfsgAfaGfaUfgAfgacAfcUfcUfuUfcsusg	15.62	1.76	65.96	7.77
4362	4363	873 - 895	AD-60482.1	GfsasAfaGfaGfuGfUfCfu(Cgn)aUfcUfuCfuUfL96	asAfsgAfaGfaUfgAfgacAfcUfcUfuUfcsusg	18.58	6.43	66.67	2.31
4364	4365	873 - 895	AD-60487.1	GfsasAfaGfaGfuGfUfCf(Tgn)CfaUfcUfuCfuUfL96	asAfsgAfaGfaUfgAfgacAfcUfcUfuUfcsusg	34.89	15.62	86.39	6.10
4366	4367	873 - 895	AD-60449.1	GfsasAfaGfaGfuGfUf(Cgn)uCfaUfcUfuCfuUfL96	asAfsgAfaGfaUfgAfgacAfcUfcUfuUfcsusg	22.65		80.09	0.75
4368	4369	873 - 895	AD-60455.1	GfsasAfaGfaGfuGf(Tgn)CfuCfaUfcUfuCfuUfL96	asAfsgAfaGfaUfgAfgacAfcUfcUfuUfcsusg	31.05	2.82	104.24	23.01
4370	4371	873 - 895	AD-60461.1	GfsasAfaGfaGfuGfUfCfuCfaUfcUfu(Cgn)uUfL96	asAfs(Ggn)AfaGfaUfgAfgacAfcUfcUfuUfcsusg	16.30	2.08	85.78	22.00
4372	4373	873 - 895	AD-60467.1	GfsasAfaGfaGfuGfUfCfuCfaUfcUf(Tgn)CfuUfL96	asAfsg(Agn)aGfaUfgAfgacAfcUfcUfuUfcsusg	17.77	8.38	82.16	18.92
4374	4375	873 - 895	AD-60473.1	GfsasAfaGfaGfuGfUfCfuCfaUfc(Tgn)uCfuUfL96	asAfsgAf(Agn)GfaUfgAfgacAfcUfcUfuUfcsusg	14.64	4.25	61.05	7.88
4376	4377	873 - 895	n/a	GfsasAfaGfaGfuGfUfCfuCfaUf(Cgn)UfuCfuUfL96	asAfsgAfa(Ggn)aUfgAfgacAfcUfcUfuUfcsusg				

4378	4379	873 - 895	AD-60483.1	GfsasAfaGfaGfuGfUfCfuCfa(Tgn)cUfuCfuUfL96	asAfsgAfaGf(Agn)UfgAfgacAfcUfcUfuUfcsusg	26.18	2.83	91.12	16.03
4380	4381	873 - 895	AD-60488.1	GfsasAfaGfaGfuGfUfCfuCf(Agn)UfcUfuCfuUfL96	asAfsgAfaGfa(Tgn)gAfgacAfcUfcUfuUfcsusg	24.74	0.56	87.66	7.90
4382	4383	873 - 895	AD-60450.1	GfsasAfaGfaGfuGfUfCfu(Cgn)aUfcUfuCfuUfL96	asAfsgAfaGfaUf(Ggn)AfgacAfcUfcUfuUfcsusg	37.09	2.89	111.90	1.97
4384	4385	873 - 895	AD-60456.1	GfsasAfaGfaGfuGfUfCf(Tgn)CfaUfcUfuCfuUfL96	asAfsgAfaGfaUfg(Agn)gacAfcUfcUfuUfcsusg	41.82	0.60	102.56	11.25
4386	4387	873 - 895	AD-60462.1	GfsasAfaGfaGfuGfUf(Cgn)uCfaUfcUfuCfuUfL96	asAfsgAfaGfaUfgAf(Ggn)acAfcUfcUfuUfcsusg	38.91	3.89	122.47	35.17
4388	4389	873 - 895	AD-60468.1	GfsasAfaGfaGfuGf(Tgn)CfuCfaUfcUfuCfuUfL96	asAfsgAfaGfaUfgAfg(Agn)cAfcUfcUfuUfcsusg	28.44	3.05	97.09	2.66
4390	4391	873 - 895	AD-60550.1	GfsasAfaGfaGfuGfUfCfuCfaUfcUfuCfuUfsL96	asAfsgAfaGfaUfgAfgacAfcUfcUfuUfcsusg	20.35	0.63	69.13	22.65
4392	4393	873 - 895	AD-60555.1	GfsasAfaGfaGfuGfUfCfuCfaUfscUfsuCfuUfsL96	asAfsgAfaGfaUfgAfgacAfcUfcUfuUfcsusg	19.17	8.76	68.86	17.72
4394	4395	873 - 895	AD-60560.1	GfsasAfaGfaGfuGfUfCfuCfaUfcUfuCfuUfsL96	asAfsgAfaGfaUfgAfgacAfcUfscUfsuUfscsusg	25.71	5.66	67.63	27.72
4396	4397	873 - 895	AD-60565.1	GfsasAfaGfaGfuGfUfCfuCfaUfscUfsuCfuUfsL96	asAfsgAfaGfaUfgAfgacAfcUfscUfsuUfscsusg	22.78	7.32	74.11	1.85

As is shown in the table above, in this in vitro screen, the siRNAs that provided the greatest ALAS1 mRNA suppression (greater than 80% suppression, such that less than 20% mRNA was remaining) at 10 nM concentration included AD-58632, AD-60472, AD-60423, AD-60445, AD-60423, AD-60417, AD-60466, AD-60473, AD-60434, AD-60448, AD-60460, AD-60411, AD-60481, AD-60486, and AD-60453, AD-60480, AD-60405, AD-60477, AD-60461, AD-60470, AD-60467, AD-60482, AD-60446, AD-60555, AD-60454, AD-60469 and AD-60463. Furthermore, in this in vitro screen, the siRNAs that provided the greatest ALAS1 mRNA suppression (greater than 30% suppression, such that less than 70% mRNA was remaining) at 0.1 nM concentration included AD-60423, AD-58632, AD-60434, AD-60423, AD-60466, AD-60419, AD-60438, AD-60448, AD-60460, AD-60473, AD-60411, AD-60405, AD-60472, AD-60477, AD-60417, AD-60480, AD-60482, AD-60421, AD-60560, AD-60433, AD-60481, AD-60475, AD-60555, AD-60437, AD-60550, AD-60415, AD-60463, and AD-60443.

As is shown in the table below, testing of further siRNAs revealed that the following duplexes provided greater than 80% suppression at 10 nM concentration: AD-58632, AD-60405, AD-60423, AD-60434, AD-60445, AD-60480, AD-60460, and AD-60466, and the following duplexes provided greater than 30% suppression at 0.1 nM concentration: AD-58632, AD-60405, AD-60423, AD-60434, AD-60419, AD-60480, AD-60460, and AD-60466.

Table 26: Further sequences and in vitro screen results for AD-58632 and AD-58632 derivative siRNAs

SEQ ID NO: (sense)	SEQ ID NO: (anti-sense)	Target sites of anti-sense seq on NM_000688.4	Duplex Name	Sense Sequence (5'-3')	Antisense (AS) Sequence (5'-3')	Avg 10 nM	SD 10 nM	Avg 0.1 nM	SD 0.1 nM
4398	4399	873 - 895	AD-58632.8	GfsasAfaGfaGfuGfUfCfuCfaUfcUfuCfuUfL96	asAfsgAfaGfaUfgAfgacAfcUfcUfuUfcsusg	11.8	2.7	46.7	4.2
4400	4401	873 - 895	AD-60405.1	GfsasAfaGfaGfuGfuCfuCfaucuuCfuuL96	asAfsgAfaGfaUfgAfgacAfcUfcUfuUfcsusg	15.6	4.5	63.5	10.5
4402	4403	873 - 895		GfsasAfaGfaGfuGfuCfuCfaucuuCfuuL96	asAfsGfAfaGfaugAfgAfcAfcucuuucsusg				
4404	4405	873 - 895		GfsasAfaGfaGfuGfuCfuCfaucuuCfuuL96	asAfsgAfaGfaugAfgAfcAfcucuuucsusg				
4406	4407	873 - 895		GfsasAfaGfaGfuGfuCfuCfaucuuCfuuL96	asAfsgAfaGfaugAfgacAfcucuuucsusg				
4408	4409	873 - 895	AD-60423.2	gsasaagaGfuGfuCfuCfaucuucuuL96	asAfsGfAfaGfaUfgAfgAfcAfcUfcUfuUfcsusg	13.8	3.4	55.9	7.9
4410	4411	873 - 895		gsasaagaGfuGfuCfuCfaucuucuuL96	asAfsgAfaGfaUfgAfgacAfcUfcUfuUfcsusg				
4412	4413	873 - 895		gsasaagaGfuGfuCfuCfaucuucuuL96	asAfsGfAfaGfaugAfgAfcAfcucuuucsusg				
4414	4415	873 - 895		gsasaagaGfuGfuCfuCfaucuucuuL96	asAfsgAfaGfaugAfgAfcAfcucuuucsusg				
4416	4417	873 - 895		gsasaagaGfuGfuCfuCfaucuucuuL96	asAfsgAfaGfaugAfgacAfcucuuucsusg				

4418	4419	873 - 895	AD-60434.1	gsasaagaGfuGfuCfucaucuucuuL96	asAfsGfAfaGfaUfgAfgAfcAfcUfcUfuUfcsusg	14.7	2.8	48.7	6.6
4420	4421	873 - 895		gsasaagaGfuGfuCfucaucuucuuL96	asAfsgAfaGfaUfgAfgacAfcUfcUfuUfcsusg				
4422	4423	873 - 895		gsasaagaGfuGfuCfucaucuucuuL96	asAfsGfAfaGfaugAfgAfcAfcucuuucsusg				
4424	4425	873 - 895		gsasaagaGfuGfuCfucaucuucuuL96	asAfsgAfaGfaugAfgAfcAfcucuuucsusg				
4426	4427	873 - 895		gsasaagaGfuGfuCfucaucuucuuL96	asAfsgAfaGfaugAfgacAfcucuuucsusg				
4428	4429	873 - 895	AD-60419.1	GfsasAfaGfAfGfuGfdTcucaucuucuuL96	asAfsGfAfaGfaugAfgAfcAfcucuuucsusg	20.8	15.1	57.8	8.3
4430	4431	873 - 895		GfsasAfaGfAfGfuGfdTcucaucuucuuL96	asAfsgAfaGfaUfgAfgacAfcUfcUfuUfcsusg				
4432	4433	873 - 895		GfsasAfaGfAfGfuGfdTcucaucuucuuL96	asAfsGfAfaGfaugAfgAfcAfcucuuucsusg				
4434	4435	873 - 895		GfsasAfaGfAfGfuGfdTcucaucuucuuL96	asAfsgAfaGfaugAfgAfcAfcucuuucsusg				
4436	4437	873 - 895		GfsasAfaGfAfGfuGfdTcucaucuucuuL96	asAfsgAfaGfaugAfgacAfcucuuucsusg				
4438	4439	873 - 895	AD-60445.1	GfsasAfaGfAfGfuGfucucauc(Tgn)ucuuL96	asAfsGfAfaGfaugAfgAfcAfcucuuucsusg	13.3	3.3	78.1	4.0
4440	4441	873 - 895		GfsasAfaGfAfGfuGfucucauc(Tgn)ucuuL96	asAfsgAfaGfaUfgAfgacAfcUfcUfuUfcsusg				
4442	4443	873 - 895		GfsasAfaGfAfGfuGfucucauc(Tgn)ucuuL96	asAfsGfAfaGfaugAfgAfcAfcucuuucsusg				
4444	4445	873 - 895		GfsasAfaGfAfGfuGfucucauc(Tgn)ucuuL96	asAfsgAfaGfaugAfgAfcAfcucuuucsusg				
4446	4447	873 - 895		GfsasAfaGfAfGfuGfucucauc(Tgn)ucuuL96	asAfsgAfaGfaugAfgacAfcucuuucsusg				
4448	4449	873 - 895	AD-60480.1	GfsasAfaGfaGfuGfUfCfuCfaUfcUfuCfuUfL96	asAfsgAf(Agn)GfaUfgAfgacAfcUfcUfuUfcsusg	15.5	1.2	66.4	14.3

4450	4451	873 - 895		gsasaagaGfuGfuCfuCfaucuucuuL96	asAfsgAf(Agn)GfaUfgAfgacAfcUfcUfuUfcsusg				
4452	4453	873 - 895		gsasaagaGfuGfuCfucaucuucuuL96	asAfsgAf(Agn)GfaUfgAfgacAfcUfcUfuUfcsusg				
4454	4455	873 - 895		GfsasAfaGfAfGfuGfdTcucaucuucuuL96	asAfsgAf(Agn)GfaUfgAfgacAfcUfcUfuUfcsusg				
4456	4457	873 - 895		GfsasAfaGfAfGfuGfucucauc(Tgn)ucuuL96	asAfsgAf(Agn)GfaUfgAfgacAfcUfcUfuUfcsusg				
4458	4459	873 - 895		GfsasAfaGfAfGfuGfucucaucs(Tgns)ucuuL96	asAfsgAfs(Agns)GfaUfgAfgacAfcUfcUfuUfcsusg				
4460	4461	873 - 895		GfsasAfaGfaGfuGfUfCfuCfaUfcUfuCfuUfL96	asAfsgAf(Agn)GfaugAfgacAfcucuuucsusg				
4462	4463	873 - 895		gsasaagaGfuGfuCfuCfaucuucuuL96	asAfsgAf(Agn)GfaugAfgacAfcucuuucsusg				
4464	4465	873 - 895		gsasaagaGfuGfuCfucaucuucuuL96	asAfsgAf(Agn)GfaugAfgacAfcucuuucsusg				
4466	4467	873 - 895		GfsasAfaGfAfGfuGfdTcucaucuucuuL96	asAfsgAf(Agn)GfaugAfgacAfcucuuucsusg				
4468	4469	873 - 895		GfsasAfaGfAfGfuGfucucauc(Tgn)ucuuL96	asAfsgAf(Agn)GfaugAfgacAfcucuuucsusg				
4470	4471	873 - 895		GfsasAfaGfAfGfuGfucucaucs(Tgns)ucuuL96	asAfsgAfs(Agns)GfaugAfgacAfcucuuucsusg				
4472	4473	873 - 895	AD-58632.8	GfsasAfaGfaGfuGfUfCfuCfaUfcUfuCfuUfL96	asAfsgAfaGfaUfgAfgacAfcUfcUfuUfcsusg	11.8	2.7	46.7	4.2
4474	4475	873 - 895		GfsasAfaGfaGfuGfdTCfuCfaUfcUfuCfuUfL96	asAfsgAfaGfaUfgAfgacAfcUfcUfuUfcsusg				
4476	4477	873 - 895		GfsasAfaGfaGfuGfUfCfuCfaUfcUfuCfuUfL96	asAfsgAfaGfaUfgAfgacAfcdTcUfuUfcsusg				
4478	4479	873 - 895		GfsasAfaGfaGfuGfdTCfuCfaUfcUfuCfuUfL96	asAfsgAfaGfaUfgAfgacAfcdTcUfuUfcsusg				
4480	4481	873 - 895		GfsasAfaGfaGfuGfdTCfuCfaUfcUfsuCfsuUfsL96	asAfsgAfaGfaUfgAfgacAfcdTcUfuUfcsusg				

4482	4483	873 - 895		GfsasAfaGfaGfuGfdTCfuCfaUfcUfsucsuUfsL96	asAfsGfAfaGfaUfgAfgacAfcdTcUfuUfcsusg				
4484	4485	873 - 895	AD-60460.1	GfsasAfaGfaGfuGfUfCfuCfaUfc(Tgn)uCfuUfL96	asAfsgAfaGfaUfgAfgacAfcUfcUfuUfcsusg	14.9	5.8	60.1	6.0
4486	4487	873 - 895		GfsasAfaGfaGfuGfdTCfuCfaUfc(Tgn)uCfuUfL96	asAfsgAfaGfaUfgAfgacAfcUfcUfuUfcsusg				
4488	4489	873 - 895		GfsasAfaGfaGfuGfUfCfuCfaUfc(Tgn)uCfuUfL96	asAfsgAfaGfaUfgAfgacAfcdTcUfuUfcsusg				
4490	4491	873 - 895		GfsasAfaGfaGfuGfdTCfuCfaUfc(Tgn)uCfuUfL96	asAfsgAfaGfaUfgAfgacAfcdTcUfuUfcsusg				
4492	4493	873 - 895		GfsasAfaGfaGfuGfdTCfuCfaUfc(Tgn)suCfsuUfsL96	asAfsgAfaGfaUfgAfgacAfcdTcUfuUfcsusg				
4494	4495	873 - 895		GfsasAfaGfaGfuGfdTCfuCfaUfc(Tgn)sucsuUfsL96	asAfsGfAfaGfaUfgAfgacAfcdTcUfuUfcsusg				
4496	4497	873 - 895	AD-60466.1	GfsasAfaGfaGfuGfUfCfuCfaUf(Cgn)UfuCfuUfL96	asAfsgAfaGfaUfgAfgacAfcUfcUfuUfcsusg	14.0	7.5	56.8	11.6
4498	4499	873 - 895		GfsasAfaGfaGfuGfdTCfuCfaUf(Cgn)UfuCfuUfL96	asAfsgAfaGfaUfgAfgacAfcUfcUfuUfcsusg				
4500	4501	873 - 895		GfsasAfaGfaGfuGfUfCfuCfaUf(Cgn)UfuCfuUfL96	asAfsgAfaGfaUfgAfgacAfcdTcUfuUfcsusg				
4502	4503	873 - 895		GfsasAfaGfaGfuGfdTCfuCfaUf(Cgn)UfuCfuUfL96	asAfsgAfaGfaUfgAfgacAfcdTcUfuUfcsusg				
4504	4505	873 - 895		GfsasAfaGfaGfuGfdTCfuCfaUf(Cgn)UfsuCfsuUfsL96	asAfsgAfaGfaUfgAfgacAfcdTcUfuUfcsusg				
4506	4507	873 - 895		GfsasAfaGfaGfuGfdTCfuCfaUf(Cgn)UfsucsuUfsL96	asAfsGfAfaGfaUfgAfgacAfcdTcUfuUfcsusg				

Table 27: Further sequences of AD-58632 derivative siRNAs

SEQ ID NO: (sense)	SEQ ID NO: (anti-sense)	Target sites of antisense sequence on NM_	Duplex Name	Sense Sequence (5'-3')	Antisense Sequence (5'-3')
4508	4509	873 - 895	AD-58632	GfsasAfaGfaGfuGfUfCfuCfaUfcUfuCfuUfL96	asAfsgAfaGfaUfgAfgacAfcUfcUfuUfcsusg
4510	4511	873 - 895	AD-60405.1	GfsasAfaGfaGfuGfuCfuCfaucuuCfuuL96	asAfsgAfaGfaUfgAfgacAfcUfcUfuUfcsusg
4512	4513	873 - 895	AD-60887	GfsasAfaGfaGfuGfuCfuCfaucuuCfuuL96	asAfsGfAfaGfaugAfgAfcAfcucuuucsusg
4514	4515	873 - 895	AD-60923	GfsasAfaGfaGfuGfuCfuCfaucuuCfuuL96	asAfsGfAfaGfaUfgAfgAfcAfcUfcUfuUfcsusg
4516	4517	873 - 895	AD-60434.1	gsasaagaGfuGfuCfucaucuucuuL96	asAfsGfAfaGfaUfgAfgAfcAfcUfcUfuUfcsusg
4518	4519	873 - 895	AD-60892	gsasaagaGfuGfuCfucaucuucuuL96	asAfsGfAfaGfaugAfgAfcAfcucuuucsusg
4520	4521	873 - 895	AD-60891	gsasaagaGfuGfuCfucaucuucuuL96	asAfsgAfaGfaUfgAfgacAfcUfcUfuUfcsusg
4522	4523	873 - 895	AD-60419.1	GfsasAfaGfAfGfuGfdTcucaucuucuuL96	asAfsGfAfaGfaugAfgAfcAfcucuuucsusg
4524	4525	873 - 895	AD-60924	GfsasAfaGfAfGfuGfdTcucaucuucuuL96	asAfsGfAfaGfaUfgAfgAfcAfcUfcUfuUfcsusg
4526	4527	873 - 895	AD-60885	GfsasAfaGfAfGfuGfdTcucaucuucuuL96	asAfsgAfaGfaUfgAfgacAfcUfcUfuUfcsusg
4528	4529	873 - 895	AD-60445.1	GfsasAfaGfAfGfuGfucucauc(Tgn)ucuuL96	asAfsGfAfaGfaugAfgAfcAfcucuuucsusg
4530	4531	873 - 895	AD-60925	GfsasAfaGfAfGfuGfucucauc(Tgn)ucuuL96	asAfsGfAfaGfaUfgAfgAfcAfcUfcUfuUfcsusg
4532	4533	873 - 895	AD-60890	GfsasAfaGfAfGfuGfucucauc(Tgn)ucuuL96	asAfsgAfaGfaUfgAfgacAfcUfcUfuUfcsusg
4534	4535	873 - 895	AD-60926	GfsasAfaGfaGfuGfUfCfuCfaUfcUfuCfuUfL96	asAfsGfAfaGfaUfgAfgAfcAfcUfcUfuUfcsusg

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IC50s based on in vitro activity

Similar to the experiments described above, further dose-response experiments were done at 10 nM, 1.66667 nM, 0.277778 nM, 0.046296 nM, 0.007716 nM, 0.001286 nM, 0.000214 nM, and 3.57E-05 nM final duplex concentration, and IC50 values were calculated.

Table 28: Further sequences and IC50s of AD-58632 and AD-58632 derivative siRNAs

SEQ ID NO: (sense)	SEQ ID NO: (anti-sense)	Target sites of anti-sense seq on NM_000688.4	Duplex Name	Sense Sequence (5'-3')	Antisense (AS) Sequence (5'-3')	IC50
4536	4537	873 - 895	AD-58632.10	GfsasAfaGfaGfuGfUfCfuCfaUfcUfuCfuUfL96	asAfsgAfaGfaUfgAfgacAfcUfcUfuUfcsusg	0.017
4538	4539	873 - 895	AD-60405.2	GfsasAfaGfaGfuGfuCfuCfaucuuCfuuL96	asAfsgAfaGfaUfgAfgacAfcUfcUfuUfcsusg	0.070
4540	4541	873 - 895	AD-60887.1	GfsasAfaGfaGfuGfuCfuCfaucuuCfuuL96	asAfsGfAfaGfaugAfgAfcAfcucuuucsusg	0.120
4542	4543	873 - 895	AD-60819.1	GfsasAfaGfaGfuGfuCfuCfaucuuCfuuL96	asAfsgAfaGfaugAfgAfcAfcucuuucsusg	0.009
4544	4545	873 - 895	AD-60823.1	GfsasAfaGfaGfuGfuCfuCfaucuuCfuuL96	asAfsgAfaGfaugAfgacAfcucuuucsusg	0.032
4546	4547	873 - 895	AD-60423.3	gsasaagaGfuGfuCfuCfaucuucuuL96	asAfsGfAfaGfaUfgAfgAfcAfcUfcUfuUfcsusg	0.020
4548	4549	873 - 895	AD-60889.1	gsasaagaGfuGfuCfuCfaucuucuuL96	asAfsGfAfaGfaugAfgAfcAfcucuuucsusg	0.242
4550	4551	873 - 895	AD-60827.1	gsasaagaGfuGfuCfuCfaucuucuuL96	asAfsgAfaGfaugAfgAfcAfcucuuucsusg	0.044
4552	4553	873 - 895	AD-60831.1	gsasaagaGfuGfuCfuCfaucuucuuL96	asAfsgAfaGfaugAfgacAfcucuuucsusg	0.077
4554	4555	873 - 895	AD-60434.2	gsasaagaGfuGfuCfucaucuucuuL96	asAfsGfAfaGfaUfgAfgAfcAfcUfcUfuUfcsusg	0.028
4556	4557	873 - 895	AD-60891.1	gsasaagaGfuGfuCfucaucuucuuL96	asAfsgAfaGfaUfgAfgacAfcUfcUfuUfcsusg	0.078
4558	4559	873 - 895	AD-60892.1	gsasaagaGfuGfuCfucaucuucuuL96	asAfsGfAfaGfaugAfgAfcAfcucuuucsusg	0.138
4560	4561	873 - 895	AD-60835.1	gsasaagaGfuGfuCfucaucuucuuL96	asAfsgAfaGfaugAfgAfcAfcucuuucsusg	0.015

4562	4563	873 - 895	AD-60839.1	gsasaagaGfuGfuCfucaucuucuuL96	asAfsgAfaGfaugAfgacAfcucuuucsusg	0.014
4564	4565	873 - 895	AD-60419.2	GfsasAfaGfAfGfuGfdTcucaucuucuuL96	asAfsGfAfaGfaugAfgAfcAfcucuuucsusg	0.014
4566	4567	873 - 895	AD-60885.1	GfsasAfaGfAfGfuGfdTcucaucuucuuL96	asAfsgAfaGfaUfgAfgacAfcUfcUfuUfcsusg	0.091
4568	4569	873 - 895	AD-60419.3	GfsasAfaGfAfGfuGfdTcucaucuucuuL96	asAfsGfAfaGfaugAfgAfcAfcucuuucsusg	0.026
4570	4571	873 - 895	AD-60843.1	GfsasAfaGfAfGfuGfdTcucaucuucuuL96	asAfsgAfaGfaugAfgAfcAfcucuuucsusg	0.004
4572	4573	873 - 895	AD-60847.1	GfsasAfaGfAfGfuGfdTcucaucuucuuL96	asAfsgAfaGfaugAfgacAfcucuuucsusg	0.012
4574	4575	873 - 895	AD-60445.2	GfsasAfaGfAfGfuGfucucauc(Tgn)ucuuL96	asAfsGfAfaGfaugAfgAfcAfcucuuucsusg	0.077
4576	4577	873 - 895	AD-60890.1	GfsasAfaGfAfGfuGfucucauc(Tgn)ucuuL96	asAfsgAfaGfaUfgAfgacAfcUfcUfuUfcsusg	1.201
4578	4579	873 - 895	AD-60445.3	GfsasAfaGfAfGfuGfucucauc(Tgn)ucuuL96	asAfsGfAfaGfaugAfgAfcAfcucuuucsusg	0.302
4580	4581	873 - 895	AD-60820.1	GfsasAfaGfAfGfuGfucucauc(Tgn)ucuuL96	asAfsgAfaGfaugAfgAfcAfcucuuucsusg	0.006
4582	4583	873 - 895	AD-60824.1	GfsasAfaGfAfGfuGfucucauc(Tgn)ucuuL96	asAfsgAfaGfaugAfgacAfcucuuucsusg	0.032
4584	4585	873 - 895	AD-60480.2	GfsasAfaGfaGfuGfUfCfuCfaUfcUfuCfuUfL96	asAfsgAf(Agn)GfaUfgAfgacAfcUfcUfuUfcsusg	0.066
4586	4587	873 - 895	AD-60893.1	gsasaagaGfuGfuCfuCfaucuucuuL96	asAfsgAf(Agn)GfaUfgAfgacAfcUfcUfuUfcsusg	0.034
4588	4589	873 - 895	AD-60884.1	gsasaagaGfuGfuCfucaucuucuuL96	asAfsgAf(Agn)GfaUfgAfgacAfcUfcUfuUfcsusg	0.157
4590	4591	873 - 895	AD-60886.1	GfsasAfaGfAfGfuGfdTcucaucuucuuL96	asAfsgAf(Agn)GfaUfgAfgacAfcUfcUfuUfcsusg	0.801
4592	4593	873 - 895	AD-60888.1	GfsasAfaGfAfGfuGfucucauc(Tgn)ucuuL96	asAfsgAf(Agn)GfaUfgAfgacAfcUfcUfuUfcsusg	0.201
4594	4595	873 - 895	AD-60828.1	GfsasAfaGfAfGfuGfucucaucs(Tgns)ucuuL96	asAfsgAfs(Agns)GfaUfgAfgacAfcUfcUfuUfcsusg	0.145
4596	4597	873 - 895	AD-60832.1	GfsasAfaGfaGfuGfUfCfuCfaUfcUfuCfuUfL96	asAfsgAf(Agn)GfaugAfgacAfcucuuucsusg	0.036
4598	4599	873 - 895	AD-60836.1	gsasaagaGfuGfuCfuCfaucuucuuL96	asAfsgAf(Agn)GfaugAfgacAfcucuuucsusg	0.076
4600	4601	873 - 895	AD-60840.1	gsasaagaGfuGfuCfucaucuucuuL96	asAfsgAf(Agn)GfaugAfgacAfcucuuucsusg	0.033
4602	4603	873 - 895	AD-60844.1	GfsasAfaGfAfGfuGfdTcucaucuucuuL96	asAfsgAf(Agn)GfaugAfgacAfcucuuucsusg	0.017
4604	4605	873 - 895	AD-60848.1	GfsasAfaGfAfGfuGfucucauc(Tgn)ucuuL96	asAfsgAf(Agn)GfaugAfgacAfcucuuucsusg	0.007

4606	4607	873 - 895	AD-60821.1	GfsasAfaGfAfGfuGfucucaucs(Tgns)ucuuL96	asAfsgAfs(Agns)GfaugAfgacAfcucuuucsusg	0.076
4608	4609	873 - 895	AD-58632.11	GfsasAfaGfaGfuGfUfCfuCfaUfcUfuCfuUfL96	asAfsgAfaGfaUfgAfgacAfcUfcUfuUfcsusg	0.063
4610	4611	873 - 895	AD-60825.1	GfsasAfaGfaGfuGfdTCfuCfaUfcUfuCfuUfL96	asAfsgAfaGfaUfgAfgacAfcUfcUfuUfcsusg	0.031
4612	4613	873 - 895	AD-60829.1	GfsasAfaGfaGfuGfUfCfuCfaUfcUfuCfuUfL96	asAfsgAfaGfaUfgAfgacAfcdTcUfuUfcsusg	0.033
4614	4615	873 - 895	AD-60833.1	GfsasAfaGfaGfuGfdTCfuCfaUfcUfuCfuUfL96	asAfsgAfaGfaUfgAfgacAfcdTcUfuUfcsusg	0.100
4616	4617	873 - 895	AD-60837.1	GfsasAfaGfaGfuGfdTCfuCfaUfcUfsuCfsuUfsL96	asAfsgAfaGfaUfgAfgacAfcdTcUfuUfcsusg	0.031
4618	4619	873 - 895	AD-60841.1	GfsasAfaGfaGfuGfdTCfuCfaUfcUfsucsuUfsL96	asAfsGfAfaGfaUfgAfgacAfcdTcUfuUfcsusg	0.010
4620	4621	873 - 895	AD-60460.2	GfsasAfaGfaGfuGfUfCfuCfaUfc(Tgn)uCfuUfL96	asAfsgAfaGfaUfgAfgacAfcUfcUfuUfcsusg	0.009
4622	4623	873 - 895	AD-60845.1	GfsasAfaGfaGfuGfdTCfuCfaUfc(Tgn)uCfuUfL96	asAfsgAfaGfaUfgAfgacAfcUfcUfuUfcsusg	0.002
4624	4625	873 - 895	AD-60849.1	GfsasAfaGfaGfuGfUfCfuCfaUfc(Tgn)uCfuUfL96	asAfsgAfaGfaUfgAfgacAfcdTcUfuUfcsusg	0.005
4626	4627	873 - 895	AD-60822.1	GfsasAfaGfaGfuGfdTCfuCfaUfc(Tgn)uCfuUfL96	asAfsgAfaGfaUfgAfgacAfcdTcUfuUfcsusg	0.007
4628	4629	873 - 895	AD-60826.1	GfsasAfaGfaGfuGfdTCfuCfaUfc(Tgn)suCfsuUfsL96	asAfsgAfaGfaUfgAfgacAfcdTcUfuUfcsusg	0.009
4630	4631	873 - 895	AD-60830.1	GfsasAfaGfaGfuGfdTCfuCfaUfc(Tgn)sucsuUfsL96	asAfsGfAfaGfaUfgAfgacAfcdTcUfuUfcsusg	0.019
4632	4633	873 - 895	AD-60466.2	GfsasAfaGfaGfuGfUfCfuCfaUf(Cgn)UfuCfuUfL96	asAfsgAfaGfaUfgAfgacAfcUfcUfuUfcsusg	0.066
4634	4635	873 - 895	AD-60834.1	GfsasAfaGfaGfuGfdTCfuCfaUf(Cgn)UfuCfuUfL96	asAfsgAfaGfaUfgAfgacAfcUfcUfuUfcsusg	0.024
4636	4637	873 - 895	AD-60838.1	GfsasAfaGfaGfuGfUfCfuCfaUf(Cgn)UfuCfuUfL96	asAfsgAfaGfaUfgAfgacAfcdTcUfuUfcsusg	0.013
4638	4639	873 - 895	AD-60842.1	GfsasAfaGfaGfuGfdTCfuCfaUf(Cgn)UfuCfuUfL96	asAfsgAfaGfaUfgAfgacAfcdTcUfuUfcsusg	0.010
4640	4641	873 - 895	AD-60846.1	GfsasAfaGfaGfuGfdTCfuCfaUf(Cgn)UfsuCfsuUfsL96	asAfsgAfaGfaUfgAfgacAfcdTcUfuUfcsusg	0.011
4642	4643	873 - 895	AD-60850.1	GfsasAfaGfaGfuGfdTCfuCfaUf(Cgn)UfsucsuUfsL96	asAfsGfAfaGfaUfgAfgacAfcdTcUfuUfcsusg	0.018

As is shown in Table 28, the following duplexes had an IC50 of less than 0.01nM: AD-60845 , AD-60843, AD-60849, AD-60820, AD-60848, AD-60822, AD-60826, AD-60819, and AD-60460.

The following duplexes had an IC50 of less than 0.02 nM: AD-60845 , AD-60843, AD-60849, AD-60820, AD-60848, AD-60822, AD-60826, AD-60819, and AD-60460, AD-60841, AD-60842, AD-60846, AD-60847, AD-60838, AD-60419, AD-60839, AD-60835, AD-586320, AD-60844, AD-60850, and AD-60830.

The following duplexes had an IC50 of less than 0.05 nM: AD-60845 , AD-60843, AD-60849, AD-60820, AD-60848, AD-60822, AD-60826, AD-60819, and AD-60460, AD-60841, AD-60842, AD-60846, AD-60847, AD-60838, AD-60419, AD-60839, AD-60835, AD-586320, AD-60844, AD-60850, AD-60830, AD-60423, AD-60834, AD-60419, AD-60434, AD-60825, AD-60837, AD-60823, AD-60824, AD-60840, AD-60829, AD-60893, AD-60832, and AD-60827.

Example 25: In vivo structure activity relationship studies of AD-58632

Derivatives of the AD-58632 parent siRNA were generated and screened in vivo in rats. The sequences of siRNAs that were screened are provided in the table below.

Table 29: Sequences of ALAS1 siRNA Duplexes

SEQ ID NO: (sense)	SEQ ID NO: (anti-sense)	Target sites of antisense sequence on NM_000688.4	Duplex Name	Sense Sequence (5'-3')	Antisense Sequence (5'-3')
4644	4645	873 - 895	AD-58632	GfsasAfaGfaGfuGfUfCfuCfaUfcUfuCfuUfL96	asAfsgAfaGfaUfgAfgacAfcUfcUfuUfcsusg
4646	4647	873 - 895	AD-60405	GfsasAfaGfaGfuGfuCfuCfaucuuCfuuL96	asAfsgAfaGfaUfgAfgacAfcUfcUfuUfcsusg

4648	4649	873 - 895	AD-60887	GfsasAfaGfaGfuGfuCfuCfaucuuCfuuL96	asAfsGfAfaGfaugAfgAfcAfcucuuucsusg
4650	4651	873 - 895	AD-60923	gsasaagaGfuGfuCfucaucuucuuL96	asAfsGfAfaGfaUfgAfgAfcAfcUfcUfuUfcsusg
4652	4653	873 - 895	AD-60434	gsasaagaGfuGfuCfucaucuucuuL96	asAfsGfAfaGfaUfgAfgAfcAfcUfcUfuUfcsusg
4654	4655	873 - 895	AD-60892	gsasaagaGfuGfuCfucaucuucuuL96	asAfsGfAfaGfaugAfgAfcAfcucuuucsusg
4656	4657	873 - 895	AD-60419	GfsasAfaGfAfGfuGfdTcucaucuucuuL96	asAfsGfAfaGfaugAfgAfcAfcucuuucsusg
4658	4659	873 - 895	AD-60924	GfsasAfaGfAfGfuGfdTcucaucuucuuL96	asAfsGfAfaGfaUfgAfgAfcAfcUfcUfuUfcsusg
4660	4661	873 - 895	AD-60445	GfsasAfaGfAfGfuGfucucauc(Tgn)ucuuL96	asAfsGfAfaGfaugAfgAfcAfcucuuucsusg
4662	4663	873 - 895	AD-60925	GfsasAfaGfAfGfuGfucucauc(Tgn)ucuuL96	asAfsGfAfaGfaUfgAfgAfcAfcUfcUfuUfcsusg
4664	4665	873 - 895	AD-60926	GfsasAfaGfaGfuGfUfCfuCfaUfcUfuCfuUfL96	asAfsGfAfaGfaUfgAfgAfcAfcUfcUfuUfcsusg

A single dose of 5 mg/kg of siRNA was administered. At 5 days following administration of the siRNA, mRNA measurements of rat ALAS1 (rALAS1) mRNA and rat GAPDH (rGAPDH) mRNA were made using bDNA assay, and tissue levels of drug were determined using qPCR. The results are provided in FIG. 33 and FIG. 34. As is shown in FIG. 33, at least ten duplexes (AD-60405, AD-60887, AD-60923, AD-60434, AD-60892, AD-60419, AD-60924, AD-60445, AD-60925, and AD-60926) that were screened showed improved

suppression of ALAS1 mRNA compared with AD-58632. Furthermore, as is shown FIG. 34, these duplexes (with the exception of AD-60926) achieved higher liver concentrations than did AD-58632.

Example 26: Efficacy of AD-60925 and AD-60926 in a rat AIP model

The therapeutic efficacy of AD-60925 and AD-60926 (described in the previous example) was investigated in a rat AIP model. The experimental design is shown in the top of FIG. 35. Rats were treated with PBS or 3 mg/kg ALAS1-GalNAc siRNA t.i.w., Phenobarbital (PB), and a PBGD siRNA in an AF11 LNP formulation (AF11-PBGD) at the times indicated in FIG. 35. Control rats received the PBGD siRNA only, without Phenobarbital induction.

The results are shown in FIG. 35, FIG. 36 and FIG. 37. Administering Phenobarbital induced ALAS1 mRNA expression and increased levels of PBG and ALA in urine, compared with the control. Treatment with a total of eight doses of 3 mg/kg of AD-60925 or AD-60926 three times per week suppressed ALAS1 mRNA (FIG. 35), urine PBG (FIG. 36 and FIG. 37, top), and urine ALA (FIG. 36 and FIG. 37, bottom) Phenobarbital induced increases in ALAS1 mRNA, urine PBG, and ALA. The time course of treatment effects is shown in FIG. 37. The arrows indicate the timepoints when PB was administered. The siRNA treatment prevented phenobarbital induced increases in in ALAS1 mRNA, urine PBG, and ALA.

Both AD-60925 and AD-60926 showed therapeutic efficacy treatment of AIP. AD-60925 was even more effective than AD-60926 in suppressing ALAS1 mRNA, urine ALA, and urine PBG.

Example 27: Further in vivo structure activity relationship studies of AD-58632

Derivatives of the AD-58632 parent siRNA were generated and screened in vivo in rats.

In vivo screen, part I

The sequences of siRNAs that were screened are provided in the table below.

Table 30: Sequences of ALAS1 siRNA Duplexes

SEQ ID NO: (sense)	SEQ ID NO: (anti-sense)	Target sites of antisense sequence on NM_000688.4	Duplex Name	Sense Sequence (5'-3')	Antisense Sequence (5'-3')
4666	4667	873 – 895	AD-58632	GfsasAfaGfaGfuGfUfCfuCfaUfcUfuCfuUfL96	asAfsgAfaGfaUfgAfgacAfcUfcUfuUfcsusg
4668	4669	873 - 895	AD-60820	GfsasAfaGfAfGfuGfucucauc(Tgn)ucuuL96	asAfsgAfaGfaugAfgAfcAfcucuuucsusg
4670	4671	873 - 895	AD-60824	GfsasAfaGfAfGfuGfucucauc(Tgn)ucuuL96	asAfsgAfaGfaugAfgacAfcucuuucsusg
4672	4673	873 - 895	AD-61137	GfsasAfaGfAfGfuGfucucauc(Tgn)ucuuL96	asAfsgAfaGfaUfgAfgAfcAfcUfcUfuUfcsusg
4674	4675	873 - 895	AD-60843	GfsasAfaGfAfGfuGfdTcucaucuucuuL96	asAfsgAfaGfaugAfgAfcAfcucuuucsusg
4676	4677	873 - 895	AD-60847	GfsasAfaGfAfGfuGfdTcucaucuucuuL96	asAfsgAfaGfaugAfgacAfcucuuucsusg
4678	4679	873 - 895	AD-61138	GfsasAfaGfAfGfuGfdTcucaucuucuuL96	asAfsgAfaGfaUfgAfgAfcAfcUfcUfuUfcsusg
4680	4681	873 - 895	AD-60819	GfsasAfaGfaGfuGfuCfuCfaucuuCfuuL96	asAfsgAfaGfaugAfgAfcAfcucuuucsusg

4682	4683	873 - 895	AD-60823	GfsasAfaGfaGfuGfuCfuCfaucuuCfuuL96	asAfsgAfaGfaugAfgacAfcucuuucsusg
4684	4685	873 - 895	AD-61139	GfsasAfaGfaGfuGfuCfuCfaucuuCfuuL96	asAfsgAfaGfaUfgAfgAfcAfcUfcUfuUfcsusg
4686	4687	873 - 895	AD-61140	GfsasAfaGfaGfuGfdTCfuCfaUfc(Tgn)uCfuUfL96	asAfsgAfaGfaugAfgAfcAfcucuuucsusg

Rats were administered four doses of 2.5 mg/kg of siRNA biweekly (two times per week) for two weeks. At 72 hours following administration of the last dose of siRNA, the animals were sacrificed and measurements of rat ALAS1 (rALAS1) mRNA and rat GAPDH (rGAPDH) mRNA levels were made using bDNA assay.

As is shown in FIG. 38, at least four of the siRNAs (AD-60820, AD-60843, AD-60819, and AD-61140) that were tested showed improved suppression of ALAS1 mRNA compared with AD-58632.

In vivo screen, part II

The sequences of the siRNAs that were screened are provided in the table below.

Table 31 Sequences of ALAS1 siRNA Duplexes

SEQ ID NO: (sense)	SEQ ID NO: (anti-sense)	Target sites of antisense sequence on NM_000688.4	Duplex Name	Sense Sequence (5'-3')	Antisense Sequence (5'-3')
4688	4689	873 - 895	AD-58632	GfsasAfaGfaGfuGfUfCfuCfaUfcUfuCfuUfL96	asAfsgAfaGfaUfgAfgacAfcUfcUfuUfcsusg

4690	4691	873 - 895	AD-61141.2	GfsasAfaGfaGfuGfdTCfuCfaUfc(Tgn)uCfuUfL96	asAfsgAfaGfaugAfgacAfcucuuucsusg
4692	4693	873 - 895	AD-61142.2	GfsasAfaGfaGfuGfdTCfuCfaUfc(Tgn)uCfuUfL96	asAfsgAfaGfaUfgAfgAfcAfcUfcUfuUfcsusg
4694	4695	873 - 895	AD-60835	gsasaagaGfuGfuCfucaucuucuuL96	asAfsgAfaGfaugAfgAfcAfcucuuucsusg
4696	4697	873 - 895	AD-60839	gsasaagaGfuGfuCfucaucuucuuL96	asAfsgAfaGfaugAfgacAfcucuuucsusg
4698	4699	873 - 895	AD-61143.2	gsasaagaGfuGfuCfucaucuucuuL96	asAfsgAfaGfaUfgAfgAfcAfcUfcUfuUfcsusg
4700	4701	873 - 895	AD-61144.1	gsasaagaGfuGfdTCfucaucuucuuL96	asAfsgAfaGfaugAfgAfcAfcucuuucsusg
4702	4703	873 - 895	AD-61145.1	gsasaagaGfuGfdTCfucaucuucuuL96	asAfsgAfaGfaugAfgacAfcucuuucsusg
4704	4705	873 - 895	AD-61146.1	gsasaagaGfuGfdTCfucaucuucuuL96	asAfsgAfaGfaUfgAfgAfcAfcUfcUfuUfcsusg

Rats were administered a single dose of 2.5 mg/kg of siRNA. At 72 hours following administration of the siRNA, mRNA measurements of rat ALAS1 (rALAS1) mRNA and rat GAPDH (rGAPDH) mRNA were made using bDNA assay.

As is shown in FIG. 39, the siRNAs AD-61141, AD-61142, AD-60835, AD-60839, AD-61143, AD-61144, AD-61145, and AD-61146 showed improved suppression of ALAS1 mRNA compared with AD-58632. The siRNA that provided the greatest suppression in this experiment was AD-60835.

Example 28: In vitro structure activity relationship studies of AD-60489

AD-60489 and siRNA derivatives of AD-60489 were generated, and some siRNAs were screened *in vitro* for activity. The *in vitro* activity of the siRNAs in suppressing ALAS1 mRNA was tested as described in Example 24. Sequences of siRNAs and results of in vitro testing are provided in the tables below.

Table 32: Sequences and in vitro screen results for AD-60489 and AD-60489 derivative siRNAs

SEQ ID NO: (sense)	SEQ ID NO: (anti-sense)	Target sites of anti-sense seq on NM_000688.4	Duplex Name*	Sense Sequence (5'-3')	Antisense (AS) Sequence (5'-3')	Avg 10 nM	SD 10 nM	Avg 0.1 nM	SD 0.1 nM
4706	4707	871 - 893	AD-60489.1	CfsasGfaAfaGfaGfUfGfuCfuCfaUfcUfuAfL96	usAfsaGfaUfgAfgAfcacUfcUfuUfcUfgsgsu	17.02	0.18	50.65	5.11
4708	4709	871 - 893	AD-60495.1	CfsasGfaAfaGfaGfuGfuCfuCfaucuuAfL96	usAfsaGfaUfgAfgAfcacUfcUfuUfcUfgsgsu	17.13	5.94	63.58	18.61
4710	4711	871 - 893	AD-60501.1	CfsasGfaAfaGfaGfuGfuCfuCfaucuuAfL96	usAfsAfGfaUfgAfgAfcAfcUfcUfuUfcUfgsgsu	11.90	0.66	45.19	8.93
4712	4713	871 - 893	AD-60507.1	CfsasgaaaGfaGfuGfuCfuCfaucuuAfL96	usAfsAfGfaUfgAfgAfcAfcUfcUfuUfcUfgsgsu	14.63	7.85	48.50	19.55
4714	4715	871 - 893	AD-60513.1	CfsasgaaaGfaGfuGfuCfuCfaucuuaL96	usAfsAfGfaUfgAfgAfcAfcUfcUfuUfcUfgsgsu	14.39	2.44	51.03	7.01
4716	4717	871 - 893	AD-60519.1	csasgaaaGfaGfuGfuCfuCfaucuuaL96	usAfsAfGfaUfgAfgAfcAfcUfcUfuUfcUfgsgsu	14.67	4.88	51.70	7.72
4718	4719	871 - 893	AD-60525.1	csasgaaaGfaGfuguCfuCfaucuuaL96	usAfsAfGfaUfgAfgAfcAfcUfcUfuUfcUfgsgsu	45.24	18.16	105.09	14.17
4720	4721	871 - 893	AD-60531.1	csasgaaaGfaGfugucuCfaucuuaL96	usAfsAfGfaUfgAfgAfcAfcUfcUfuUfcUfgsgsu	62.19	12.47	107.18	1.37
4722	4723	871 - 893	AD-60490.1	csasgaaaGfaGfugucucaucuuaL96	usAfsAfGfaUfgAfgAfcAfcUfcUfuUfcUfgsgsu	39.06	10.92	82.70	10.67
4724	4725	871 - 893	AD-60496.1	csasgaaaGfaGfugucucaucuuaL96	usAfsAfGfaUfgAfgAfcAfcUfcUfuUfcugsgsu	48.90	1.85	71.55	5.47
4726	4727	871 - 893	AD-60502.1	csasgaaaGfaGfugucucaucuuaL96	usAfsAfGfaUfgAfgAfcAfcUfcUfuucugsgsu	51.83	12.62	81.85	4.25
4728	4729	871 - 893	AD-60508.1	csasgaaaGfaGfugucucaucuuaL96	usAfsAfGfaUfgAfgAfcAfcUfcuuucugsgsu	65.34	21.75	89.23	11.08

4730	4731	871 - 893	AD-60514.1	csasgaaaGfaGfugucucaucuuaL96	usAfsAfGfaUfgAfgAfcAfcucuuucugsgsu	97.13	3.47	102.94	7.36
4732	4733	871 - 893	AD-60520.1	csasgaaaGfaGfugucucaucuuaL96	usAfsAfGfaugAfgAfcAfcucuuucugsgsu	104.97	0.93	126.75	20.47
4734	4735	871 - 893	AD-60526.1	csasgaaaGfAfGfugucucaucuuaL96	usAfsAfGfaugAfgAfcAfcucuuucugsgsu	61.48	15.44	81.28	7.99
4736	4737	871 - 893	AD-60532.1	csasgaaaGfAfGfugucucaucuuaL96	usAfsAfGfaugAfgAfcAfcdTcuuucugsgsu	83.47	13.36	94.13	8.11
4738	4739	871 - 893	AD-60491.1	csasgaAfaGfAfGfugucucaucuuaL96	usAfsAfGfaugAfgAfcAfcdTcuuucugsgsu	109.05	0.43	97.85	9.77
4740	4741	871 - 893	AD-60497.1	CfsasgaAfaGfAfGfugucucaucuuaL96	usAfsAfGfaugAfgAfcAfcdTcuuucugsgsu	73.81	10.17	95.01	5.40
4742	4743	871 - 893	AD-60503.1	csasgaaaGfAfGfudGucucaucuuaL96	usAfsAfGfaugAfgAfcAfcdTcuuucugsgsu	75.30	11.29	96.03	6.30
4744	4745	871 - 893	AD-60509.1	csasgaaaGfAfGfugudCucaucuuaL96	usAfsAfGfaugAfgAfcAfcdTcuuucugsgsu	71.37	24.41	104.36	18.62
4746	4747	871 - 893	AD-60515.1	csasgaaaGfAfGfugucudCaucuuaL96	usAfsAfGfaugAfgAfcAfcdTcuuucugsgsu	64.16	13.95	98.80	0.92
4748	4749	871 - 893	AD-60521.1	csasgaaaGfAfGfugucucadTcuuaL96	usAfsAfGfaugAfgAfcAfcdTcuuucugsgsu	73.99	36.39	99.96	7.29
4750	4751	871 - 893	AD-60527.1	csasgaaaGfAfGfugucucaucdTuaL96	usAfsAfGfaugAfgAfcAfcdTcuuucugsgsu	82.69	26.56	114.13	4.81
4752	4753	871 - 893	AD-60533.1	csasgaaaGfAfGfudGudCucaucuuaL96	usAfsAfGfaugAfgAfcAfcdTcuuucugsgsu	89.41	4.69	107.40	9.67
4754	4755	871 - 893	AD-60492.1	csasgaaaGfAfGfudGucudCaucuuaL96	usAfsAfGfaugAfgAfcAfcdTcuuucugsgsu	83.52	1.56	100.70	0.79
4756	4757	871 - 893	AD-60498.1	csasgaaaGfAfGfudGucucadTcuuaL96	usAfsAfGfaugAfgAfcAfcdTcuuucugsgsu	64.64	16.21	98.41	19.60
4758	4759	871 - 893	AD-60504.1	csasgaaaGfAfGfudGudCucadTcuuaL96	usAfsAfGfaugAfgAfcAfcdTcuuucugsgsu	57.55	5.13	93.14	0.41
4760	4761	871 - 893	AD-60510.1	csasgaaaGfAfGfudGudCudCadTcuuaL96	usAfsAfGfaugAfgAfcAfcdTcuuucugsgsu	58.88	25.48	91.39	2.06

4762	4763	871 - 893	AD-60516.1	csasgaaaGfAfGfugucucaucuuaL96	usAfsAfGfadTgAfgAfcAfcdTcuuucugsgsu	53.24	6.56	84.40	2.73
4764	4765	871 - 893	AD-60522.1	csasgaAfaGfAfGfugucucaucuuaL96	usAfsAfGfadTgAfgAfcAfcdTcuuucugsgsu	67.17	6.48	91.62	5.43
4766	4767	871 - 893	AD-60528.1	CfsasgaAfaGfAfGfugucucaucuuaL96	usAfsAfGfadTgAfgAfcAfcdTcuuucugsgsu	81.44	37.04	89.71	8.60
4768	4769	871 - 893	AD-60534.1	csasgaaaGfAfGfugucucaucuuaL96	usAfsAfGfaugAfgAfcAfcdTcdTuucugsgsu	62.63	20.55	99.62	7.37
4770	4771	871 - 893	AD-60493.1	csasgaaaGfAfGfugucucaucuuaL96	usAfsAfGfaugAfgAfcAfcdTcuudTcugsgsu	107.18	0.11	87.98	2.41
4772	4773	871 - 893	AD-60499.1	csasgaaaGfAfGfugucucaucuuaL96	usAfsAfGfadTgAfgAfcAfcdTcuudTcugsgsu	35.92	13.78	77.52	6.68
4774	4775	871 - 893	AD-60505.1	csasgaaaGfAfGfugucucaucuuaL96	usAfsAfGfaugAfgAfcAfc(Tgn)cuuucugsgsu	66.77	16.45	109.82	2.48
4776	4777	871 - 893	AD-60511.1	csasgaAfaGfAfGfugucucaucuuaL96	usAfsAfGfaugAfgAfcAfc(Tgn)cuuucugsgsu	82.10	10.11	95.50	14.73
4778	4779	871 - 893	AD-60517.1	CfsasgaAfaGfAfGfugucucaucuuaL96	usAfsAfGfaugAfgAfcAfc(Tgn)cuuucugsgsu	80.83	29.69	93.57	18.41
4780	4781	871 - 893	AD-60523.1	GfsasGfaAfaGfaGfUfGfuCfuCfaUfcUfuAfL96	usAfsaGfaUfgAfgAfcacUfcUfuUfcUfcscsa	29.60	1.64	67.16	8.40
4782	4783	871 - 893	AD-60529.1	GfsusGfaAfaGfaGfUfGfuCfuCfaUfcUfuAfL96	usAfsaGfaUfgAfgAfcacUfcUfuUfcAfcscsa	21.15	1.21	79.02	28.99
4784	4785	871 - 893	AD-60535.1	GfsusCfaAfaGfaGfUfGfuCfuCfaUfcUfuAfL96	usAfsaGfaUfgAfgAfcacUfcUfuUfgAfcscsa	31.78	13.54	79.36	26.56
4786	4787	871 - 893	AD-60494.1	GfsusCfaAfaGfaGfUfGfuCfuCfaUfcUfuAfL96	usAfsaGfaUfgAfgAfcacUfcUfuUfgAfcsgsu	30.14	3.42	81.46	12.88
4788	4789	871 - 893	AD-60500.1	CfsusCfuAfaGfaGfUfGfuCfuCfaUfcUfuAfL96	usAfsaGfaUfgAfgAfcacUfcUfuAfgAfgsgsu	29.85	11.78	72.14	4.56
4790	4791	871 - 893	AD-60506.1	CfsusCfuUfaGfaGfUfGfuCfuCfaUfcUfuAfL96	usAfsaGfaUfgAfgAfcacUfcUfaAfgUfgsgsu	19.06	1.98	88.93	11.13
4792	4793	871 - 893	AD-60512.1	CfsusGfuUfuGfaGfUfGfuCfuCfaUfcUfuAfL96	usAfsaGfaUfgAfgAfcacUfcAfaAfcUfgsgsu	29.71	1.79	86.32	17.69

4794	4795	871 - 893	AD-60518.1	CfsasGfaAfaGfaGfUfGfuCfuCfaUfcUfuAfL96	(Tgns)AfsaGfaUfgAfgAfcacUfcUfuUfcUfgsgsu	27.47	2.59	81.03	10.93
4796	4797	871 - 893	AD-60524.1	CfsasGfaAfaGfaGfUfGfuCfuCfaUfcUfuAfL96	usAfs(Agn)GfaUfgAfgAfcacUfcUfuUfcUfgsgsu	17.02	1.39	71.65	17.42
4798	4799	871 - 893	n/a	CfsasGfaAfaGfaGfUfGfuCfuCfaUfcUfuAfL96	usAfsa(Ggn)aUfgAfgAfcacUfcUfuUfcUfgsgsu				
4800	4801	871 - 893	AD-60536.1	CfsasGfaAfaGfaGfUfGfuCfuCfaUfcUfuAfL96	usAfsaGf(Agn)aUfgAfgAfcacUfcUfuUfcUfgsgsu	61.77	10.19	91.55	2.20
4802	4803	871 - 893	AD-60541.1	CfsasGfaAfaGfaGfUfGfuCfuCfaUfcUfuAfL96	usAfsaGfa(Tgn)gAfgAfcacUfcUfuUfcUfgsgsu	25.47	2.83	54.36	15.02
4804	4805	871 - 893	AD-60546.1	CfsasGfaAfaGfaGfUfGfuCfuCfaUfcUfuAfL96	usAfsaGfaUf(Ggn)AfgAfcacUfcUfuUfcUfgsgsu	49.32	1.50	90.79	11.36
4806	4807	871 - 893	AD-60551.1	CfsasGfaAfaGfaGfUfGfuCfuCfaUfcUfuAfL96	usAfsaGfaUfg(Agn)gAfcacUfcUfuUfcUfgsgsu	24.37	1.11	76.80	24.99
4808	4809	871 - 893	AD-60556.1	CfsasGfaAfaGfaGfUfGfuCfuCfaUfcUfuAfL96	usAfsaGfaUfgAf(Ggn)AfcacUfcUfuUfcUfgsgsu	21.43	4.71	61.90	5.64
4810	4811	871 - 893	AD-60561.1	CfsasGfaAfaGfaGfUfGfuCfuCfaUfcUfuAfL96	usAfsaGfaUfgAfg(Agn)cacUfcUfuUfcUfgsgsu	28.25	6.41	71.84	27.01
4812	4813	871 - 893	AD-60566.1	CfsasGfaAfaGfaGfUfGfuCfuCfaUfcUfuAfL96	usAfsaGfaUfgAfgAf(Cgn)acUfcUfuUfcUfgsgsu	27.57	4.87	67.91	18.28
4814	4815	871 - 893	AD-60570.1	CfsasGfaAfaGfaGfUfGfuCfuCfaUfcUfuAfL96	usAfsaGfaUfgAfgAfc(Agn)cUfcUfuUfcUfgsgsu	24.11	0.04	58.75	21.02
4816	4817	871 - 893	AD-60583.1	CfsasGfaAfaGfaGfUfGfuCfuCfaUfcUfu(Agn)L96	asAfsgAfaGfaUfgAfgacAfcUfcUfuUfcsusg	15.32	4.78	42.01	9.51
4818	4819	871 - 893	AD-60585.1	CfsasGfaAfaGfaGfUfGfuCfuCfaUfcUf(Tgn)AfL96	asAfsgAfaGfaUfgAfgacAfcUfcUfuUfcsusg	21.15	4.14	49.54	13.34
4820	4821	871 - 893	AD-60587.1	CfsasGfaAfaGfaGfUfGfuCfuCfaUfc(Tgn)uAfL96	asAfsgAfaGfaUfgAfgacAfcUfcUfuUfcsusg	14.66	1.73	41.47	13.20
4822	4823	871 - 893	AD-60589.1	CfsasGfaAfaGfaGfUfGfuCfuCfaUf(Cgn)UfuAfL96	asAfsgAfaGfaUfgAfgacAfcUfcUfuUfcsusg	20.77	5.12	43.97	15.63
4824	4825	871 - 893	AD-60591.1	CfsasGfaAfaGfaGfUfGfuCfuCfa(Tgn)cUfuAfL96	asAfsgAfaGfaUfgAfgacAfcUfcUfuUfcsusg	13.66	0.05	36.42	16.94

4826	4827	871 - 893	AD-60592.1	CfsasGfaAfaGfaGfUfGfuCfuCf(Agn)UfcUfuAfL96	asAfsgAfaGfaUfgAfgacAfcUfcUfuUfcsusg	13.35	4.11	35.43	11.36
4828	4829	871 - 893	AD-60593.1	CfsasGfaAfaGfaGfUfGfuCfu(Cgn)aUfcUfuAfL96	asAfsgAfaGfaUfgAfgacAfcUfcUfuUfcsusg	15.13	0.28	39.99	19.39
4830	4831	871 - 893	AD-60582.1	CfsasGfaAfaGfaGfUfGfuCf(Tgn)CfaUfcUfuAfL96	asAfsgAfaGfaUfgAfgacAfcUfcUfuUfcsusg	19.56	8.17	51.59	1.11
4832	4833	871 - 893	AD-60584.1	CfsasGfaAfaGfaGfUfGfu(Cgn)uCfaUfcUfuAfL96	asAfsgAfaGfaUfgAfgacAfcUfcUfuUfcsusg	23.36	6.63	58.79	10.35
4834	4835	871 - 893	AD-60586.1	CfsasGfaAfaGfaGfUfGf(Tgn)CfuCfaUfcUfuAfL96	asAfsgAfaGfaUfgAfgacAfcUfcUfuUfcsusg	27.78	2.20	76.86	6.81
4836	4837	871 - 893	AD-60588.1	CfsasGfaAfaGfaGfUf(Ggn)uCfuCfaUfcUfuAfL96	asAfsgAfaGfaUfgAfgacAfcUfcUfuUfcsusg	105.27	14.25	99.26	19.62
4838	4839	871 - 893	AD-60590.1	CfsasGfaAfaGfaGf(Tgn)GfuCfuCfaUfcUfuAfL96	asAfsgAfaGfaUfgAfgacAfcUfcUfuUfcsusg	28.74	1.66	81.88	22.04
4840	4841	871 - 893	AD-60558.1	CfsasGfaAfaGfaGfUfGfuCfuCfaUfc(Tgn)uAfL96	usAfs(Agn)GfaUfgAfgAfcacUfcUfuUfcUfgsgsu	22.74	14.85	60.42	19.38
4842	4843	871 - 893	n/a	CfsasGfaAfaGfaGfUfGfuCfuCfaUf(Cgn)UfuAfL96	usAfsa(Ggn)aUfgAfgAfcacUfcUfuUfcUfgsgsu				
4844	4845	871 - 893	AD-60568.1	CfsasGfaAfaGfaGfUfGfuCfuCfa(Tgn)cUfuAfL96	usAfsaGf(Agn)aUfgAfgAfcacUfcUfuUfcUfgsgsu	86.66	21.93	110.05	16.44
4846	4847	871 - 893	AD-60572.1	CfsasGfaAfaGfaGfUfGfuCfuCf(Agn)UfcUfuAfL96	usAfsaGfa(Tgn)gAfgAfcacUfcUfuUfcUfgsgsu	22.37	4.86	71.24	22.19
4848	4849	871 - 893	AD-60539.1	CfsasGfaAfaGfaGfUfGfuCfu(Cgn)aUfcUfuAfL96	usAfsaGfaUf(Ggn)AfgAfcacUfcUfuUfcUfgsgsu	43.35	14.53	104.44	2.51
4850	4851	871 - 893	AD-60544.1	CfsasGfaAfaGfaGfUfGfuCf(Tgn)CfaUfcUfuAfL96	usAfsaGfaUfg(Agn)gAfcacUfcUfuUfcUfgsgsu	25.85	1.18	69.98	5.86
4852	4853	871 - 893	AD-60549.1	CfsasGfaAfaGfaGfUfGfu(Cgn)uCfaUfcUfuAfL96	usAfsaGfaUfgAf(Ggn)AfcacUfcUfuUfcUfgsgsu	29.40	4.62	72.98	20.16
4854	4855	871 - 893	AD-60554.1	CfsasGfaAfaGfaGfUfGf(Tgn)CfuCfaUfcUfuAfL96	usAfsaGfaUfgAfg(Agn)cacUfcUfuUfcUfgsgsu	33.74	0.45	75.36	19.39
4856	4857	871 - 893	n/a	CfsasGfaAfaGfaGfUf(Ggn)uCfuCfaUfcUfuAfL96	usAfsaGfaUfgAfgAf(Cgn)acUfcUfuUfcUfgsgsu				

4858	4859	871 - 893	AD-60564.1	CfsasGfaAfaGfaGf(Tgn)GfuCfuCfaUfcUfuAfL96	usAfsaGfaUfgAfgAfc(Agn)cUfcUfuUfcUfgsgsu	27.77	10.34	77.01	11.51
4860	4861	871 - 893	AD-60569.1	CfsasGfaAfaGfaGfUfGfuCfuCfaUfcUfuAfsL96	usAfsaGfaUfgAfgAfcacUfcUfuUfcUfgsgsu	20.34	5.29	59.40	19.74
4862	4863	871 - 893	AD-60573.1	CfsasGfaAfaGfaGfUfGfuCfuCfaUfscUfsuAfsL96	usAfsaGfaUfgAfgAfcacUfcUfuUfcUfgsgsu	20.07	3.83	60.80	14.93
4864	4865	871 - 893	AD-60540.1	CfsasGfaAfaGfaGfUfGfuCfuCfaUfcUfuAfsL96	usAfsaGfaUfsgAfgAfcacUfcUfsuUfscUfgsgsu	24.36	0.56	75.75	14.97
4866	4867	871 - 893	n/a	CfsasGfaAfaGfaGfUfGfuCfuCfaUfscUfsuAfsL96	usAfsaGfaUfsgAfgAfcacUfcUfsuUfscUfgsgsu				

In the in vitro screen for which the results are shown in the table above, the siRNAs that provided the greatest ALAS1 mRNA suppression (greater than 80% suppression, such that less than 20% mRNA was remaining) at 10 nM concentration included AD-60501, AD-60592, AD-60591, AD-60513, AD-60507, AD-60587, AD-60519, AD-60593, AD-60583, AD-60524, AD-60489, AD-60495, AD-60506, and AD-60582.

In the in vitro screen for which the results are shown in the table above, the siRNAs that provided the greatest ALAS1 mRNA suppression (greater than 30% suppression, such that less than 70% mRNA was remaining) at 0.1 nM concentration included AD-60592, AD-60591, AD-60593, AD-60587, AD-60583, AD-60589, AD-60501, AD-60507, AD-60585, AD-60489, AD-60513, AD-60582, AD-60519, AD-60541, AD-60570, AD-60584, AD-60569, AD-60558, AD-60573, AD-60556, AD-60495, AD-60523, AD-60566, and AD-60544.

As is shown in the table below, testing of further siRNAs revealed that the following duplexes provided greater than 80% suppression at 10 nM concentration: AD-60489, AD-60495, AD-60501, AD-60507, AD-60513, AD-60519, AD-60583, AD-60591, AD-60592, and AD-60593, and the following duplexes provided greater than 30% suppression at 0.1 nM concentration: AD-60489, AD-60495, AD-60501, AD-60507, AD-60513, AD-60519, AD-60583, AD-60591, AD-60592, and AD-60593.

Table 33: Sequences and in vitro screen results for AD-60489 and AD-60489 derivative siRNAs

SEQ ID NO: (sense)	SEQ ID NO: (anti-sense)	Target sites of anti-sense seq on NM_000688.4	Duplex Name	Sense Sequence (5'-3')	Antisense (AS) Sequence (5'-3')	Avg 10 nM	SD 10 nM	Avg 0.1 nM	SD 0.1 nM
4868	4869	871 - 893	AD-60489.1	CfsasGfaAfaGfaGfUfGfuCfuCfaUfcUfuAfL96	usAfsaGfaUfgAfgAfcacUfcUfuUfcUfgsgsu	17.0	0.2	50.7	5.1
4870	4871	871 - 893	AD-60495.1	CfsasGfaAfaGfaGfuGfuCfuCfaucuuAfL96	usAfsaGfaUfgAfgAfcacUfcUfuUfcUfgsgsu	17.1	5.9	63.6	18.6
4872	4873	871 - 893	AD-60501.1	CfsasGfaAfaGfaGfuGfuCfuCfaucuuAfL96	usAfsAfGfaUfgAfgAfcAfcUfcUfuUfcUfgsgsu	11.9	0.7	45.2	8.9
4874	4875	871 - 893		CfsasGfaAfaGfaGfuGfuCfuCfaucuuAfL96	usAfsAfGfaugAfgAfcAfcucuuucugsgsu				
4876	4877	871 - 893		CfsasGfaAfaGfaGfuGfuCfuCfaucuuAfL96	usAfsAfGfadTgAfgAfcAfcdTcuudTcugsgsu				
4878	4879	871 - 893		CfsasGfaAfaGfaGfuGfuCfuCfaucuuAfL96	usAfsaGfadTgAfgAfcAfcdTcuudTcugsgsu				
4880	4881	871 - 893		CfsasGfaAfaGfaGfuGfuCfuCfaucuuAfL96	usAfsAfGfadTgAfgAfcacdTcuudTcugsgsu				
4882	4883	871 - 893	AD-60507.1	CfsasgaaaGfaGfuGfuCfuCfaucuuAfL96	usAfsAfGfaUfgAfgAfcAfcUfcUfuUfcUfgsgsu	14.6	7.8	48.5	19.6
4884	4885	871 - 893		CfsasgaaaGfaGfuGfuCfuCfaucuuAfL96	usAfsaGfaUfgAfgAfcacUfcUfuUfcUfgsgsu				
4886	4887	871 - 893		CfsasgaaaGfaGfuGfuCfuCfaucuuAfL96	usAfsAfGfaugAfgAfcAfcucuuucugsgsu				
4888	4889	871 - 893		CfsasgaaaGfaGfuGfuCfuCfaucuuAfL96	usAfsAfGfadTgAfgAfcAfcdTcuudTcugsgsu				
4890	4891	871 - 893		CfsasgaaaGfaGfuGfuCfuCfaucuuAfL96	usAfsaGfadTgAfgAfcAfcdTcuudTcugsgsu				
4892	4893	871 - 893		CfsasgaaaGfaGfuGfuCfuCfaucuuAfL96	usAfsAfGfadTgAfgAfcacdTcuudTcugsgsu				
4894	4895	871 - 893	AD-60513.1	CfsasgaaaGfaGfuGfuCfuCfaucuuaL96	usAfsAfGfaUfgAfgAfcAfcUfcUfuUfcUfgsgsu	14.4	2.4	51.0	7.0
4896	4897	871 - 893		CfsasgaaaGfaGfuGfuCfuCfaucuuaL96	usAfsaGfaUfgAfgAfcacUfcUfuUfcUfgsgsu				
4898	4899	871 - 893		CfsasgaaaGfaGfuGfuCfuCfaucuuaL96	usAfsAfGfaugAfgAfcAfcucuuucugsgsu				
4900	4901	871 - 893		CfsasgaaaGfaGfuGfuCfuCfaucuuaL96	usAfsAfGfadTgAfgAfcAfcdTcuudTcugsgsu				
4902	4903	871 - 893		CfsasgaaaGfaGfuGfuCfuCfaucuuaL96	usAfsaGfadTgAfgAfcAfcdTcuudTcugsgsu				
4904	4905	871 - 893		CfsasgaaaGfaGfuGfuCfuCfaucuuaL96	usAfsAfGfadTgAfgAfcacdTcuudTcugsgsu				
4906	4907	871 - 893	AD-60519.1	csasgaaaGfaGfuGfuCfuCfaucuuaL96	usAfsAfGfaUfgAfgAfcAfcUfcUfuUfcUfgsgsu	14.7	4.9	51.7	7.7
4908	4909	871 - 893		csasgaaaGfaGfuGfuCfuCfaucuuaL96	usAfsaGfaUfgAfgAfcacUfcUfuUfcUfgsgsu				
4910	4911	871 - 893		csasgaaaGfaGfuGfuCfuCfaucuuaL96	usAfsAfGfaugAfgAfcAfcucuuucugsgsu				
4912	4913	871 - 893		csasgaaaGfaGfuGfuCfuCfaucuuaL96	usAfsAfGfadTgAfgAfcAfcdTcuudTcugsgsu				
4914	4915	871 - 893		csasgaaaGfaGfuGfuCfuCfaucuuaL96	usAfsaGfadTgAfgAfcAfcdTcuudTcugsgsu				

4916	4917	871 - 893		csasgaaaGfaGfuGfuCfuCfaucuuaL96	usAfsAfGfadTgAfgAfcacdTcuudTcugsgsu				
4918	4919	871 - 893	AD-60499.1	csasgaaaGfAfGfugucucaucuuaL96	usAfsAfGfadTgAfgAfcAfcdTcuudTcugsgsu	35.9	13.8	77.5	6.7
4920	4921	871 - 893		csasgaaaGfAfGfugucucaucuuaL96	usAfsaGfaUfgAfgAfcacUfcUfuUfcUfgsgsu				
4922	4923	871 - 893		csasgaaaGfAfGfugucucaucuuaL96	usAfsAfGfaUfgAfgAfcAfcUfcUfuUfcUfgsgsu				
4924	4925	871 - 893		csasgaaaGfAfGfugucucaucuuaL96	usAfsAfGfaugAfgAfcAfcucuuucugsgsu				
4926	4927	871 - 893		csasgaaaGfAfGfugucucaucuuaL96	usAfsaGfadTgAfgAfcAfcdTcuudTcugsgsu				
4928	4929	871 - 893		csasgaaaGfAfGfugucucaucuuaL96	usAfsAfGfadTgAfgAfcacdTcuudTcugsgsu				
4930	4931	871 - 893		csasgaaaGfAfGfugucuca(Tgn)cuuaL96	usAfsaGfaUfgAfgAfcacUfcUfuUfcUfgsgsu				
4932	4933	871 - 893		csasgaaaGfAfGfugucuca(Tgn)cuuaL96	usAfsAfGfaUfgAfgAfcAfcUfcUfuUfcUfgsgsu				
4934	4935	871 - 893		csasgaaaGfAfGfugucuca(Tgn)cuuaL96	usAfsAfGfaugAfgAfcAfcucuuucugsgsu				
4936	4937	871 - 893		csasgaaaGfAfGfugucuca(Tgn)cuuaL96	usAfsAfGfadTgAfgAfcAfcdTcuudTcugsgsu				
4938	4939	871 - 893		csasgaaaGfAfGfugucuca(Tgn)cuuaL96	usAfsaGfadTgAfgAfcAfcdTcuudTcugsgsu				
4940	4941	871 - 893		csasgaaaGfAfGfugucuca(Tgn)cuuaL96	usAfsAfGfadTgAfgAfcacdTcuudTcugsgsu				
4942	4943	871 - 893	AD-60583.1	CfsasGfaAfaGfaGfUfGfuCfuCfaUfcUfu(Agn)L96	asAfsgAfaGfaUfgAfgacAfcUfcUfuUfcsusg	15.3	4.8	42.0	9.5
4944	4945	871 - 893	AD-60591.1	CfsasGfaAfaGfaGfUfGfuCfuCfa(Tgn)cUfuAfL96	asAfsgAfaGfaUfgAfgacAfcUfcUfuUfcsusg	13.7	0.0	36.4	16.9
4946	4947	871 - 893	AD-60592.1	CfsasGfaAfaGfaGfUfGfuCfuCf(Agn)UfcUfuAfL96	asAfsgAfaGfaUfgAfgacAfcUfcUfuUfcsusg	13.4	4.1	35.4	11.4
4948	4949	871 - 893	AD-60593.1	CfsasGfaAfaGfaGfUfGfuCfu(Cgn)aUfcUfuAfL96	asAfsgAfaGfaUfgAfgacAfcUfcUfuUfcsusg	15.1	0.3	40.0	19.4
4950	4951	871 - 893	AD-60489.1	CfsasGfaAfaGfaGfUfGfuCfuCfaUfcUfuAfL96	usAfsaGfaUfgAfgAfcacUfcUfuUfcUfgsgsu	17.0	0.2	50.7	5.1
4952	4953	871 - 893		CfsasGfaAfaGfaGfdTGfuCfuCfaUfcUfuAfL96	usAfsaGfaUfgAfgAfcacUfcUfuUfcUfgsgsu				
4954	4955	871 - 893		CfsasGfaAfaGfaGfUfGfuCfuCfaUfcUfuAfL96	usAfsaGfaUfgAfgAfcacUfcdTuUfcUfgsgsu				
4956	4957	871 - 893		CfsasGfaAfaGfaGfdTGfuCfuCfaUfcUfuAfL96	usAfsaGfaUfgAfgAfcacUfcdTuUfcUfgsgsu				
4958	4959	871 - 893		CfsasGfaAfaGfaGfdTGfuCfuCfaUfscUfsuAfsL96	usAfsaGfaUfgAfgAfcacUfcdTuUfcUfgsgsu				
4960	4961	871 - 893		CfsasGfaAfaGfaGfdTGfuCfuCfaUfscusuAfsL96	usAfsAfGfaUfgAfgAfcacUfcdTuUfcUfgsgsu				
4962	4963	871 - 893	AD-60591.1	CfsasGfaAfaGfaGfUfGfuCfuCfa(Tgn)cUfuAfL96	asAfsgAfaGfaUfgAfgacAfcUfcUfuUfcsusg	13.7	0.0	36.4	16.9
4964	4965	871 - 893		CfsasGfaAfaGfaGfdTGfuCfuCfa(Tgn)cUfuAfL96	asAfsgAfaGfaUfgAfgacAfcUfcUfuUfcsusg				
4966	4967	871 - 893		CfsasGfaAfaGfaGfUfGfuCfuCfa(Tgn)cUfuAfL96	usAfsaGfaUfgAfgAfcacUfcdTuUfcUfgsgsu				
4968	4969	871 - 893		CfsasGfaAfaGfaGfdTGfuCfuCfa(Tgn)cUfuAfL96	usAfsaGfaUfgAfgAfcacUfcdTuUfcUfgsgsu				
4970	4971	871 - 893		CfsasGfaAfaGfaGfdTGfuCfuCfa(Tgn)scUfsuAfsL96	usAfsaGfaUfgAfgAfcacUfcdTuUfcUfgsgsu				
4972	4973	871 - 893		CfsasGfaAfaGfaGfdTGfuCfuCfa(Tgn)scusuAfsL96	usAfsAfGfaUfgAfgAfcacUfcdTuUfcUfgsgsu				

4974	4975	871 - 893	AD-60592.1	CfsasGfaAfaGfaGfUfGfuCfuCf(Agn)UfcUfuAfL96	asAfsgAfaGfaUfgAfgacAfcUfcUfuUfcsusg	13.4	4.1	35.4	11.4
4976	4977	871 - 893		CfsasGfaAfaGfaGfdTGfuCfuCf(Agn)UfcUfuAfL96	asAfsgAfaGfaUfgAfgacAfcUfcUfuUfcsusg				
4978	4979	871 - 893		CfsasGfaAfaGfaGfUfGfuCfuCf(Agn)UfcUfuAfL96	usAfsaGfaUfgAfgAfcacUfcdTuUfcUfgsgsu				
4980	4981	871 - 893		CfsasGfaAfaGfaGfdTGfuCfuCf(Agn)UfcUfuAfL96	usAfsaGfaUfgAfgAfcacUfcdTuUfcUfgsgsu				
4982	4983	871 - 893		CfsasGfaAfaGfaGfdTGfuCfuCf(Agn)UfscUfsuAfsL96	usAfsaGfaUfgAfgAfcacUfcdTuUfcUfgsgsu				
4984	4985	871 - 893		CfsasGfaAfaGfaGfdTGfuCfuCf(Agn)UfscusuAfsL96	usAfsAfGfaUfgAfgAfcacUfcdTuUfcUfgsgsu				
4986	4987	871 - 893		CfsasGfaAfaGfaGfdTGfuCfuCfs(Agns)UfscUfsuAfsL96	usAfsaGfaUfgAfgAfcacUfcdTuUfcUfgsgsu				
4988	4989	871 - 893		CfsasGfaAfaGfaGfdTGfuCfuCfs(Agns)UfscusuAfsL96	usAfsAfGfaUfgAfgAfcacUfcdTuUfcUfgsgsu				

Table 34: Further sequences of AD-60489 derivative siRNAs

SEQ ID NO: (sense)	SEQ ID NO: (anti-sense)	Target sites of antisense sequence on NM_000688.4	Duplex Name	Sense Sequence (5'-3')	Antisense Sequence (5'-3')
4990	4991	871 - 893	AD-60489	CfsasGfaAfaGfaGfUfGfuCfuCfaUfcUfuAfL96	usAfsaGfaUfgAfgAfcacUfcUfuUfcUfgsgsu
4992	4993	871 - 893	AD-60501.1	CfsasGfaAfaGfaGfuGfuCfuCfaucuuAfL96	usAfsAfGfaUfgAfgAfcAfcUfcUfuUfcUfgsgsu
4994	4995	871 - 893	AD-60900.1	CfsasGfaAfaGfaGfuGfuCfuCfaucuuAfL96	usAfsAfGfadTgAfgAfcAfcdTcuudTcugsgsu
4996	4997	871 - 893	AD-60519.1	csasgaaaGfaGfuGfuCfuCfaucuuaL96	usAfsAfGfaUfgAfgAfcAfcUfcUfuUfcUfgsgsu
4998	4999	871 - 893	AD-60905.1	csasgaaaGfaGfuGfuCfuCfaucuuaL96	usAfsAfGfadTgAfgAfcAfcdTcuudTcugsgsu
5000	5001	871 - 893	AD-60901.1	csasgaaaGfaGfuGfuCfuCfaucuuaL96	usAfsaGfaUfgAfgAfcacUfcUfuUfcUfgsgsu
5002	5003	871 - 893	AD-60495.2	csasgaaaGfaGfuGfuCfuCfaucuuaL96	usAfsaGfaUfgAfgAfcacUfcUfuUfcUfgsgsu
5004	5005	871 - 893	AD-60935.1	CfsasGfaAfaGfaGfUfGfuCfuCfaUfcUfuAfL96	usAfsAfGfaUfgAfgAfcAfcUfcUfuUfcUfgsgsu

Table 35: Further sequences and IC50s of AD-60489 and AD-60489 derivative siRNAs

SEQ ID NO: (sense)	SEQ ID NO: (anti-sense)	Target sites of anti-sense seq on NM_000688.4	Duplex Name*	Sense Sequence (5'-3')	Antisense (AS) Sequence (5'-3')	IC50
5006	5007	871 - 893	AD-60489.3	CfsasGfaAfaGfaGfUfGfuCfuCfaUfcUfuAfL96	usAfsaGfaUfgAfgAfcacUfcUfuUfcUfgsgsu	0.010
5008	5009	871 - 893	AD-60495.2	CfsasGfaAfaGfaGfuGfuCfuCfaucuuAfL96	usAfsaGfaUfgAfgAfcacUfcUfuUfcUfgsgsu	0.254
5010	5011	871 - 893	AD-60501.2	CfsasGfaAfaGfaGfuGfuCfuCfaucuuAfL96	usAfsAfGfaUfgAfgAfcAfcUfcUfuUfcUfgsgsu	0.006
5012	5013	871 - 893	AD-60898.1	CfsasGfaAfaGfaGfuGfuCfuCfaucuuAfL96	usAfsAfGfaugAfgAfcAfcucuuucugsgsu	na
5014	5015	871 - 893	AD-60900.1	CfsasGfaAfaGfaGfuGfuCfuCfaucuuAfL96	usAfsAfGfadTgAfgAfcAfcdTcuudTcugsgsu	0.151
5016	5017	871 - 893	AD-60851.1	CfsasGfaAfaGfaGfuGfuCfuCfaucuuAfL96	usAfsaGfadTgAfgAfcAfcdTcuudTcugsgsu	0.033
5018	5019	871 - 893	AD-60855.1	CfsasGfaAfaGfaGfuGfuCfuCfaucuuAfL96	usAfsAfGfadTgAfgAfcacdTcuudTcugsgsu	0.065
5020	5021	871 - 893	AD-60507.2	CfsasgaaaGfaGfuGfuCfuCfaucuuAfL96	usAfsAfGfaUfgAfgAfcAfcUfcUfuUfcUfgsgsu	0.029
5022	5023	871 - 893	AD-60902.1	CfsasgaaaGfaGfuGfuCfuCfaucuuAfL96	usAfsaGfaUfgAfgAfcacUfcUfuUfcUfgsgsu	0.024
5024	5025	871 - 893	AD-60904.1	CfsasgaaaGfaGfuGfuCfuCfaucuuAfL96	usAfsAfGfaugAfgAfcAfcucuuucugsgsu	na
5026	5027	871 - 893	AD-60894.1	CfsasgaaaGfaGfuGfuCfuCfaucuuAfL96	usAfsAfGfadTgAfgAfcAfcdTcuudTcugsgsu	0.011
5028	5029	871 - 893	AD-60860.1	CfsasgaaaGfaGfuGfuCfuCfaucuuAfL96	usAfsaGfadTgAfgAfcAfcdTcuudTcugsgsu	0.249
5030	5031	871 - 893	AD-60864.1	CfsasgaaaGfaGfuGfuCfuCfaucuuAfL96	usAfsAfGfadTgAfgAfcacdTcuudTcugsgsu	1.899
5032	5033	871 - 893	AD-60513.2	CfsasgaaaGfaGfuGfuCfuCfaucuuaL96	usAfsAfGfaUfgAfgAfcAfcUfcUfuUfcUfgsgsu	0.019
5034	5035	871 - 893	AD-60896.1	CfsasgaaaGfaGfuGfuCfuCfaucuuaL96	usAfsaGfaUfgAfgAfcacUfcUfuUfcUfgsgsu	0.034
5036	5037	871 - 893	AD-60899.1	CfsasgaaaGfaGfuGfuCfuCfaucuuaL96	usAfsAfGfaugAfgAfcAfcucuuucugsgsu	na
5038	5039	871 - 893	AD-60868.1	CfsasgaaaGfaGfuGfuCfuCfaucuuaL96	usAfsaGfadTgAfgAfcAfcdTcuudTcugsgsu	0.249
5040	5041	871 - 893	AD-60872.1	CfsasgaaaGfaGfuGfuCfuCfaucuuaL96	usAfsAfGfadTgAfgAfcacdTcuudTcugsgsu	0.014
5042	5043	871 - 893	AD-60519.2	csasgaaaGfaGfuGfuCfuCfaucuuaL96	usAfsAfGfaUfgAfgAfcAfcUfcUfuUfcUfgsgsu	0.026

5044	5045	871 - 893	AD-60901.1	csasgaaaGfaGfuGfuCfuCfaucuuaL96	usAfsaGfaUfgAfgAfcacUfcUfuUfcUfgsgsu	0.080
5046	5047	871 - 893	AD-60903.1	csasgaaaGfaGfuGfuCfuCfaucuuaL96	usAfsAfGfaugAfgAfcAfcucuuucugsgsu	na
5048	5049	871 - 893	AD-60905.1	csasgaaaGfaGfuGfuCfuCfaucuuaL96	usAfsAfGfadTgAfgAfcAfcdTcuudTcugsgsu	0.018
5050	5051	871 - 893	AD-60876.1	csasgaaaGfaGfuGfuCfuCfaucuuaL96	usAfsaGfadTgAfgAfcAfcdTcuudTcugsgsu	0.091
5052	5053	871 - 893	AD-60880.1	csasgaaaGfaGfuGfuCfuCfaucuuaL96	usAfsAfGfadTgAfgAfcacdTcuudTcugsgsu	0.131
5054	5055	871 - 893	AD-60499.2	csasgaaaGfAfGfugucucaucuuaL96	usAfsAfGfadTgAfgAfcAfcdTcuudTcugsgsu	na
5056	5057	871 - 893	AD-60895.1	csasgaaaGfAfGfugucucaucuuaL96	usAfsaGfaUfgAfgAfcacUfcUfuUfcUfgsgsu	na
5058	5059	871 - 893	AD-60897.1	csasgaaaGfAfGfugucucaucuuaL96	usAfsAfGfaUfgAfgAfcAfcUfcUfuUfcUfgsgsu	na
5060	5061	871 - 893	AD-60526.2	csasgaaaGfAfGfugucucaucuuaL96	usAfsAfGfaugAfgAfcAfcucuuucugsgsu	na
5062	5063	871 - 893	AD-60852.1	csasgaaaGfAfGfugucucaucuuaL96	usAfsaGfadTgAfgAfcAfcdTcuudTcugsgsu	4.970
5064	5065	871 - 893	AD-60856.1	csasgaaaGfAfGfugucucaucuuaL96	usAfsAfGfadTgAfgAfcacdTcuudTcugsgsu	na
5066	5067	871 - 893	AD-60861.1	csasgaaaGfAfGfugucuca(Tgn)cuuaL96	usAfsaGfaUfgAfgAfcacUfcUfuUfcUfgsgsu	0.019
5068	5069	871 - 893	AD-60865.1	csasgaaaGfAfGfugucuca(Tgn)cuuaL96	usAfsAfGfaUfgAfgAfcAfcUfcUfuUfcUfgsgsu	0.061
5070	5071	871 - 893	AD-60869.1	csasgaaaGfAfGfugucuca(Tgn)cuuaL96	usAfsAfGfaugAfgAfcAfcucuuucugsgsu	na
5072	5073	871 - 893	AD-60873.1	csasgaaaGfAfGfugucuca(Tgn)cuuaL96	usAfsAfGfadTgAfgAfcAfcdTcuudTcugsgsu	0.009
5074	5075	871 - 893	AD-60877.1	csasgaaaGfAfGfugucuca(Tgn)cuuaL96	usAfsaGfadTgAfgAfcAfcdTcuudTcugsgsu	0.008
5076	5077	871 - 893	AD-60881.1	csasgaaaGfAfGfugucuca(Tgn)cuuaL96	usAfsAfGfadTgAfgAfcacdTcuudTcugsgsu	0.025
5078	5079	871 - 893	AD-60583.2	CfsasGfaAfaGfaGfUfGfuCfuCfaUfcUfu(Agn)L96	usAfsaGfaUfgAfgAfcacUfcUfuUfcUfgsgsu	0.022
5080	5081	871 - 893	AD-60591.3	CfsasGfaAfaGfaGfUfGfuCfuCfa(Tgn)cUfuAfL96	usAfsaGfaUfgAfgAfcacUfcUfuUfcUfgsgsu	0.031
5082	5083	871 - 893	AD-60592.3	CfsasGfaAfaGfaGfUfGfuCfuCf(Agn)UfcUfuAfL96	usAfsaGfaUfgAfgAfcacUfcUfuUfcUfgsgsu	0.010
5084	5085	871 - 893	AD-60593.2	CfsasGfaAfaGfaGfUfGfuCfu(Cgn)aUfcUfuAfL96	usAfsaGfaUfgAfgAfcacUfcUfuUfcUfgsgsu	0.006
5086	5087	871 - 893	AD-60489.4	CfsasGfaAfaGfaGfUfGfuCfuCfaUfcUfuAfL96	usAfsaGfaUfgAfgAfcacUfcUfuUfcUfgsgsu	0.011
5088	5089	871 - 893	AD-60857.1	CfsasGfaAfaGfaGfdTGfuCfuCfaUfcUfuAfL96	usAfsaGfaUfgAfgAfcacUfcUfuUfcUfgsgsu	0.019

5090	5091	871 - 893	AD-60862.1	CfsasGfaAfaGfaGfUfGfuCfuCfaUfcUfuAfL96	usAfsaGfaUfgAfgAfcacUfcdTuUfcUfgsgsu	0.012
5092	5093	871 - 893	AD-60866.1	CfsasGfaAfaGfaGfdTGfuCfuCfaUfcUfuAfL96	usAfsaGfaUfgAfgAfcacUfcdTuUfcUfgsgsu	0.015
5094	5095	871 - 893	AD-60870.1	CfsasGfaAfaGfaGfdTGfuCfuCfaUfscUfsuAfsL96	usAfsaGfaUfgAfgAfcacUfcdTuUfcUfgsgsu	0.012
5096	5097	871 - 893	AD-60874.1	CfsasGfaAfaGfaGfdTGfuCfuCfaUfscusuAfsL96	usAfsAfGfaUfgAfgAfcacUfcdTuUfcUfgsgsu	0.003
5098	5099	871 - 893	AD-60591.2	CfsasGfaAfaGfaGfUfGfuCfuCfa(Tgn)cUfuAfL96	asAfsgAfaGfaUfgAfgacAfcUfcUfuUfcsusg	0.014
5100	5101	871 - 893	AD-60878.1	CfsasGfaAfaGfaGfdTGfuCfuCfa(Tgn)cUfuAfL96	asAfsgAfaGfaUfgAfgacAfcUfcUfuUfcsusg	0.008
5102	5103	871 - 893	AD-60882.1	CfsasGfaAfaGfaGfUfGfuCfuCfa(Tgn)cUfuAfL96	usAfsaGfaUfgAfgAfcacUfcdTuUfcUfgsgsu	0.003
5104	5105	871 - 893	AD-60853.1	CfsasGfaAfaGfaGfdTGfuCfuCfa(Tgn)cUfuAfL96	usAfsaGfaUfgAfgAfcacUfcdTuUfcUfgsgsu	0.007
5106	5107	871 - 893	AD-60858.1	CfsasGfaAfaGfaGfdTGfuCfuCfa(Tgn)scUfsuAfsL96	usAfsaGfaUfgAfgAfcacUfcdTuUfcUfgsgsu	0.013
5108	5109	871 - 893	AD-60863.1	CfsasGfaAfaGfaGfdTGfuCfuCfa(Tgn)scusuAfsL96	usAfsAfGfaUfgAfgAfcacUfcdTuUfcUfgsgsu	0.002
5110	5111	871 - 893	AD-60592.2	CfsasGfaAfaGfaGfUfGfuCfuCf(Agn)UfcUfuAfL96	asAfsgAfaGfaUfgAfgacAfcUfcUfuUfcsusg	0.013
5112	5113	871 - 893	AD-60867.1	CfsasGfaAfaGfaGfdTGfuCfuCf(Agn)UfcUfuAfL96	asAfsgAfaGfaUfgAfgacAfcUfcUfuUfcsusg	0.084
5114	5115	871 - 893	AD-60871.1	CfsasGfaAfaGfaGfUfGfuCfuCf(Agn)UfcUfuAfL96	usAfsaGfaUfgAfgAfcacUfcdTuUfcUfgsgsu	0.008
5116	5117	871 - 893	AD-60875.1	CfsasGfaAfaGfaGfdTGfuCfuCf(Agn)UfcUfuAfL96	usAfsaGfaUfgAfgAfcacUfcdTuUfcUfgsgsu	0.004
5118	5119	871 - 893	AD-60879.1	CfsasGfaAfaGfaGfdTGfuCfuCf(Agn)UfscUfsuAfsL96	usAfsaGfaUfgAfgAfcacUfcdTuUfcUfgsgsu	0.001
5120	5121	871 - 893	AD-60883.1	CfsasGfaAfaGfaGfdTGfuCfuCf(Agn)UfscusuAfsL96	usAfsAfGfaUfgAfgAfcacUfcdTuUfcUfgsgsu	0.004
5122	5123	871 - 893	AD-60854.1	CfsasGfaAfaGfaGfdTGfuCfuCfs(Agns)UfscUfsuAfsL96	usAfsaGfaUfgAfgAfcacUfcdTuUfcUfgsgsu	0.002
5124	5125	871 - 893	AD-60859.1	CfsasGfaAfaGfaGfdTGfuCfuCfs(Agns)UfscusuAfsL96	usAfsAfGfaUfgAfgAfcacUfcdTuUfcUfgsgsu	0.002

As is shown in the table above, a number of duplexes showed efficacy in suppressing ALAS1 mRNA. The following duplexes had an IC50 of less than 0.01 nM: AD-60879, AD-60859, AD-60863, AD-60854, AD-60882, AD-60874, AD-60883, AD-60875, AD-60501, AD-60593, AD-60853, AD-60877, AD-60878, AD-60871, and AD-60873. The following duplexes had an IC50 of less than 0.02 nM: AD-60879, AD-60859, AD-60863, AD-60854, AD-60882, AD-60874 , AD-60883, AD-60875, AD-60501, AD-60593, AD-60853, AD-60877, AD-60878, AD-60871, AD-60873, AD-60489, AD-60592, AD-60894, AD-60489, AD-60870, AD-60862, AD-60858, AD-60592, AD-60591, AD-60872, AD-60866, AD-60905, AD-60857, AD-60513, and AD-60861. The following duplexes had an IC50 of less than 0.05 nM: AD-60879, AD-60859, AD-60863, AD-60854, AD-60882, AD-60874 , AD-60883, AD-60875, AD-60501, AD-60593, AD-60853, AD-60877, AD-60878, AD-60871, AD-60873, AD-60489, AD-60592, AD-60894, AD-60489, AD-60870, AD-60862, AD-60858, AD-60592, AD-60591, AD-60872, AD-60866, AD-60905, AD-60857, AD-60513, AD-60861, AD-60583.2, AD-60902.1, AD-60881.1, AD-60519.2, AD-60507.2, AD-60591.3, AD-60851.1, AD-60896.1, and AD-60537.2.

Example 29: In vivo structure activity relationship studies of AD-60489

Derivatives of the AD-60489 parent siRNA were generated and screened in vivo in rats.

In vivo screen 1 of AD-60489 derivatives

The sequences of the siRNAs that were screened are provided in the table below.

Table 36: Sequences of ALAS1 siRNA Duplexes

SEQ ID NO: (sense)	SEQ ID NO: (anti-sense)	Target sites of antisense sequence on NM_000688.4	Duplex Name	Sense Sequence (5'-3')	Antisense Sequence (5'-3')
5126	5127	871 - 893	AD-60489	CfsasGfaAfaGfaGfUfGfuCfuCfaUfcUfuAfL96	usAfsaGfaUfgAfgAfcacUfcUfuUfcUfgsgsu

5128	5129	871 - 893	AD-60501.2	CfsasGfaAfaGfaGfuGfuCfuCfaucuuAfL96	usAfsAfGfaUfgAfgAfcAfcUfcUfuUfcUfgsgsu
5130	5131	871 - 893	AD-60519.2	csasgaaaGfaGfuGfuCfuCfaucuuaL96	usAfsAfGfaUfgAfgAfcAfcUfcUfuUfcUfgsgsu
5132	5133	871 - 893	AD-60901.1	csasgaaaGfaGfuGfuCfuCfaucuuaL96	usAfsaGfaUfgAfgAfcacUfcUfuUfcUfgsgsu
5134	5135	871 - 893	AD-60495.2	CfsasGfaAfaGfaGfuGfuCfuCfaucuuAfL96	usAfsaGfaUfgAfgAfcacUfcUfuUfcUfgsgsu
5136	5137	871 - 893	AD-60900.1	CfsasGfaAfaGfaGfuGfuCfuCfaucuuAfL96	usAfsAfGfadTgAfgAfcAfcdTcuudTcugsgsu
5138	5139	871 - 893	AD-60935.1	CfsasGfaAfaGfaGfUfGfuCfuCfaUfcUfuAfL96	usAfsAfGfaUfgAfgAfcAfcUfcUfuUfcUfgsgsu
5140	5141	871 - 893	AD-60905.1	csasgaaaGfaGfuGfuCfuCfaucuuaL96	usAfsAfGfadTgAfgAfcAfcdTcuudTcugsgsu

Rats were administered a single dose of 3 mg/kg of siRNA. At 5 days following administration of the siRNA, mRNA measurements of rat ALAS1 (rALAS1) mRNA and rat GAPDH (rGAPDH) mRNA were made using bDNA assay, and tissue levels of drug (siRNA) were determined using qPCR.

As is shown in FIG. 40 (top), the siRNAs AD-60501, AD-60519, AD-60901, AD-60495, AD-60900, and AD-60935 showed improved suppression of ALAS1 mRNA compared with AD-60489. The siRNAs AD-60519, AD-60901, AD-60495, and AD-60935 achieved higher liver levels than did AD-60489 (see FIG. 40, bottom). Thus, most of the duplexes that provided improved suppression of ALAS1 mRNA also achieved higher liver levels.

At least for the duplexes AD-60489, AD-60519, and AD-60901, efficacy correlated with liver levels of the siRNA (see FIG. 41), such that a higher level of siRNA in liver was associated with greater ALAS1 mRNA suppression.

In vivo screen 2 of AD-60489 derivatives

The sequences of the siRNAs that were screened are provided in the table below.

Table 37: Sequences of ALAS1 siRNA Duplexes

SEQ ID NO: (sense)	SEQ ID NO: (anti-sense)	Target sites of antisense sequence on NM_000688.4	Duplex Name	Sense Sequence (5'-3')	Antisense Sequence (5'-3')
5142	5143	871 - 893	AD-60489	CfsasGfaAfaGfaGfUfGfuCfuCfaUfcUfuAfL96	usAfsaGfaUfgAfgAfcacUfcUfuUfcUfgsgsu
5144	5145	871 - 893	AD-60879	CfsasGfaAfaGfaGfdTGfuCfuCf(Agn)UfscUfsuAfsL96	usAfsaGfaUfgAfgAfcacUfcdTuUfcUfgsgsu
5146	5147	871 - 893	AD-61190	CfsasGfaAfaGfaGfdTGfuCfuCf(Agn)UfscUfsuAfsL96	usAfsAfGfaUfgAfgAfcAfcUfcUfuUfcUfgsgsu
5148	5149	871 - 893	AD-61191	CfsasGfaAfaGfaGfdTGfuCfuCf(Agn)UfscUfsuAfsL96	usAfsAfGfaUfgAfgAfcAfcUfcUfuUfcUfgsgsu
5150	5151	871 - 893	AD-60877	csasgaaaGfAfGfugucuca(Tgn)cuuaL96	usAfsaGfadTgAfgAfcAfcdTcuudTcugsgsu

5152	5153	871 - 893	AD-61192	csasgaaaGfAfGfugucuca(Tgn)cuuaL96	usAfsaGfadTgAfgAfcAfcdTcuudTcugsgsu
5154	5155	871 - 893	AD-60865	csasgaaaGfaGfuGfuCfuCfaucuuaL96	usAfsaGfadTgAfgAfcAfcdTcuudTcugsgsu
5156	5157	871 - 893	AD-60861	csasgaaaGfAfGfugucuca(Tgn)cuuaL96	usAfsaGfaUfgAfgAfcacUfcUfuUfcUfgsgsu
5158	5159	871 - 893	AD-60876	csasgaaaGfaGfuGfuCfuCfaucuuaL96	usAfsaGfadTgAfgAfcAfcdTcuudTcugsgsu
5160	5161	871 - 893	AD-61193	csasgaaaGfaGfuGfuCfuCfaucuuaL96	usAfsaGfaUfgAfgAfcacUfcdTuUfcUfgsgsu
5162	5163	871 - 893	AD-60519	csasgaaaGfaGfuGfuCfuCfaucuuaL96	usAfsAfGfaUfgAfgAfcAfcUfcUfuUfcUfgsgsu

Rats were administered a single dose of 2.5 mg/kg of siRNA. At 5 days following administration of the siRNA, mRNA measurements of rat ALAS1 (rALAS1) mRNA and rat GAPDH (rGAPDH) mRNA were made using bDNA assay.

As is shown in FIG. 42, the siRNAs AD-60879, AD-61190, AD-61191, AD-60865, AD-60861, AD-60876, AD-61193, and AD-60519 showed improved suppression of ALAS1 mRNA compared with AD-60489.

Example 30: Multidosing improves potency

To investigate the effects of administering multiple doses of siRNA, rats (n=3 per group) were administered PBS or an siRNA (AD-58632, AD-60925, AD-60419, AD-60445, AD-60892, AD-60489, AD-60519, or AD-60901) at a dose of 2.5 mg/kg twice per week for 2 weeks. The levels of rat ALAS1 (rALAS1) mRNA and rat GAPDH (rGAPDH) mRNA were assessed using bDNA assay.

Table 38: Sequences of ALAS1 siRNA Duplexes

SEQ ID NO: (sense)	SEQ ID NO: (anti-sense)	Target sites of antisense sequence on NM_000688.4	Duplex Name	Sense Sequence (5'-3')	Antisense Sequence (5'-3')
5164	5165	873 - 895	AD-58632	GfsasAfaGfaGfuGfUfCfuCfaUfcUfuCfuUfL96	asAfsgAfaGfaUfgAfgacAfcUfcUfuUfcsusg
5166	5167	873 - 895	AD-60925	GfsasAfaGfAfGfuGfucucauc(Tgn)ucuuL96	asAfsGfAfaGfaUfgAfgAfcAfcUfcUfuUfcsusg
5168	5169	873 - 895	AD-60419	GfsasAfaGfAfGfuGfdTcucaucuucuuL96	asAfsGfAfaGfaugAfgAfcAfcucuuucsusg
5170	5171	873 - 895	AD-60445	GfsasAfaGfAfGfuGfucucauc(Tgn)ucuuL96	asAfsGfAfaGfaugAfgAfcAfcucuuucsusg
5172	5173	873 - 895	AD-60892	gsasaagaGfuGfuCfucaucuucuuL96	asAfsGfAfaGfaugAfgAfcAfcucuuucsusg
5174	5175	871 - 893	AD-60489	CfsasGfaAfaGfaGfUfGfuCfuCfaUfcUfuAfL96	usAfsaGfaUfgAfgAfcacUfcUfuUfcUfgsgsu
5176	5177	871 - 893	AD-60519.2	csasgaaaGfaGfuGfuCfuCfaucuuaL96	usAfsAfGfaUfgAfgAfcAfcUfcUfuUfcUfgsgsu
5178	5179	871 - 893	AD-60901.1	csasgaaaGfaGfuGfuCfuCfaucuuaL96	usAfsaGfaUfgAfgAfcacUfcUfuUfcUfgsgsu

As is shown in FIG. 43, the AD-58632 derivative siRNAs AD-60892, AD-60419, AD-60445, and AD-60925 showed improved suppression of ALAS1 mRNA compared with the parent AD-58632. In addition, the AD-60489 derivative siRNAs AD-60519 and AD-60901 showed improved suppression of ALAS1 mRNA compared with the parent AD-60489.

Example 31: Multidosing studies with AD-60519 and AD-60489

The therapeutic efficacy of AD-60519 was investigated in a rat AIP model. The experimental design is shown in FIG. 44 (top). Rats were treated with PBS or ALAS1-GalNAc siRNA at either 2.5 mg/kg or 5 mg/kg two times per week for three weeks. Phenobarbital (Phenobarb) and a PBGD siRNA in an AF11 LNP formulation was administered at the times indicated in FIG. 44. A control group received PBS and the PBGD siRNA only, without Phenobarbital induction. Urine was collected at days 18-19 of the study.

The results are shown in FIG. 44 (bottom). Administering phenobarbital and PBS induced ALAS1 mRNA expression and increased levels of PBG and ALA in urine (see FIG. 44), compared with PBS only. Treatment with a total of six doses of 2.5 or 5 mg/kg of AD-60519 twice per week suppressed the phenobarbital induced increases in urine PBG and urine ALA (FIG. 44). These results demonstrate that AD-60519 is effective in suppressing ALA and PBG when repeated doses as low as 2.5 mg/kg are administered. In particular, AD-60519 was effective in reducing increases in urine levels of PBG and ALA associated with acute attacks in the rat AIP model.

In further studies using the same experimental design but in a mouse model (see schematic at top of FIG. 44), the therapeutic efficacy of AD-60519 and AD-60489 in suppressing phenobarbital induced increases in serum PBG and ALA was investigated. In the PBS ("Saline") control group, administration of phenobarbital increased levels of PBG and ALA in serum (see FIG. 45), compared with PBS only. Treatment with a total of six doses of 2.5 or 5 mg/kg of AD-60519 or AD-60489 twice per week suppressed the phenobarbital induced increases in serum PBG and serum ALA (FIG. 44). These results demonstrate that both AD-60519 and AD-60489 are effective in suppressing ALA and PBG when repeated doses as low as

2.5 mg/kg are administered. In particular, AD-60519 and AD-60489 were effective in reducing increases in serum PBG and ALA associated with acute attacks.

Because treatments in this example were administered prior to the phenobarbital induction, these results indicate that AD-60519 and AD-60489 have prophylactic effects.

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Example 32: Further siRNA sequences

The following AD-58632 derivative (Table 39) and AD-60489 derivative (Table 40) siRNA sequences have also been generated.

Table 39: AD-58632 derivative sequences

SEQ ID NO: (sense)	SEQ ID NO: (anti-sense)	Target sites of antisense sequence on NM_000688.4	Duplex Name	Sense Sequence (5'-3')	Antisense Sequence (5'-3')
5180	5181	873 - 895	AD-60802	GfsasAfaGfAfGfuGfucucauc(Tgn)ucuuL96	asAfsgAfaGfaugAfgAfcAfcucuuucsusg
5182	5183	873 - 895	AD-60824	GfsasAfaGfAfGfuGfucucauc(Tgn)ucuuL96	asAfsgAfaGfaugAfgacAfcucuuucsusg
5184	5185	873 - 895		GfsasAfaGfAfGfuGfucucauc(Tgn)ucuuL96	asAfsgAfaGfaUfgAfgAfcAfcUfcUfuUfcsusg
5186	5187	873 - 895	AD-60843	GfsasAfaGfAfGfuGfdTcucaucuucuuL96	asAfsgAfaGfaugAfgAfcAfcucuuucsusg
5188	5189	873 - 895	AD-60847	GfsasAfaGfAfGfuGfdTcucaucuucuuL96	asAfsgAfaGfaugAfgacAfcucuuucsusg
5190	5191	873 - 895		GfsasAfaGfAfGfuGfdTcucaucuucuuL96	asAfsgAfaGfaUfgAfgAfcAfcUfcUfuUfcsusg
5192	5193	873 - 895	AD-60819	GfsasAfaGfaGfuGfuCfuCfaucuuCfuuL96	asAfsgAfaGfaugAfgAfcAfcucuuucsusg
5194	5195	873 - 895	AD-60823	GfsasAfaGfaGfuGfuCfuCfaucuuCfuuL96	asAfsgAfaGfaugAfgacAfcucuuucsusg
5196	5197	873 - 895		GfsasAfaGfaGfuGfuCfuCfaucuuCfuuL96	asAfsgAfaGfaUfgAfgAfcAfcUfcUfuUfcsusg
5198	5199	873 - 895		GfsasAfaGfaGfuGfdTCfuCfaUfc(Tgn)uCfuUfL96	asAfsgAfaGfaugAfgAfcAfcucuuucsusg
5200	5201	873 - 895		GfsasAfaGfaGfuGfdTCfuCfaUfc(Tgn)uCfuUfL96	asAfsgAfaGfaugAfgacAfcucuuucsusg
5202	5203	873 - 895		GfsasAfaGfaGfuGfdTCfuCfaUfc(Tgn)uCfuUfL96	asAfsgAfaGfaUfgAfgAfcAfcUfcUfuUfcsusg
5204	5205	873 - 895	AD-60853	gsasaagaGfuGfuCfucaucuucuuL96	asAfsgAfaGfaugAfgAfcAfcucuuucsusg

5206	5207	873 - 895	AD-60839	gsasaagaGfuGfuCfucaucuucuuL96	asAfsgAfaGfaugAfgacAfcucuuucsusg
5208	5209	873 - 895		gsasaagaGfuGfuCfucaucuucuuL96	asAfsgAfaGfaUfgAfgAfcAfcUfcUfuUfcsusg
5210	5211	873 - 895		gsasaagaGfuGfdTCfucaucuucuuL96	asAfsgAfaGfaugAfgAfcAfcucuuucsusg
5212	5213	873 - 895		gsasaagaGfuGfdTCfucaucuucuuL96	asAfsgAfaGfaugAfgacAfcucuuucsusg
5214	5215	873 - 895		gsasaagaGfuGfdTCfucaucuucuuL96	asAfsgAfaGfaUfgAfgAfcAfcUfcUfuUfcsusg

Table 40: AD-60489 derivative sequences

SEQ ID NO: (sense)	SEQ ID NO: (anti-sense)	Target sites of antisense sequence on NM_000688.4	Duplex Name	Sense Sequence (5'-3')	Antisense Sequence (5'-3')
5216	5217	871 - 893		CfsasGfaAfaGfaGfdTGfuCfuCf(Agn)UfscUfsuAfsL96	usAfsaGfadTgAfgAfcAfcdTcuudTcugsgsu
5218	5219	871 - 893	AD-60879	CfsasGfaAfaGfaGfdTGfuCfuCf(Agn)UfscUfsuAfsL96	usAfsaGfaUfgAfgAfcacUfcdTuUfcUfgsgsu
5220	5221	871 - 893		CfsasGfaAfaGfaGfdTGfuCfuCf(Agn)UfscUfsuAfsL96	usAfsAfGfaUfgAfgAfcAfcUfcUfuUfcUfgsgsu
5222	5223	871 - 893		CfsasGfaAfaGfaGfdTGfuCfuCf(Agn)UfscUfsuAfsL96	usAfsaGfaUfgAfgAfcacUfcUfuUfcUfgsgsu
5224	5225	871 - 893	AD-60877	csasgaaaGfAfGfugucuca(Tgn)cuuaL96	usAfsaGfadTgAfgAfcAfcdTcuudTcugsgsu
5226	5227	871 - 893		csasgaaaGfAfGfugucuca(Tgn)cuuaL96	usAfsaGfaUfgAfgAfcacUfcdTuUfcUfgsgsu
5228	5229	871 - 893	AD-60865	csasgaaaGfAfGfugucuca(Tgn)cuuaL96	usAfsAfGfaUfgAfgAfcAfcUfcUfuUfcUfgsgsu
5230	5231	871 - 893	AD-60861	csasgaaaGfAfGfugucuca(Tgn)cuuaL96	usAfsaGfaUfgAfgAfcacUfcUfuUfcUfgsgsu
5232	5233	871 - 893	AD-60876	csasgaaaGfaGfuGfuCfuCfaucuuaL96	usAfsaGfadTgAfgAfcAfcdTcuudTcugsgsu
5234	5235	871 - 893		csasgaaaGfaGfuGfuCfuCfaucuuaL96	usAfsaGfaUfgAfgAfcacUfcdTuUfcUfgsgsu
5236	5237	871 - 893	AD-60519	csasgaaaGfaGfuGfuCfuCfaucuuaL96	usAfsAfGfaUfgAfgAfcAfcUfcUfuUfcUfgsgsu

Example 33: Further multidose studies with AD-60519

The therapeutic efficacy of AD-60519 was investigated in a rat AIP model like that used in Example 31. The experimental design is shown in FIG. 46 (top). Rats were treated with PBS or ALAS1-GalNAc siRNA at 3 mg/kg, 1 mg/kg, or 0.3 mg/kg once per week for four weeks (treatment on day 0, day 7, day 14, and day 21). Phenobarbital (Phenobarb) and a PBGD siRNA in an AF11 LNP formulation were administered at the times indicated in FIG. 46. A control group received PBS and the PBGD siRNA only, without phenobarbital induction. Urine was collected at day 25 of the study.

The results are shown in FIG. 46 (bottom) and in FIG. 47. Administering phenobarbital and PBS induced ALAS1 mRNA expression and increased levels of PBG and ALA in urine, compared with PBS only. Treatment with a total of four doses of 3 mg/kg, 1 mg/kg, or 0.3 mg/kg of AD-60519 once per week suppressed phenobarbital induced increases in levels of rat ALAS1 mRNA in liver in a dose-dependent manner (see FIG. 46). (The levels of rat liver ALAS1 (rALAS1) mRNA are expressed relative to the levels of rat GAPDH mRNA.) The levels of urine PBG and urine ALA also showed dose-dependent treatment effects.

Repeated weekly doses of AD-60519 were effective in suppressing ALAS1 mRNA expression and in reducing elevated levels of ALA and PBG associated with induced acute attacks in a rat AIP model. These treatment effects were dose dependent. These results illustrate that AD-60519 can act prophylactically when dosed prior to an attack.

Example 34: Multidose effects of ALAS1 siRNA GalNAc conjugates in non-human primates

The effects of ALAS1 siRNA GalNAc conjugates in suppressing liver ALAS1 mRNA and circulating ALAS1 mRNA was investigated in a non-human primate (NHP) study. The GalNAc conjugates AD-58632, AD-60519, AD-61193, and AD-60819 were employed. The study design is shown in Table 41 and in FIG. 48.

Table 41: NHP study design

Test Article	Group #	N	Dose Level (mg/kg)	Dose Conc (mg/mL)	Dose Frequency	Dose Days	Dose Volume (mL/kg)	Dose Route
AD-58632	1	3	2.5	1.25	QDx5 WK1, BIW WK2-4	1, 2, 3, 4, 5, 8, 11, 15, 18, 22, 25	2	SC
AD-60519	2	3	1.25	0.625	QDx5 WK1, BIW WK2-4			
	3	3	2.5	1.25	QDx5 WK1, BIW WK2-4			
	4	3	2.5	1.25	QDx5 WK1, QW WK2-4	1, 2, 3, 4, 5, 11, 18, 25		
	5	3	5	2.5	QDx5 WK1, QW WK2-4			
AD-61193	6	3	2.5	1.25	QDx5 WK1, BIW WK2-4	1, 2, 3, 4, 5, 8, 11, 15, 18, 22, 25		
AD-60819	7	3	2.5	1.25	QDx5 WK1, BIW WK2-4	1, 2, 3, 4, 5, 8, 11, 15, 18, 22, 25		

Each group received multiple subcutaneous doses of an ALAS1 siRNA GalNAc conjugate at a dose volume of 2 mg/ml. Group 1 (n=3) received 2.5 mg/kg of 1.25 mg/ml AD-58632 on days 1, 2, 3, 4, 5, 8, 11, 15, 18, 22, and 25. Group 2 (n=3) received 1.25 mg/kg of 0.625 mg/ml AD-60519 on 1, 2, 3, 4, 5, 8, 11, 15, 18, 22, and 25. Group 3 (n=3) received 2.5 mg/kg of 1.25 mg/ml AD-60519 on days 1, 2, 3, 4, 5, 8, 11, 15, 18, 22, and 25. Group 4 (n=3) received 2.5 mg/kg of 1.25 mg/ml AD-60519 on days 1, 2, 3, 4, 5, 11, 18, and 25. Group 5 (n=3) received 5 mg/kg of 2.5 mg/ml AD-60519 on days 1, 2, 3, 4, 5, 11, 18, and 25. Group 6 (n=3) received 2.5 mg/kg of 1.25 mg/ml AD-61193 on days 1, 2, 3, 4, 5, 8, 11, 15, 18, 22, and 25. Group 7 (n=3) received 2.5 mg/kg of 1.25 mg/ml of AD-60819 on days 1, 2, 3, 4, 5, 8, 11, 15, 18, 22, and 25.

Serum samples for the circulating extracellular RNA detection (cERD) assay (see Example 21) were collected on days -3, 7, 13, 21, 27, 39, 46, and 60 (in FIG. 48, "PD Draws" indicates the days on which serum was collected). Serum was collected for a clinical chemistry panel on days -3 and 6. The clinical chemistry panel included assessment of the levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP).

ALAS1 mRNA silencing was evaluated in liver tissue obtained from a liver biopsy taken on day 21 (see FIG. 48). The biopsy was taken after a serum draw.

Suppression of ALAS1 mRNA levels in liver

The liver ALAS 1 mRNA levels at study day 21 are shown in FIG. 49. The results are shown as a percentage of the average level observed in a control group treated with PBS. Results are shown as the average values for each treatment group.

These results presented in FIG. 49 demonstrate that compared with control animals that received PBS treatment, all of the treatment conditions were effective in suppressing liver levels of ALAS1 mRNA. The treatments achieved mRNA silencing ranging from about 20% to 80% (corresponding to ALAS1 mRNA levels ranging from about 80% to 20% of control levels). Individual animals that received AD-58632 showed silencing of about 20-50%, with the average level of silencing being about 40% (ALAS1 mRNA levels were on average about 60% of control levels). With all of the dosing schedules employed, AD-60519 was highly effective in suppressing ALAS1 mRNA levels. Individual animals that received AD-60519 showed silencing of between about 60% and 80% (ALAS1 mRNA levels were about 20% to 40% of control levels). On average, AD-60519 treatment regimens achieved silencing of between about 65% and 75%. As is disclosed herein AD-60519 is a derivative of AD-60489. Similar results for AD-60489 are described in Example 20 and shown in FIG. 30. Furthermore, AD-61193 (a derivative of AD-60489) and AD-60819 (a derivative of AD-58632) also achieved silencing of more than 50%. It is noteworthy that the levels of silencing reported in this example and in Example 20 (e.g., about 20% to 80%) were achieved even in a "non-induced" state; it is anticipated that in an induced state, e.g., when levels of ALAS1 are acutely or chronically elevated (e.g., in a patient having or at risk for a porphyria, e.g., an acute hepatic porphyria, e.g., AIP), lower levels of silencing, e.g., reduction of ALAS1 mRNA levels to normal or pre-attack levels, can suffice to achieve therapeutic efficacy.

Suppression of circulating extracellular ALAS1 mRNA levels

FIG. 50 shows circulating extracellular ALAS 1 mRNA levels (means and standard deviations) at each timepoint throughout the study when serum samples were obtained. The

circulating extracellular ALAS1 mRNA results demonstrate efficacy of mRNA silencing following multidose treatment with each of the siRNAs studied (AD60519, AD-61193, AD-60819, and AD-58632). In all groups, the greatest suppression effect on circulating ALAS1 mRNA was observed on day 27, following the final dose of siRNA on day 25. In all treatment groups, during the weeks after the treatment ceased, ALAS1 mRNA levels gradually increased and returned to baseline by the final measurement on day 60.

The most pronounced suppression of circulating ALAS1 mRNA (maximal silencing of nearly 80%) was observed in Group 3 (2.5 mg/kg AD-60519 QDx5, BIWx3) and Group 5 (5 mg/kg AD-60519, QDx5, QWx3). Group 2 (1.25 mg/kg AD-60519, QDx5, BIWx3), Group 4 (2.5 mg/kg AD-60519, QDx5, QWx3), Group 7 (2.5 mg/kg AD-60819, QDx5, BIWx3), and Group 6 (2.5 mg/kg AD-61193, QDx5, BIWx3) also showed excellent suppression, with maximal silencing (day 27) of greater than 50%. In group 1, notable silencing (more than 30% on day 27) was also achieved.

These results are consistent with the liver ALAS1 mRNA results and confirm the potent activity of AD-60519. At dose levels as low as 1.25 mg/kg, AD-60519 provided 65-75% silencing.

Correlation between circulating and liver ALAS1 mRNA levels

FIG. 27 shows the levels of the ALAS1 mRNA in liver (left bars) and in serum (right bars). There is a good correlation between the relative ALAS1 mRNA levels measured in liver and in serum, indicating that these measurements provide consistent results.

Example 35: Rat single dose study of AD-60519 and AD-60589 using a urine cERD assay to monitor the duration of ALAS1 mRNA suppression

A single dose study was conducted in rats using the ALAS1 siRNA GalNAc conjugates AD-60489 and AD-60519. The efficacy of these GalNAc conjugates in inhibiting expression of ALAS1 mRNA was monitored using assessments of urine with a circulating extracellular RNA detection assay. The assay was similar to the assay used in Examples 21 and 34, except that urine samples were used. The urine samples were lyophilized to concentrate it. Lyophilized urine was resuspended in 4 ml dH2O and vortexed. Then the sample was centrifuged at 4,000xg

for 10-20 minutes to pellet any debris. Remaining steps were similar to those described in Example 21.

Groups of rats were administered a single dose of 10 mg/kg of AD-60489 or AD-60519. The normalized levels of ALAS1 mRNA at various timepoints throughout the study are shown in FIG. 51. The timepoint indicated as "0 hours" is for the baseline pre-dose urine sample drawn just prior to administration of the ALAS1 mRNA. Results for subsequent timepoints are expressed as a fraction of the pre-dose level.

As can be seen from the results shown in FIG. 51, AD-60519 provided improved potency compared with AD-60489. At its maximum, the single dose of AD-60519 provided a suppression of up to about 80%, whereas the suppression provided by AD-60489 was about 60%. The effect of a single 10 mg/kg dose of these ALAS1 siRNAs in suppressing ALAS1 mRNA lasted about 21 days. These results demonstrate the validity of the urine cERD assay for monitoring ALAS1 mRNA levels.

Example 36: Pharmacological effects of AD-60519 in non-human primates

A further study of the effects of the ALAS1 siRNA GalNAc conjugate AD-60519 was conducted in a non-human primates. The study investigated the effect of weeky versus biweekly dosing, use of a loading dose versus no loading dose, and the kinetics of ALAS1 mRNA silencing following a single dose. The design of the study is shown in Table 42 and in FIG. 52.

Table 42: Pharmacology Study Design for Study with AD-60519

Group #	N	Dose Level (mg/kg)	Dose Frequency	Material Needs (mg)*	Dose Vol (mL/kg)	Dose Days	Dose Conc (mg/mL)	Dose Route
1	3	2.5	QWx8Wks	210	0.125	1, 8, 15, 22, 29, 36, 43, 50	20	SC
2	3	5	QWx8Wks	420	0.25			
3	3	Load-QDx3@5mg/kg, maint-5mg/kg	Load D1-D3, QWx7Wks	525	0.25/25	1, 2, 3, 8, 15, 22, 29, 36, 43, 50		
4	3	Load-QDx3@5mg/kg, maint-2.5mg/kg	Load D1-D3, QWx7Wks	270	0.25/0.125			
5	3	5	BIWx8Wks	924	0.25	1, 4, 8, 11, 15, 18, 22 25, 29, 32, 36, 39, 43, 46, 50, 53		
6	3	1	Single Dose	12	0.05	1		
7	3	10	Single Dose	116	0.5	1		

Each group received one or more subcutaneous doses of AD-60519 as provided in Table 42. Group 1 (n=3) received 2.5 mg/kg at a dose volume of 0.125 ml/kg once per week for 8 weeks (doses were administered on dose days 1, 8, 15, 22, 29, 36, 43, and 50). Group 2 (n=3) received 5 mg/kg at a dose volume of 0.25 mg/ml once per week for 8 weeks (doses were administered on dose days 1, 8, 15, 22, 29, 36, 43, and 50). Group 3 (n=3) received a loading dose of 5 mg/kg at a dose volume of 0.25 ml/kg once per day for three days followed by a maintenance dose of 5 mg/kg at a dose volume of 0.25 ml/kg once per week for 7 weeks (doses were administered on days 1, 2, 3, 8, 15, 22, 29, 36, 43, and 50). Group 4 (n=3) received a loading dose of 5 mg/kg at a dose volume of 0.25 ml/kg once per day for three days followed by a maintenance dose of 2.5 mg/kg at a dose volume of 0.125 ml/kg once per week for 7 weeks (doses were administered on days 1, 2, 3, 8, 15, 22, 29, 36, 43, and 50). Group 5 (n=3) received 5 mg/kg at a dose volume of 0.25 ml/kg twice per week for 8 weeks (doses were administered on

dose days 1, 4, 8, 11, 15, 18, 22, 25, 29, 32, 36, 39, 43, 46, 50, and 53). Group 6 (n=3) received a single dose of 1 mg/kg at a dose volume of 0.05 ml/kg on day 1. Group 7 (n=3) received a single dose of 10 mg/kg at a dose volume of 0.5 ml/kg on day 1.

Serum samples (listed as "PD draws" in FIG 52), plasma samples (listed as "PK draws" in FIG 52) and urine samples were collected as indicated in FIG. 52 and in Table 43. The urine and serum samples were subjected to the cERD assay. All blood and urine samples collected on the day of a liver biopsy were collected prior to the liver biopsy.

Table 43: Sample Collection Schedule

<table>
<tr><th colspan="2">Serum for mRNA Detection</th><th colspan="2">Liver Biopsy</th><th colspan="2">Urine for exploratory mRNA Detection **</th><th colspan="2">Plasma for PK</th></tr>
<tr><td>Gps 1-4</td><td>Day -3, and Days 5, 10, 17, 24, 31, 38, 45, 52, 57, 64, 78, 92</td><td>Groups 1-5</td><td>Day 24*</td><td rowspan="3">Gps 1-5</td><td rowspan="3">Day -3, and Days 24*, 52</td><td rowspan="4">Gp7</td><td rowspan="4">Day -3, and Day 1 @ 0.25, 0.5, 1, 2, 4, 8, 12 hours and Days 2, 3, 4, 5, 6, 8, 9, 15, 22, 29, 36</td></tr>
<tr><td>Gps 5</td><td>Day -3, and Days 3, 10, 17, 24, 31, 38, 45, 52, 60, 67, 81, 95</td><td rowspan="2">Groups 6-7</td><td rowspan="2">Day 4*</td></tr>
<tr><td>Gps 6-7</td><td>Day -3, and Days 2, 4, 6, 9, 15, 22, 29, 36</td><td>Gps 6-7</td><td>Day -3, and Days 4*, 36</td></tr>
<tr><td colspan="8">*All blood samples and urine were collected prior to the liver biopsy
**First Morning Urine collection</td></tr>
</table>

The liver ALAS1 mRNA results are shown in FIG. 53. Significant ALAS1 mRNA suppression was achieved in all study conditions. Up to 75-80% ALAS1 silencing was achieved across multi-dose regimens using AD-60519. At three days after a single dose, silencing of about 15% was achieved with a single dose of 1 mg/kg, and silencing of about 70% was achieved with a single dose of 10 mg/kg (see FIG. 53). Comparison of the data from groups 1 and 7 reveals a slight difference in kinetics after a single dose (in group 7) versus multiple doses

(group 1) of the same cumulative amount (30 mg administered), as assessed 3 days post-dose in group 7 and at two days after the fourth dose in group 1. In particular, greater silencing was observed after a single dose (silencing of about 70% on average in group 7 versus silencing of about 45% on average in group 1). See FIG. 54. This type of result was also observed in rat studies.

The serum ALAS1 mRNA results through day 22 are shown in FIG. 54 (top). The correlation between liver ALAS1 mRNA, serum ALAS1 mRNA, and plasma ALAS1 mRNA is shown in FIG. 55. These results demonstrate a good correlation between liver, serum, and urine ALAS1 mRNA levels. The results also provide further evidence demonstrating potent activity of AD-60519. Silencing of 55-75% was observed at at all dose levels across all multi-dose dosing regimens. Administering loading doses (once per day for 3 days, as in groups 3 and 4) resulted in slightly more rapid down-regulation of ALAS1 mRNA. The groups that received weekly (groups 1 and 2) or biweekly doses (group 5) doses ultimately showed comparable levels of ALAS1 mRNA suppression, indicating that accumulation over time provides sustained knockdown.

Results showing the kinetics of ALAS1 mRNA silencing after a single dose are shown in FIG. 54 (bottom). In the 1 mg/kg group, an ALAS1 mRNA suppression of about 20% was observed by day 6. In the 10 mg/kg group, there was a rapid, about 70% ALAS1 mRNA reduction by day 4, with recovery to within 20% of baseline at day 22 (21 days post-dose). Levels of serum ALAS1 mRNA returned to baseline after about 2 weeks or 4 weeks following a 1mg/kg or 10mg/kg single dose, respectively.

The full time course of the serum ALAS mRNA up to 8 weeks after administration of the AD-60519 is shown in FIG. 56. All groups reached a maximum of 80% ALAS1 mRNA suppression following 5 to 8 weeks of ALN-AS1 dosing. Groups with three daily doses in week 1 (QDx3) had a faster onset of ALAS1 mRNA suppression than those just dosed once in the first week (QWx8). All animals returned to baseline ALAS1 levels, approximately 30-40 days post the last dose

Example 37: Production of an siRNA Drug Product

ALN-60519 (FIG. 57) is a chemically synthesized double stranded oligonucleotide covalently linked to a ligand containing three N-acetylgalactosamine (GalNAc) residues. All nucleotides are 2'-OMe or 2'-F modified and are connected through 3'-5' phosphodiester linkages, thus forming the sugar-phosphate backbone of the oligonucleotide. The sense strand and the antisense strand contain 21 and 23 nucleotides, respectively. The 3'-end of the sense strand is conjugated to the triantennary GalNAc moiety (referred to as L96) through a phosphodiester linkage. The antisense strand contains four phosphorothioate linkages - two at the 3' end and two at the 5' end. The sense strand contains two phosphorothioate linkages at the 5' end. The 21 nucleotides of the sense strand hybridize with the complementary 21 nucleotides of the antisense strand, thus forming 21 nucleotide base pairs and a two-base overhang at the 3'-end of the antisense strand. The two single strands, the sense strand and the antisense strand, were synthesized by conventional solid phase oligonucleotide synthesis, employing standard phosphoramidite chemistry with the 5'-hydroxyl group protected as dimethoxytriphenylmethyl (DMT) ether. Each strand was assembled from the 3' to the 5' terminus by sequential addition of protected nucleoside phosphoramidites.

AD-60519, also referred to herein as ALN-60519, was formulated as a solution for Injection for subcutaneous use, referred to herein as ALN-AS1. ALN-60519 was dissolved in water for injection (WFI) and the pH was adjusted (target 7.0). The concentration of ALN-60519 was determined and adjusted by adding WFI. The solution with a final concentration of approximately 200 mg/mL was then filter sterilized and filled into 2 mL Type I glass vials. A fill volume of approximately 0.55 mL was chosen to permit complete withdrawal of 0.5 mL of drug product.

Example 38: Measurement of Serum or Urine ALAS1 mRNA Levels in AIP Patients or Healthy Volunteers Using cERD Method

Non-human primate pharmacology studies with ALN-AS1 indicated that the circulating extracellular RNA detection (cERD) method for measuring the ALAS1 mRNA in serum or urine was robust and reproducible. The cERD assay was also used to measure ALAS1 mRNA levels in serum and urine from AIP patients and healthy volunteers. Serum ALAS1 mRNA levels were

generally increased in AIP patients relative to healthy volunteers, consistent with the role ALAS1 induction plays in disease pathophysiology (FIG. 58). Importantly, levels of ALAS1 in serum and urine within the same patient correlated with each other. In two patients that had repeat collections of urine and serum the ALAS1 mRNA level was consistent over time. Collectively, these data indicate that ALAS1 mRNA can be measured in serum and urine samples from human subjects including AIP patients, and the cERD method is useful for tracking the pharmacodynamic activity of ALN-AS1.

Example 39: Exemplary Clinical Studies

A human study can be conducted to determine the safety and tolerability of ALN-AS1 when administered as a single dose and multiple doses to AIP patients that are asymptomatic high excreters (ASHE) (patients who have elevated levels of ALA and/or PBG, as described herein) or AIP patients who have recurrent attacks.

Secondary objectives include the characterization of plasma and urine PK for ALN-AS1 as well as post-dose assessment of the impact of ALN-AS1 on both plasma and urinary ALA and PBG levels. The cERD assay that measures mRNA in exosomes is used to measure serum (or plasma) and urinary 5-aminolevulinate synthase (ALAS-1 mRNA).

In the asymptomatic high excreters, ALN-AS1 is administered at single doses, e.g., at 0.1, 0.35 1.0, or 2.5 mg/kg, or in repeated weekly doses, e.g., of 1 and 2.5 mg/kg, for several weeks (e.g., for 4 weeks). As a comparison, a control (e.g., placebo) treatment is administered. The safety, pharmacokinetics and effects of the drug on ALA and PBG levels is assessed. A dose of ALN-AS1 that lowers ALA and PBG to within the normal reference range (e.g., a dose that normalizes ALA and/or PBG to levels below 2x the upper reference value) can be selected for subsequent studies, e.g., in AIP patients.

In the AIP patients, the attack rate and baseline symptoms are assessed during a pre-dosing run-in period (e.g., of 12 weeks). Patients are administered ALN-AS1, e.g., at a dose of 1-2.5 mg/kg weekly.The safety, pharmacokinetics and effects of the drug on ALA and PBG levels are assessed. In addition, changes in attack number, heme use, pain medication use, and hospitalization are monitored.

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 ALAS1, wherein said dsRNA comprises a sense strand and an antisense strand, the antisense strand comprising a region of complementarity to an ALAS1 RNA transcript (e.g., SEQ ID NO:1), which antisense strand comprises at least 20 contiguous nucleotides from the antisense sequence of UAAGAUGAGACACUCUUUCUGGU (SEQ ID NO: 4153) or UAAGAUGAGACACUCTUUCUGGU (SEQ ID NO: 4154).

2. A double-stranded ribonucleic acid (dsRNA) for inhibiting expression of ALAS1, wherein said dsRNA comprises a sense strand and an antisense strand, the antisense strand comprising a region of complementarity to an ALAS1 RNA transcript (e.g., SEQ ID NO:1), which antisense strand comprises at least 20 contiguous nucleotides from (i) an antisense sequence listed in any one of Tables 21 to 40, or (ii) an unmodified version of an antisense sequence listed in any one of Tables 21 to 40 (SEQ ID NOs: 4172 to 5237).

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

4. The dsRNA of any one of the preceding claims, wherein the duplex region is 17-23 nucleotide pairs in length.

5. The dsRNA of any one of the preceding claims, wherein at least one strand comprises a 3' overhang of at least 2 nucleotides.

6. The dsRNA of any one of the preceding claims, wherein each strand is no more than 26 nucleotides in length.

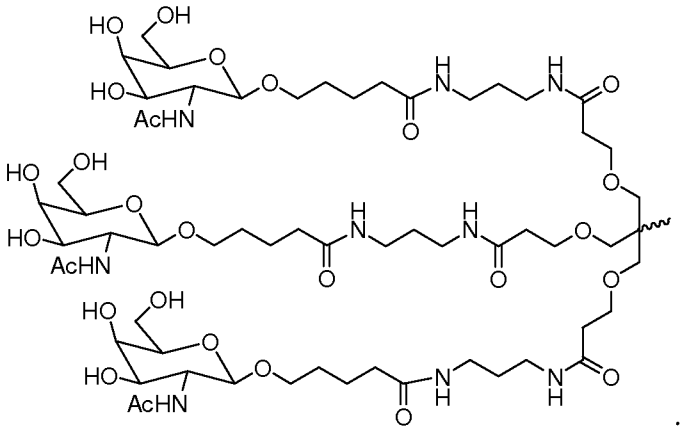
7. The dsRNA of claim 3, wherein at least one modified nucleotide is chosen from a 2′-O-

methyl, a 2′-fluoro modified nucleotide, and optionally one or more 5'-phosphorothioate groups, or any combination thereof.

8. The dsRNA of any one of the preceding claims, further comprising a ligand, optionally wherein the ligand is conjugated to the 3' end of the sense strand of the dsRNA.

9. The dsRNA of claim 8, wherein the ligand comprises a carbohydrate, optionally wherein the ligand is a GalNAc ligand.

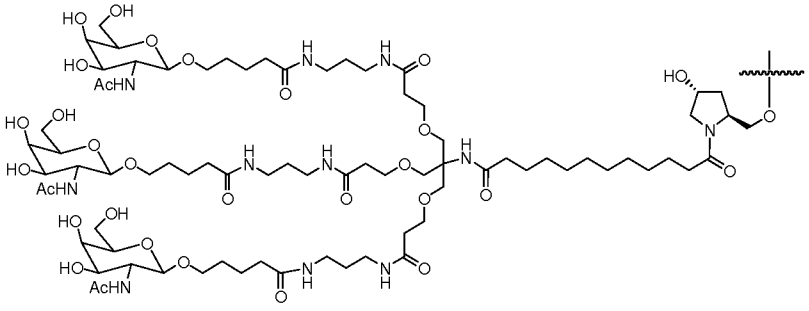
10. The dsRNA of claim 9, wherein the ligand is



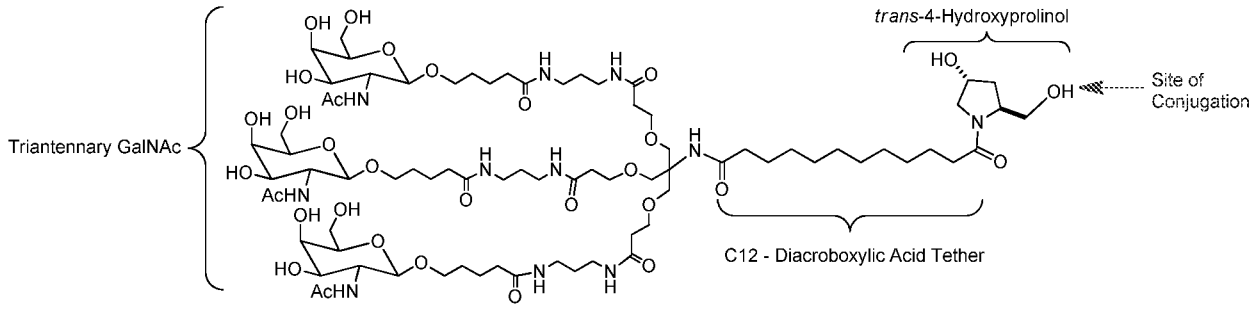
.

11. The dsRNA of any one of claims 8-10, wherein the ligand is attached via a bivalent or trivalent branched linker.

12. The dsRNA of claim 11, wherein the ligand and linker are as shown in Formula XXIV:



13. The dsRNA of claim 8, wherein the dsRNA is conjugated to ligand L96 via a linker as shown below



14. The dsRNA of any one of claims 8 to 13, wherein the ligand targets the dsRNA to hepatocytes.

15. The dsRNA of any one of the preceding claims, wherein the dsRNA comprises a sense strand consisting of a sense sequence selected from the sense sequences listed in Tables 21 to 40, and an antisense strand consisting of an antisense sequence selected from the antisense sequences listed in Tables 21 to 40.

16. The dsRNA of any one of the preceding claims, wherein the dsRNA has an IC_{50} of less than 1 nM, less than 0.05 nM, less than 0.02 nM, or less than 0.01 nM.

17. The dsRNA of any one of the preceding claims, wherein the dsRNA has a single dose ED50 of less than about 10 mg/kg or less than about 5 mg/kg.

18. The dsRNA of any one of the preceding claims, wherein the dsRNA shows improved activity compared with AD-58632 or AD-60489, optionally wherein the dsRNA is selected from the dsRNAs listed in Tables 21 to 40.

19. The dsRNA of any one of the preceding claims, wherein the sense strand comprises or consists of the sequence of CAGAAAGAGUGUCUCAUCUUA (SEQ ID NO: 4155).

20. The dsRNA of claim 1 or 2, wherein:

(i) the antisense strand comprises the antisense sequence of AD-60519, wherein the antisense sequence comprises all of the modified nucleotides of AD-60519;

(ii) the antisense strand consists of the antisense sequence of AD-60519, wherein the antisense sequence comprises all of the modified nucleotides of AD-60519;

(iii) the sense strand comprises the sense sequence of AD-60519, wherein the sense sequence comprises all of the modified nucleotides of AD-60519;

(iv) the sense strand consists of the sense sequence of AD-60519, wherein the sense sequence comprises all of the modified nucleotides of AD-60519;

(v) the sense strand comprises the sense sequence of AD-60519, and the antisense strand comprises the antisense sequence of AD-60519, wherein the sense and antisense sequences comprise all of the modified nucleotides of AD-60519, or

(vi) the sense strand consists of the sense sequence of AD-60519, and the antisense strand consists of the antisense sequence of AD-60519, wherein the sense and antisense sequences comprise all of the modified nucleotides of AD-60519.

21. The dsRNA of claim 1 or 2, wherein:

(i) the antisense strand comprises the antisense sequence of AD-60489, and/or the sense strand comprises the sense sequence of AD-60489, wherein the antisense and/or sense sequence comprises all of the modified nucleotides of AD-60489, or

(ii) the antisense strand consists of the antisense sequence of AD-60489, and/or the sense strand consists of the sense sequence of AD-60489, wherein the antisense and/or sense sequence comprises all of the modified nucleotides of AD-60489.

22. The dsRNA of claim 1 or 2, wherein:

(i) the antisense strand comprises the antisense sequence of AD-61193, and/or the sense strand comprises the sense sequence of AD-61193, wherein the antisense and/or sense sequence comprises all of the modified nucleotides of AD-61193, or
(ii) the antisense strand consists of the antisense sequence of AD-61193, and/or the sense strand consists of the sense sequence of AD-61193, wherein the antisense and/or sense sequence comprises all of the modified nucleotides of AD-61193.

23. The dsRNA of claim 2, wherein:
(i) the antisense strand comprises the antisense sequence of AD-60819, and/or the sense strand comprises the sense sequence of AD-60819, wherein the antisense and/or sense sequence comprises all of the modified nucleotides of AD-60819, or
(ii) the antisense strand consists of the antisense sequence of AD-60819, and/or the sense strand consists of the sense sequence of AD-60819, wherein the antisense and/or sense sequence comprises all of the modified nucleotides of AD-60819.

24. A vector encoding at least one strand of a dsRNA of any one of claims 1 to 23.

25. A cell comprising the dsRNA of any one of claims 1 to 23 or the vector of claim 24.

26. A pharmaceutical composition for inhibiting expression of an ALAS1 gene, the composition comprising the dsRNA of any one of claims 1 to 23.

27. The pharmaceutical composition of claim 26, wherein dsRNA is administered in an unbuffered saline or water solution.

28. The pharmaceutical composition of claim 26 or 27, wherein said composition is suitable for subcutaneous administration.

29. A method of inhibiting ALAS1 expression in a cell, the method comprising:

(a) introducing into the cell the dsRNA of any one of claims 1 to 23, and

(b) maintaining the cell of step (a) for a time sufficient to obtain degradation of the mRNA transcript of an ALAS1 gene, thereby inhibiting expression of the ALAS1 gene in the cell, optionally wherein the expression of ALAS1 is inhibited by at least 20% or at least 30%.

30. A method for decreasing a level of a porphyrin or a porphyrin precursor (e.g., ALA or PBG) in a cell (e.g., a hepatocyte), comprising contacting the cell with the dsRNA of any one of claims 1 to 23, in an amount effective to decrease the level of the porphyrin or the porphyrin precursor in the cell.

31. A method of treating a porphyria, the method comprising administering to a subject in need of such treatment a therapeutically effective amount of
(i) the dsRNA of any one of claims 1 to 23 or
(ii) the composition of any one of claims 26 to 28,
thereby treating the porphyria.

32. The method of claim 31, wherein the subject is at risk for developing, or is diagnosed with, a porphyria.

33. The method of claim 31 or 32, wherein the porphyria is acute intermittent porphyria or ALA-dehydratase deficiency porphyria.

34. The method of any one of claims 31 to 33, wherein (i) the dsRNA or composition comprising dsRNA is administered after an acute attack of porphyria, (ii) the dsRNA or composition comprising dsRNA is administered during an acute attack of porphyria, or (iii) the dsRNA or composition comprising dsRNA is administered prophylactically to prevent an acute attack of porphyria.

35. The method of any one of claims 31 to 34, wherein the dsRNA is administered at a dose of 0.05 to 50 mg/kg bodyweight of the subject, e.g, at a dose of 0.01 mg/kg to 5 mg/kg bodyweight of the subject.

36. The method of any one of claims 31 to 35, wherein the method
(i) decreases a level of a porphyrin or a porphyrin precursor (e.g., δ-aminolevulinic acid (ALA) or porphopilinogen (PBG)) in the subject, optionally wherein the level is decreased by at least 30% and/or
(ii) inhibits ALAS1 expression in the subject.

37. The method of any one of claims 31 to 36, wherein said method (i) ameliorates a symptom associated with an ALAS1 related disorder (e.g., a porphyria), (ii) decreases frequency of acute attacks of symptoms associated with a porphyria in the subject, and/or (iii) decreases incidence of acute attacks of symptoms associated with a porphyria in the subject when the subject is exposed to a precipitating factor, e.g., the premenstrual phase.

38. The method of claim any one of claims 31 to 37, wherein the dsRNA or composition comprising the dsRNA is administered according to a dosing regimen, e.g., weekly, biweekly, or monthly.

39. The method of any one of claims 31 to 38, wherein the dsRNA is administered before an acute attack of porphyria, e.g., during a prodrome.

40. The method of claim any one of claims 31 to 39, wherein the subject has an elevated level (e.g., plasma or urine level) of ALA and/or PBG and optionally wherein the subject suffers from chronic pain.

41. The method of any one of claims 31 to 40, wherein the method decreases the elevated level of ALA and/or PBG.

42. The method of any one of claims 31 to 41, wherein the method decreases or prevents pain, neuropathy, and/or nerve damage.

43. The method of any one of claims 31 to 42, wherein the method prevents acute attacks of porphyria.

44. The method of any one of claims 31 to 43, wherein the dsRNA or composition comprising the dsRNA is administered repeatedly.

45. A method of treating a subject with an elevated level of ALA and/or PBG, the method comprising administering to a subject in need of such treatment a therapeutically effective amount of

(i) the dsRNA of any one of claims 1 to 23 or

(ii) the composition of any one of claims 26 to 28.

optionally, wherein the method is effective to decrease the level of ALA and/or PBG.

46. A method of treating a subject having an increased level of ALA and/or PBG, the method comprising administering the dsRNA of any one of claims 1 to 23 at 1 mg/kg, 2.5 mg/kg, or 5 mg/kg once per week for at least ten weeks, thereby reducing the level of ALA and/or PBG in said subject.

47. A method of treating a human patient with AIP who has suffered from multiple recurrent attacks, the method comprising administering the dsRNA of any one of claims 1 to 23 at a dose of 2.5 mg/kg for at least 6 months, thereby treating said patient,

optionally wherein said method

(i) reduces the frequency of attacks,

(ii) reduces hematin use,

(iii) reduces hospitalization, and/or

(iv) improves quality of life.

48. A method for assaying the level of circulating extracellular ALAS1 mRNA in a subject, said method comprising:

detecting (*e.g.*, measuring) the level of ALAS1 mRNA in a biological fluid sample (e.g., a blood sample, a plasma sample, a serum sample, or a urine sample) from the subject, said biological fluid sample comprising the ALAS1 mRNA,

thereby assaying the level of circulating extracellular ALAS1 mRNA in the subject.

49. A method for assaying the level of circulating extracellular ALAS1 mRNA in a subject, said method comprising

(i) providing RNA (*e.g.*, extracellular RNA) from a biological fluid sample (e.g., a blood sample, a plasma sample, a serum sample, or urine sample) from the subject, said biological fluid sample comprising the ALAS1 mRNA;

(ii) obtaining an ALAS1 cDNA from the ALAS1 mRNA;

(iii) contacting the ALAS1 cDNA with a nucleic acid complementary (e.g., probe and/or primer) to the ALAS1 cDNA or a portion thereof, thereby producing a reaction mix; and

(iv) detecting (e.g., measuring) the level of ALAS1 cDNA in the reaction mix, wherein the ALAS1 cDNA level is indicative of the ALAS1 mRNA level,

thereby assaying the level of circulating extracellular ALAS1 mRNA in the subject,

optionally wherein

(a) the method comprises PCR, qPCR or 5'-RACE,

(b) the nucleic acid is a probe or primer, and/or

(c) the nucleic acid comprises a detectable moiety and the level of ALAS1 mRNA is determined by detection of the amount of the detectable moiety.

50. The method of claim 49, wherein the efficacy of a porphyria treatment is assessed based on a comparison of the level of circulating extracellular ALAS1 mRNA in the subject with a reference value.

51. The method of claim 49, wherein a decrease in the level of of circulating extracellular ALAS1 mRNA in the subject in response to the porphyria treatment, relative to the reference value, indicates that the porphyria treatment is efficacious.

52. The method of any one of claims 49 to 51, further comprising acquiring the biological fluid sample from a subject, optionally wherein the biological fluid sample is separate from the tissue, and wherein the biological fluid sample contains exosomes.

53. A composition comprising the dsRNA of claim 20 and water for injection.

54. The composition of claim 53, comprising about 200 mg/mL of the dsRNA of claim 20.

55. The composition of claim 53 or 54, wherein the composition has a pH of 6.0-7.5, e.g., about 7.0.

56. The composition of any one of claims 53 to 55, wherein the composition is formulated for subcutaneous injection.

57. A method of treating a subject having a porphyria (e.g., AIP) or an elevated level of ALA and/or PBG, the method comprising subcutaneously administering the composition of any one of claims 53 to 56 to the subject.

58. The method of claim 57, wherein the composition is administered at a dose of 0 to 5 mg/kg, e.g., at a dose of 2.5 mg/kg or less or at a dose of 1 to 2.5 mg/kg.

59 The method of claim 58, wherein the composition is administered weekly.